Characterisation of actin cytoskeletal functions and interactions during planar polarity formation in *Arabidopsis*

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Tillägnad mina nära och kära

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Abstract

The establishment of cell and tissue polarity is essential for the development of eukaryotic organisms including plants and animals. When cell polarity is uniformly organised within a plane of a single tissue layer, it is referred to as planar polarity. In the model plant *Arabidopsis thaliana*, the polarly organised formation of root hairs towards the basal (root tip-oriented) ends of epidermal cells provides an ideal model system to study planar polarity formation. The instructive cue which uniformly organises tissue polarity in the *Arabidopsis* root epidermis is a concentration gradient of the plant hormone auxin. This concentration gradient leads to the polar recruitment of Rho-of-plant proteins to membrane sites where root hairs will form, making them the earliest cellular markers of planar polarity. Despite having identified several of the components required for planar polarity formation and the cellular read-out of it, little is known about how planar polarity is perceived and stably executed on the single cell level. Recently, a role for *ACTIN2* in polar hair positioning was described, suggesting that actin may be involved in the cellular read-out of planar polarity.

In the work presented here, we further investigate the contribution of the actin cytoskeleton to polar hair positioning. We identify a role for actin upstream of polar Rho-of-plant (ROP) positioning and describe *ACTIN7* and *ACTIN-INTERACTING PROTEIN1-2* (*AIP1-2*) as components required for planar polarity formation which interact directly in yeast and *in vitro* and genetically during planar polarity formation. *AIP1-2* expression proves hair cell file-specific and depends on *WEREWOLF* function, revealing a link between planar polarity and cell fate patterning in the root. In addition we find that *ACT7* genetically interacts with *SABRE* (*SAB*) and that actin and SAB co-localise at the cell plate and plasma membrane domains during cell division. Furthermore, we show that actin is not only involved in the positioning, but also in the organisation of the polar membrane domain which marks where a root hair will form. Among the components which localise at this site are the phosphoinositide-biosynthetic enzyme PHOSPHATIDYLINOSITOL 4-PHOSPHATE 5-KINASE3 (PIP5K3), the DYNAMIN-RELATED PROTEINs (DRPs) DRP1A and DRP2B, the D6 PROTEIN KINASE (D6PK) and membrane sterols. For several of these components, we report a function in planar polarity formation and in the organisation of the hair initiation site.

With this work, we provide deeper insight into the function of the actin cytoskeleton and its interactions during planar polarity formation and identify additional components that contribute to the process. Moreover, we report AIP1-2 placement under control of the cell fate patterning system during establishment of planar polarity. Our results provide stepping stones for future studies aimed at investigating the mechanistic processes that contribute to planar polarity formation in more molecular and cellular detail.

Sammanfattning

Etableringen av cell- och vävnadspolaritet är avgörande för utvecklingen av eukaryota organismer, inklusive växter och djur. När cellpolaritet likformigt organiseras inom ett plan av ett vävnadsskikt, kallas det planpolaritet. I modellväxten *Arabidopsis thaliana* föreställer det polärt organiserade bildandet av rothår som är riktat emot basala (rot spets orienterade) ändar av epidermalceller ett idealiskt modellsystem för att studera utformandet av planpolaritet. Den instruktiva signalen som enhetligt anordnar vävnads polariteten i *Arabidopsis* rot epidermis är en koncentrationsgradient av växthormonet auxin. Denna koncentrationsgradient leder till den polära rekryteringen av Rho-of-plant proteiner till membran platser där rothår bildas, vilket gör dem till de tidigaste cellulära markörerna för planpolaritet. Trots att flera komponenter som krävs för att planpolaritet ska kunna bildas och utläsas på cell nivå har identifierats, är lite känt om hur planpolaritet förnimmas och stabilt exekveras i enstaka celler. Nyligen beskrevs en roll för *ACTIN2* i den polära hår positioneringen, vilket tyder på att aktin kan vara involverad i den cellulära avläsningen av planpolaritet.

I arbetet som presenteras här vidare undersökte vi aktin cytoskelettets bidrag till den polära hår positioneringen. Vi identifierar en funktion för aktin uppströms av den polära Rho-of-plant (ROP) positioneringen och beskriver *ACTIN7* (*ACT7*) och (*ACTIN-INTERACTING PROTEIN1-2*) (*AIP1-2*) som två komponenter nödvändiga för utformandet av planpolaritet och som interagerar i jäst, *in vitro* och genetiskt under utformandet av planpolaritet. *AIP1-2* expressionen visar sig vara hår cell linie specifik och beroende på *WER* funktion, vilket avslöjar en länk mellan planpolaritetsutvecklingen och mönstringen av cell öden i roten. Dessutom upptäcker vi att ACT7 interagerar genetiskt med *SABRE* (*SAB*) och att aktin och SAB kolokaliserar vid cell plattor och plasma membran domäner under celldelningen. Därutöver visar vi att aktin inte bara deltar i placeringen, men också i organisationen av den polära membrandomän som markerar var ett rothår ska bildas. Bland de komponenter som vi lokaliserade på denna plats är fosfoinositid-biosyntetiska enzymet PHOSPHATIDYLINOSITOL 4-PHOSPHATE 5-KINASE3 (PIP5K3), de dynamin-relaterade proteinerna DYNAMIN-RELATED PROTEIN (DRP)1A och DRP2B, D6 PROTEIN KINASEn (D6PK) och membran steroler. För flera av dessa komponenter, beskriver vi en funktion i utformandet av planpolaritet och i organiseringen av rothårs initiationsstället.

Genom det här arbetet ger vi djupare insikt i funktionen av aktin cytoskelettet och dess interaktioner under planpolaritetens bildning och identifierar ytterligare komponenter som bidrar till processen. Vår data representerar en språngbräda för framtida studier som syftar till att undersöka de mekanistiska processerna som bidrar till planpolaritetens utbildning i ytterligare molekylär och cellulär detalj.

List of Manuscripts

I

Christian S. Kiefer, Andrea R. Claes, Jean-Claude Nzayisenga, Stefano Pietra, Thomas Stanislas, Anke Hüser, Yoshihisa Ikeda and Markus Grebe.
Arabidopsis AIP1-2 restricted by *WER*-mediated patterning modulates planar polarity. Manuscript pending revision at **Development**.

II

Stefano Pietra, Anna Gustavsson, **Christian Kiefer**, Lothar Kalmbach, Per Hörstedt, Yoshihisa Ikeda, Anna N. Stepanova, Jose M. Alonso and Markus Grebe.
Arabidopsis SABRE and *CLASP* interact to stabilize cell division plane orientation and planar polarity. **Nature Communications** 2013, **4**:2779

III

Thomas Stanislas*, Anke Hüser*, **Christian S. Kiefer**, Klaus Brackmann, Inês C.R. Barbosa, Anna Gustavsson, Stefano Pietra, Claus Schwechheimer and Markus Grebe.
D6PK AGCVIII kinase is a lipid domain-dependent mediator of *Arabidopsis* planar polarity. Manuscript pending submission.

*These authors contributed equally to this work.

In the following text the manuscripts will be referred to by their Roman numbers.

Related publications of Christian Kiefer not included in the thesis:

Moritaka Nakamura, **Christian S. Kiefer** and Markus Grebe.
Planar polarity, tissue polarity and planar morphogenesis in plants. **Current Opinion in Plant Biology** 2012, **15**:593-600

Author Contributions

I

Christian Kiefer made the largest experimental contribution to this manuscript. He analysed the organisation of the actin cytoskeleton during root hair initiation (Fig. 1J-K and supplementary Fig. 1G-J), generated and analysed the *act7*$^{-6/-7}$ transheterozygous line and results displayed in supplementary Fig. 1B-C, including root hair positioning analysis of *act8-2* (supplementary Fig. 1D). He cloned the *AIP1-2* and *ACT7* constructs for the yeast two-hybrid experiment and supervised Andrea Claes during the cloning of the ACT1, ACT2, ACT8 and AIP1-1 constructs. He and Andrea Claes performed the yeast two-hybrid analyses displayed in Fig. 2A and supplementary Fig. 2A. Christian Kiefer cloned all constructs used for *in vitro* pull down experiments and together with Markus Grebe co-supervised Jean-Claude Nzayisenga during the execution of the pull down experiments (Fig. 2B-C; supplementary Fig. 2D-F). Christian Kiefer generated and analysed all crosses including *aip1-2* alleles, generated and analysed all transgenic *AIP1-2* constructs and corresponding single, double and triple transgenic lines used in the manuscript and performed all experiments displayed in Figs. 4, 6, 7 and supplementary Fig. 4. He contributed to the analysis of all experiments, assembled the figures and wrote the first manuscript draft which he subsequently revised together with Markus Grebe.

II

Christian Kiefer performed SAB-3xYpet localisation in paraformaldehyde-fixed *mCherry-TUA5;sab-5;SAB-3xYpet* seedlings and SAB-3xYpet localisation in *sab-5;SAB-3xYpet* seedlings labelled with rhodamine phalloidin (Fig. 9I-T; supplementary Figure S5). He read and commented on the manuscript prior to submission and re-submission.
In the continuation of this work on the characterisation of *SABRE*, Christian Kiefer crossed *act7-6* with *sab-5;SAB-3xYpet* and brought it to the F2 stage, thereby contributing to the data displayed in Fig. 10 in Pietra, 2014.

III

Christian Kiefer localised YFP-D6PK in *act7-6;35S:YFP-D6PK* seedlings, *35S:YFP-D6PK* seedlings grown on medium containing

DMSO, lovastatin, fenpropimorph, and *35S:YFP-D6PK* seedlings treated with DMSO or latrunculin B (Fig. 4I-K and Fig. 5A-D). He localised filipin-sterol complexes at hair initiation sites in Col-0, *act7-6* and *GFP-ABD2-GFP* seedlings (Fig. 5E-M). Christian Kiefer localised Bodipy FL phallacidin labelled actin in Col-0 and *cpi1-1* (Fig. 5N-Q). He read and commented on the manuscript.

Abbreviations

ABP	actin-binding protein
ABP1	AUXIN BINDING PROTEIN1
ACC	1-aminocyclopropane-1-carboxylic acid
ACT	ACTIN
ADF	ACTIN-DEPOLYMERISING FACTOR
ADP	Adenosine diphosphate
ADZ	actin-depleted zone
AGD1	ARF-GAP DOMAIN1
AIP1	ACTIN-INTERACTING PROTEIN1
ANX	ANXUR
APT1	ABERRANT POLLEN TRANSMISSION1
ARF	ADP-ribosylation factor
ARK1	ARMADILLO REPEAT-CONTAINING KINESIN1
ARP	ACTIN-RELATED PROTEIN
ATP	Adenosine triphosphate
AUX1	AUXIN RESISTANT1
BFA	Brefeldin A
CAP1	CYCLASE-ASSOCIATED PROTEIN1
CDS	cortical division site
CDZ	cortical division zone
CESA6	CELLULOSE SYNTHASE6
CLASP	CYTOPLASMIC LINKER ASSOCIATED PROTEIN
CLC	CLATHRIN LIGHT CHAIN
COW1	CAN OF WORMS1
CP	CAPPING PROTEIN
CPC	CAPRICE
CPI1	CYCLOPROPYLSTEROL ISOMERASE1
CTR1	CONSTITUTIVE TRIPLE RESPONSE1
CYCD3;1	CYCLIND3;1
CytD	Cytochalasin D
2,4-D	2,4-Dichlorophenoxyacetic acid
D6PK	D6 PROTEIN KINASE
DAG	diacylglycerol
DIM	detergent insoluble membrane
DRP	DYNAMIN-RELATED PROTEIN
EE	early endosome
EGL3	ENHANCER OF GLABRA3
EIN2	ETHYLENE INSENSITIVE2
ER	endoplasmic reticulum
ETC1	ENHANCER OF TRY AND CPC1
FAD	flavin adenine dinucleotide
F-actin	filamentous actin

FH	FORMIN HOMOLOGY
G-actin	globular actin
GAP	GTPase-activating protein
GDI	GDP dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GL1	GLABRA1
GL2	GLABRA2
GL3	GLABRA3
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
HG	homogalacturonan
IAA	indole-3-acetic acid
JKD	JACKDAW
KCA1	KINESIN CDKA;1-ASSOCIATED1
KIP	KINKY POLLEN
KTN	KATANIN
kuq	kreuz und queer
LatB	Latrunculin B
MDP25	MICROTUBULE-DESTABILISING PROTEIN25
MS	manuscript
MT	microtubule
mya	million years ago
MYO	MYOSIN
1-NAA	1-Naphthaleneacetic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NH	non hair
PA	phosphatidic acid
PFN	PROFILIN
PH	pleckstrin homology
PI4K	PHOSPHATIDYLINOSITOL 4-KINASE
PIN	PIN-FORMED
PIP5K	PHOSPHATIDYLINOSITOL 4-PHOSPHATE 5-KINASE
PLC2	PHOSPHOLIPASE C2
PME	PECTIN METHYL ESTERASE
PPB	preprophase band
PRC1	PROCUSTE1
PRK2	POLLEN RECEPTOR KINASE2
PtdIns(4)P	phosphatidylinositol 4-phosphate
PtdIns(4,5)P_2	phosphatidylinositol 4,5-bisphosphate
RBOH	RESPIRATORY BURST OXIDATIVE HOMOLOG
REN1	ROP ENHANCER1
RH	root hair

RHD	ROOT HAIR DEFECTIVE
RIC	ROP-INTERACTIVE CRIB-MOTIVE CONTAINING PROTEIN
RLK	receptor-like kinase
ROP	Rho-of-plant
ROS	reactive oxygen species
RSL4	ROOT HAIR DEFECTIVE 6-LIKE4
SAB	SABRE
SCAR	SUPPRESSOR OF cAMP RECEPTOR
SCM	SCRAMBLED
SCN1	SUPERCENTIPEDE1
SIM	SIAMESE
SMT1	STEROL METHYLTRANSFERASE1
SPK1	SPIKE1
TGN	trans-Golgi network
TMK1	TRANSMEMBRANE KINASE1
TRY	TRIPTYCHON
TTG1	TRANSPARENT TESTA GLABRA1
TUA6	α-tubulin6
VLN	VILLIN
W/SRC	WAVE/SCAR regulatory complex
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP family verprolin homologous
WEI	WEAK ETHYLENE INSENSITIVE
WER	WEREWOLF

Introduction

Cell polarity – a persistent break in cellular symmetry

Cell polarity occurs when the unequal distribution of molecules or functions in an initially unpolarised and symmetric cell lead to a persistent break in symmetry. The break in symmetry gives the cell two faces, which together define an axis of polarity (Cove, 2000; Johnson et al., 2011). Acquisition and maintenance of cell polarity is important for cell division, growth and morphogenesis of unicellular organisms such as bacteria and yeast (Brown et al., 2011; Mauriello, 2010; Lybarger and Maddock, 2001; Drubin and Nelson, 1996; Chant, 1999), as well as multicellular organisms such as animals and plants (Ahringer, 2003; Bryant and Mostov, 2008; Yang, 2008). Mechanistically, cell polarity formation can be divided into three steps: (1) initial induction of a cellular asymmetry by a polarising cue, (2) establishment/alignment of a polar axis and (3) its maintenance/regulation (Cove, 2000). While each of the three steps can be regulated in diverse ways in different organisms and developmental stages, they share a subset of conceptual features.

The first cellular asymmetry can be induced by either internal or external factors that can be of varying nature, for example light, gravity, hormones or stochastic fluctuations of molecular activity distribution (Cove, 2000). Independent of the polarising cue, the initially symmetric cell needs to be able to perceive and respond to the initial signal, thereby transiently inducing a cellular asymmetry (a polar face) (Figure 1a). In eukaryotic systems the response involves the local activation of small Rho-family guanosine triphosphatases (GTPases). Since their activity is regulated by the binding of guanosine triphosphate (GTP) or its hydrolysis to guanosine diphosphate (GDP), GTPases can act as molecular switches (Chant and Stowers, 1995). In their active state (GTP-bound) they mark the polar domain (Johnson, 1999).

To establish the polar axis, the signal coming from the transiently induced polar face has to be amplified via a positive feedback mechanism. This reinforcement produces a stable polar assembly of molecules and functions, also called a polarity patch (Altschuler et al., 2008). At the same time the cell has to ensure that only one patch is formed in order to avoid mistargeting of cellular components to false second sites. To better understand how these processes occur, mathematical modelling is becoming an increasingly powerful tool not only to describe, but also to anticipate and predict the behaviour

of biological systems (Mogilner et al., 2012). The value of a model depends mainly on what can be learned from it and not on how complex it is, supporting model simplicity. The positive feedback and spatial exclusion of multiple patch formation can be mathematically explained by a simple autocatalytic process consisting of a slowly diffusing polarity patch activator, and a quickly diffusing inhibitor or substrate of the activator (Turing, 1952; Meinhardt and Gierer, 1974). This model is commonly referred to as a Turing-type system, named after its 'inventor' (Turing, 1952). The activator positively regulates its own activity as well as the activity of the inhibitor. Slow diffusion of the activator spatially restricts the spreading of the polar patch, while the more quickly moving inhibitor diffuses farther or the highly mobile substrate becomes deprived at far distances. These kinetic properties of the components lead to inhibition of further patch formation at a distance from the first patch (Meinhardt and Gierer, 1974). Local recruitment and activation of small GTPases at the polar face are thought to ultimately define the polar axis in eukaryotic cells (Johnson et al., 2011) and mathematical modelling based on work in budding yeast showed that small GTPase-mediated signaling fulfills the requirements of Turing-type systems (Goryachev and Pokhilko, 2008), supporting the validity of such a mechanism *in vivo*.

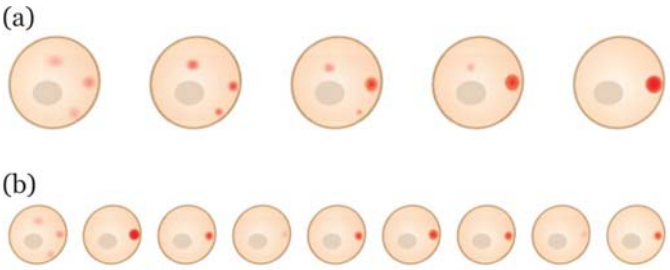

Figure 1: Polar patch formation and behaviour in yeast. (a) Induction of an initial asymmetry and establishment of a polar patch (red). (b) Dynamic behaviour observed for polar patches (red) in yeast. Image adapted from Wu and Lew, 2013 with permission of the publisher.

While simple positive-feedback models can sufficiently explain the ultimate establishment of one polar axis (Goryachev and Pokhilko, 2008), they fail to describe the oscillations in activator complex activity occurring during maintenance/regulation of the polar axis in yeast or plants (Figure 1b) (Howell et al., 2012; Hwang et al., 2005). Also, the coincidence of two transient polar faces, as frequently observed in yeast, would be expected to lead to the formation of two temporarily coexisting patches (Howell et al., 2012; Wu and Lew, 2013), which are only sporadically observed in polarity mutants

(Howell et al., 2009). To accommodate for these observations, more advanced models anticipate the existence of negative feedback circuits (Howell et al., 2012; Das et al., 2012). Recently, a negative feedback acting to modulate small GTPase activity was unraveled in yeast (Kuo et al., 2014). Considering the theoretical requirement for negative feedback regulation to explain the behaviour of established polarity patches, it is tempting to assume a role for this mechanism during maintenance/regulation of the polar axis. However, this still remains to be experimentally verified and work aimed at identifying further components of possible negative, as well as further positive feedback mechanisms contributing to a robust establishment of cell polarity is of rising interest.

Theoretical and experimental studies, especially on single-celled model systems such as dividing yeast cells or growing pollen tubes, have substantially broadened our understanding of the mechanisms and molecular components contributing to cell polarity formation. The coordination of cell polarity within a tissue is a fascinating question that further requires the use of multicellular model systems.

Tissue and planar polarity – putting cell polarity into context

Tissue polarity describes the uniformly coordinated polarity of cells in a tissue-wide context (Sachs, 1991; Grebe, 2004). When the tissue is formed by a single layer of cells, all oriented along a single polar axis within the plane of this layer, it is more specifically referred to as planar polarity (Nübler-Jung, 1987; Grebe, 2004). This means that the polar axes of the individual cells contained in the tissue must be aligned along a unifying axis defined by the tissue's polarity. Mechanistically, this requires (1) an initial polarising cue, (2) the competence of cells to sense and respond to this cue and (3) the ability to communicate the polarising signal to cells at a farther distance from the polarising cue (Bryant and Mostov, 2008).

Tissue polarities are often manifested during later stages of development when functional organs are starting to form, but the orientation of the polar axes is already predetermined by local asymmetries occurring during early embryo development. In plants, the asymmetric division of the zygote predicts the positions of the shoot and root apical meristems, thereby determining the future apical-basal (shoot-root) axis of the plant body (Zhang and Laux, 2011). In the fruitfly *Drosophila melanogaster*, differences in the distribution of a subset of mRNAs in the oocyte specify the anterior (head and thorax) and posterior (abdomen) regions along the

anterior-posterior axis of the embryo, while the asymmetric position of the nucleus in the oocyte determines the dorso-ventral (front-back) body axis of the egg and embryo, thereby defining the main body axes of the adult animal (Roth and Lynch, 2009). Once a polar axis has been aligned, the information must be translated into a signal which can transmit the polar cue throughout the tissue layer. Such a position-dependent signal may be based on cell-cell contacts, whereby the initially polarised cell induces the neighbouring cell to polarise equally, creating a domino-like system which progressively polarises the entire tissue. Alternatively, it may depend on a mobile signal such as a diffusible compound. To provide directionality for the uniform alignment of the cell polarities in a tissue, this compound needs to form a gradient of activity, which in biological systems can easily be achieved by differences in concentration. Both above-mentioned mechanisms have been shown to occur in nature (Fischer et al., 2006; Chin and Mlodzik, 2013; Wu et al., 2013), and are sometimes even thought to act in concert to firmly establish a uniform tissue polarity (Ezan and Montcouquiol, 2013).

The vascularisation of plants provides an excellent example of tissue polarity (Sachs, 1991; Sauer et al., 2006), and demonstrates its importance for the evolution of land plants. After their first occurrence, land plants acquired rooting and vascular systems to conduct water and nutrients from the soil to the aerial regions (Raven and Edwards, 2001). These inventions helped to pave the way for the vast spread of vascular plants and their colonisation of the continents that we still observe today (Knoll et al., 1984).

In the animal kingdom tissue polarity has been intensely studied amongst others in the fly wing epithelium, where hairs are uniformly initiated and oriented along the proximo-distal axis of the wing, as well as in the cochlean epithelium of mammals, where hair bundles are polarly positioned towards the apical sides of hair cells (Ezan and Montcouquiol, 2013). Considering the importance of functional wings or auditory systems for flies or mammals, respectively, this further underpins the importance of robust tissue polarity formation for the evolution and development of multicellular organisms.

The last common ancestor between animals and plants is believed to be a unicellular eukaryote (Meyerowitz, 1999). Consistently, the core components of cell polarity formation, including small GTPases and the cytoskeleton, are evolutionary conserved. However, assuming a single-celled eukaryote as the last common ancestor, the mechanisms regulating tissue formation and cell-cell communication are expected to have developed independently in animal and plant lineages (Meyerowitz, 2002). Indeed, the upstream pathways acting

to provide directional cues for tissue polarity formation differ. In animals the frizzled and fat/dachsous pathways coordinate the formation of planar cell polarity (Goodrich and Strutt, 2011; Wu et al., 2013), whereas this is mediated by signalling of the phytohormone auxin in plants (Nakamura et al., 2012). Interestingly, increasing evidence shows that some of the core components acting during cell polarity establishment are furthermore required for the establishment of tissue polarity. This suggests either that the polarity of the whole tissue depends on the polarity of every cell (non-cell autonomous polarisation of cells in the tissue), or alternatively that preexisting cell polarity modules were recruited into parallel pathways to facilitate the establishment of planar polarity (cell-autonomous polarisation of cells in the tissue), representing a convergent evolution of genetic mechanisms. To better understand the mechanistic connection between tissue and cell polarity, further investigation of these phenomena is essential.

A very easily accessible model system to study the formation of planar polarity in plants is the root epidermis of the flowering plant *Arabidopsis thaliana* (hereafter simply referred to as *Arabidopsis*), where root hairs are uniformly initiated towards the root tip-oriented (basal) end of root hair-forming cells.

The *Arabidopsis* root epidermis

The roots of vascular plants are required for anchoring the plant, as well as for uptake and transport of nutrients and water (Raven and Edwards, 2001; Grierson and Schiefelbein, 2002). The *Arabidopsis* root consists of a radial pattern of distinct tissues that surround a central vascular system (Figure 2). The tissues are formed by asymmetric divisions of mother cells in the root apical meristem, which each generate one daughter cell that maintains stem cell activity and one that further differentiates to provide cells for the diverse tissues. This mechanism of continuous tissue proliferation ensures that newly formed cells are connected to the pre-existing ones, thereby forming a continuous layer of cells (Dolan and Scheres, 1998).

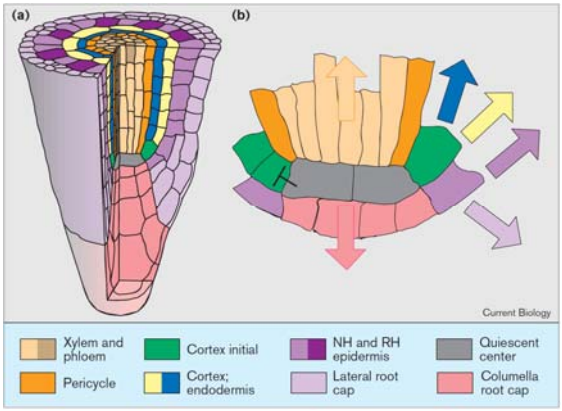

Figure 2: Schematic overview of the *Arabidopsis* root tip. (a) Overview of the tissue pattern in the root tip. (b) Overview of stem cell organisation in the root apical meristem. Arrows indicate the direction of tissue expansion. Black crossbar indicates possible different division planes of the cortex initial. NH = non-hair; RH = root hair. Image reproduced from Benfey and Scheres, 2000 with permission of the publisher.

The outermost layer of the mature root is the root epidermis, which constitutes the interface between plant and soil where the uptake of water and nutrients occurs. In order to increase the contact surface with the environment, *Arabidopsis* like many other plants forms root hairs, which are tubular protrusions from specific hair-forming epidermal cells (trichoblasts). Besides the decrease in plant fitness described in plants defective in root hair formation (Minorsky, 2002), the mere presence of root hairs in all major vascular plant lineages (Jones and Dolan, 2012) indicates the evolutionary importance of these structures for plant development. In *Arabidopsis*, the energy-consuming formation of root hairs is spatially controlled by at least two principally different mechanisms, together ensuring equal spacing between hairs in the epidermal layer. One is referred to as pattern formation, and leads to the systematic placement of hairs on every second or third cell file only. The other is referred to as planar polarity, and basally orients hair initiation on root hair-forming cells, thereby placing hairs at a longitudinal distance from each other corresponding approximately to the hair cell length.

Patterning of root hair fate – the WEREWOLF pathway

The *Arabidopsis* root epidermis consists of alternating files of cells which acquire either root hair (RH) or non-hair (NH) fate (Cormack, 1962). This decision is dependent on positional cues derived from the underlying cortex: epidermal cells overlying the cleft between two cortical cell files are considered as occupying an RH position and will acquire RH fate, while cells in contact with only one cortical cell file are considered as being in an NH position and will acquire NH fate (Figure 2 and Figure 3) (Berger et al., 1998). The central component

ultimately determining which fate an epidermal cell will acquire is the relative abundance of a protein complex containing the MYB-domain transcription factor WEREWOLF (WER), the WD-repeat protein TRANSPARENT TESTA GLABRA 1 (TTG1) and either of the redundantly acting basic helix-loop-helix (bHLH) proteins GLABRA3 (GL3) or ENHANCER OF GLABRA3 (EGL3) (Lee and Schiefelbein, 1999; Galway et al., 1994; Payne et al., 2000; Bernhardt et al., 2003). The more WER-GL3/EGL3-TTG1 complexes are formed, the more RH fate is actively repressed and consequently NH fate acquired. When sufficiently abundant, the complex induces the expression of the homeodomain-leucine-zipper transcription factor GLABRA2 (GL2), a repressor of transcription of the hair-fate promoting bHLH transcription factor ROOT HAIR DEFECTIVE 6 (RHD6) (Masucci and Schiefelbein, 1994), thereby leading to the expression of non-hair cell-specific genes and specification of NH fate (Masucci et al., 1996; Bruex et al., 2012). The WER-GL3/EGL3-TTG1 complex can hence be considered an activator of NH fate. To stabilise the NH fate once the decision has been made, the activator induces the expression of MYB23, the MYB protein most closely related to WER with a partly redundant function (Kang et al., 2009). This activation of an activator provides a positive feedback mechanism, which can act to maintain relatively high levels of the activator complex (Schiefelbein et al., 2014).

Conversely, while the activator complex induces the expression of NH fate activators to specify the NH fate, it also induces the expression of the NH-fate-repressing single-repeat MYB-domain proteins CAPRICE (CPC), TRIPTYCHON (TRY) and ENHANCER OF TRY AND CPC1 (ETC1) (Zhao et al., 2008; Morohashi and Grotewold, 2009). By binding to GL3 or EGL3, they form an inactive MYB-GL3/EGL3-TTG1 complex, thereby competing with the function of the NH fate activators WER and MYB23 (Lee and Schiefelbein, 2002). After being induced and transcribed mainly in cells in NH positions (Bruex et al., 2012), the inhibitor proteins are thought to move laterally and accumulate in RH positions as this has been shown for CPC (Wada et al., 2002; Kurata et al., 2005). As a result, these cells fail to accumulate sufficient WER/MYB23-GL3/EGL3-TTG1 complex, which in turn leads to the expression of RHD6 and the subsequent transcription of a multitude of root hair cell-specific genes and specification of RH fate. The activation of a highly mobile inhibitor is a strategy referred to as lateral inhibition (Lee and Schiefelbein, 2002) and serves to establish specific cell fates in an initially homogeneous field of cells (Schiefelbein et al., 2014). Curiously, *GL3* and *EGL3*, elements of the NH fate activator complex,

are preferentially transcribed in RH positions (Bernhardt et al., 2005). The respective proteins however accumulate in NH positions, suggesting that they move from RH to NH cells. Since the repression of GL3 and EGL3 expression in NH cells is due to negative transcriptional regulation mediated by the WER-GL3/EGL3-TTG1 complex, this pathway represents a negative feedback mechanism which is believed to be required for reinforcement of the patterning system (Schiefelbein et al., 2014).

While the WER patterning system can regulate itself once it has been set up, the initial cue for the acquisition of NH and RH fate comes from the underlying cortical tissue layer. An interesting question arising from the fate acquisition depending on the position of the cells relative to the underlying cortex, is how this positional cue is transformed into the accumulation of WER-GL3/EGL3-TTG1 in NH cells. This potentially involves signalling via the leucine-rich repeat receptor-like kinase SCRAMBLED (SCM). SCM accumulates specifically in RH positions where it negatively affects WER expression (Kwak and Schiefelbein, 2007; Kwak and Schiefelbein, 2008), thereby confining its accumulation to NH cells. In turn, SCM itself is negatively regulated by downstream transcription factors of WER-dependent signalling, providing a further positive feedback circuit that potentially acts to promote robust fate patterning in the root epidermis. Signalling via SCM is likely to act downstream of the zinc finger protein JACKDAW (JKD) in control of epidermal fate patterning (Hassan et al., 2010). JKD, however, is expressed only in the cortical and endodermal tissues and is hence likely to exert a non-cell autonomous effect on root hair patterning (Hassan et al., 2010). The non-cell autonomous effect of the underlying tissue layer has so far only been demonstrated for the patterning of RH fate in *Arabidopsis* but not for the patterning of trichome fate, which otherwise shares several molecular components that, however, have opposite functions in shoot and root (Grebe, 2012). Another mechanistic difference between patterning of RH and trichome fate appears to be the maintenance of cell fate. During trichome patterning, multiple endoreplication steps have been suggested to not only be required for trichome morphogenesis, but also for patterning itself (Bramsiepe et al., 2010; Grebe, 2012). Proteins promoting or inhibiting endoreplication such as SIAMESE (SIM) or CYCLIND3;1 (CYCD3;1) opposingly affect trichome patterning (Bramsiepe et al., 2010). SIM likely acts downstream of and is positively regulated by GL3 and GLABRA1 (GL1), which similar to WER during RH/NH patterning in the root is the activating MYB-domain transcription factor during trichome patterning (Oppenheimer et al., 1991; Kirik et

al., 2005). Activation of SIM and induction of endoreplication might hence provide another example of a positive feedback circuit acting to promote robust establishment of fate pattern.

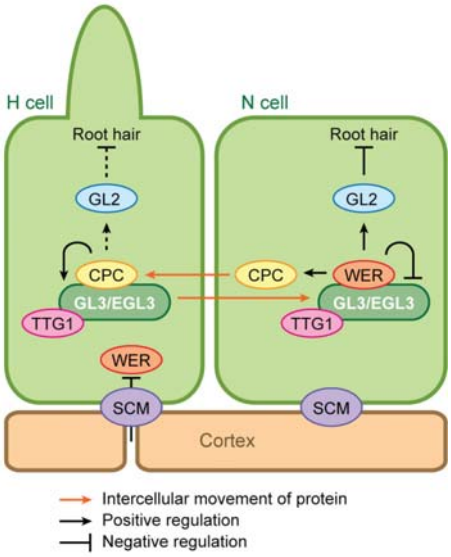

Figure 3: Pattern formation in the *Arabidopsis* root epidermis. The MYB-domain protein WER forms a complex with bHLH proteins GL3 or EGL3 and the WD-repeat protein TTG1 to promote the expression of the homeodomain-leucine-zipper transcription factor GL2 in NH cells, leading to the specification of NH fate. The WER-GL3/EGL3-TTG1 complex further induces the expression of the single-repeat MYB protein CPC, which move laterally into RH cells where they compete with WER for binding to GL3 or EGL3, leading to an inactive complex and the specification of RH fate. SCM maximum activity is thought to occur in RH cells, where it represses WER function. Image reproduced from Ishida et al., 2008 with permission of the publisher.

Remarkably, the regulation of cell fate and cell polarity formation are reminiscent of each other, in that they employ similar mechanistic pathways to firmly establish a specific outcome. For example Wingless-dependent signaling contributes to both patterning and planar cell polarity formation in the wing (Nüsslein-Vollhard et al., 1984; Wu et al., 2013). Interestingly, expression of the auxin influx carrier AUXIN RESISTANT1 (AUX1), a modulator of planar polarity formation in *Arabidopsis*, is transcriptionally dependent on WER and MYB23 (Jones et al., 2009; Ikeda et al., 2009; see following chapter), and mutants of *RHD6* are defective in polar root hair positioning (Masucci and Schiefelbein, 1994), suggesting the existence of a direct crosstalk between the two pathways for which a developmental function has not been revealed, yet.

Planar polarity of root hair positioning

Once RH fate is determined, RH-specific genes that promote root hair formation are expressed. The positioning of root hairs in the root

epidermis is uniformly oriented towards the basal ends of trichoblasts in *Arabidopsis* wild type (Figure 4). Interference with planar polarity alters root hair position, making it an excellent model system in which to study the formation of planar polarity in plants (Masucci and Schiefelbein, 1994; Grebe et al., 2002; Fischer et al., 2006).

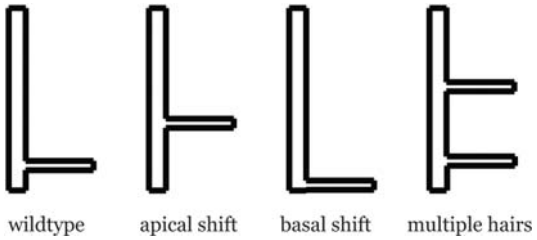

Figure 4: Hair positioning on root hair cells of *Arabidopsis thaliana*. Phenotypic description of root hair position is indicated below for each trichoblast. Image adapted from Fischer et al., 2007 with permission of the publisher.

Providing a directional cue for planar polarity

Early work already indicated a possible role for the phytohormones auxin and ethylene in the regulation of planar polarity, since addition of indole-3-acetic acid (IAA) or 1-aminocyclopropane-1-carboxylic acid (ACC) to the growth medium altered root hair positioning. Moreover, the auxin-response defective *auxin resistant2-1* mutant caused an apical shift of hair positioning (Masucci and Schiefelbein, 1994). This was further supported by the observation that *aux1* mutants, defective in an auxin influx carrier (Marchant et al., 1999), displayed an apical shift of root hair positioning, providing first evidence that auxin influx activity is required for the polarisation of cells (Grebe et al., 2002). More recently, it was revealed that vectorial information directing the positioning of root hairs comes from a concentration gradient of auxin (Fischer et al., 2006). Combined disruption of AUX1, ETHYLENE INSENSITIVE2 (EIN2), a central component of ethylene signalling pathway (Alonso et al., 1999), and reduced activity of GNOM, an ADP-ribosylation factor (ARF)-guanine nucleotide exchange factor (GEF) involved in endocytic recycling (Steinmann et al., 1999; Geldner et al., 2001; Geldner et al., 2003), abolished the generation of an apical-basal auxin concentration gradient in the root, concomitant with a complete loss of polar root hair positioning (Fischer et al., 2006). Introduction of an artificial auxin gradient, however, was able to reestablish a directional bias of hair positioning, ultimately revealing an auxin concentration gradient as the vectorial cue to coordinate planar polarity in the root epidermis (Figure 5).

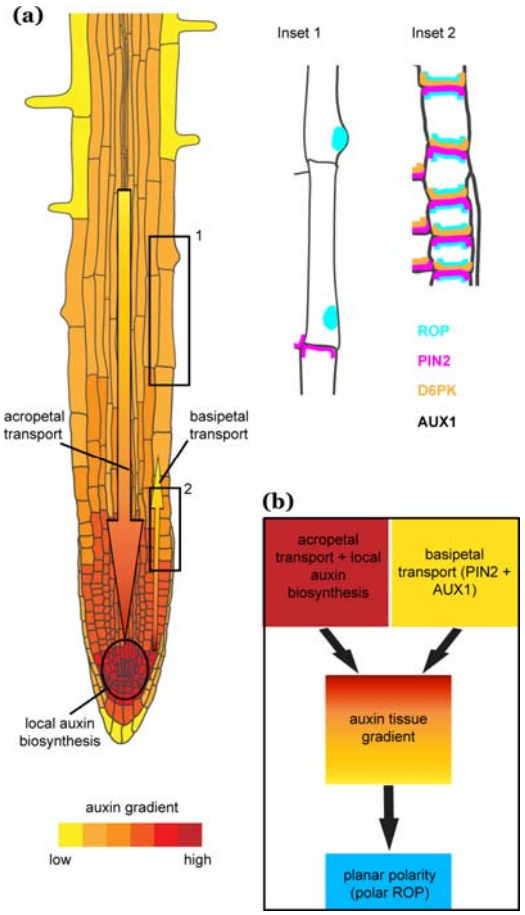

Figure 5: Epidermal cells and planar polarity in the *Arabidopsis* root.
(a) Auxin transport and concentration gradient are indicated. In the vasculature, transport orients 'downward' (acropetal) and in the epidermis 'upward' (basipetal). Insets display the polar cellular localisation of proteins in the late (Inset 1) and early (Inset 2) elongation zone. (b) Schematic presentation of mechanisms contributing to auxin distribution and planar polarity. Image adapted from Nakamura et al., 2012 with permission of the publisher.

Generation and maintenance of an auxin concentration gradient

The generation of an auxin concentration gradient *in vivo* depends on the formation of a local auxin biosynthesis maximum in the root tip (Petersson et al., 2009; Brunoud et al., 2012; Ikeda et al., 2009) and the redistribution of auxin via influx and efflux carrier-mediated transport (Fischer et al., 2006; Ikeda et al., 2009). Tip-localised biosynthesis depends on the function of the auxin precursor synthesis enzymes WEAK ETHYLENE INSENSITIVE2 (WEI2) and WEI7 (Ikeda et al., 2009). Expression of *WEI2* and *WEI7* is negatively regulated by signalling downstream of CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) which acts via EIN2 during hair positioning

(Ikeda et al., 2009). This was revealed by the epistasy of *ein2* over *ctr1* in multiple mutant combinations and the recent finding that EIN2 protein is a direct target for phosphorylation catalysed by the CTR1 Raf-like kinase (Ikeda et al., 2009; Ju et al., 2012). Loss of CTR1 function leads to a local increase in auxin concentration in the root tip, steepening of the concentration gradient over the root and hyper-polar positioning of root hairs at the basal-most ends of cells (Ikeda et al., 2009). Analysis of triple mutants defective in *CTR1*, *AUX1* and *PIN-FORMED 2* (*PIN2*), encoding an auxin efflux carrier which localises to the apical membrane of epidermis cells (Müller et al., 1998), further revealed the requirement of both auxin transport facilitators downstream of ethylene signalling in planar polarity formation (Ikeda et al., 2009). The hyper-polar hair positioning and steepened auxin gradient of *ctr1* mutants is restored to wild-type levels when combined with the *aux1;pin2* double mutant, which displays a flattened auxin concentration gradient. This suggests that AUX1 and PIN2 act downstream of CTR1 via transport of auxin (Ikeda et al., 2009). Interestingly, while mutation of *AUX1* affects polar root hair positioning and root hair formation in RH cells, a functional fusion of AUX1 is found to accumulate mainly in NH cells (Jones et al., 2009; Ikeda et al., 2009), suggesting a largely non-cell autonomous mode of action. The NH cell-specific enrichment of AUX1 is thought to be required for long-distance transport of auxin, raising its concentration in the differentiation zone high enough to promote root hair formation upon lateral distribution to RH cells (Jones et al., 2009). The finding that AUX1 is not expressed in the epidermis of a *wer;myb23* double mutant (Jones et al., 2009), which fails to specify the NH fate (Bruex et al., 2012), further revealed AUX1 as a molecular link between pattern and planar polarity formation. The mechanisms leading to NH file-specific AUX1 enrichment however remain enigmatic, considering the absence of *AUX1* in transcriptomic studies comparing the expression profiles of RH and NH files (Deal and Henikoff, 2010; Bruex et al., 2012).

As mentioned before, interference with GNOM function in an *aux1;ein2;gnom* triple mutant leads to a loss of planar polarity in the epidermis. The first indication for the requirement of an ARF-GEF during planar polarity formation came from the observation that application of the vesicle trafficking inhibitor brefeldin A (BFA), which targets large ARF-GEFs including GNOM (Steinmann et al., 1999), induced apical shifts in root hair positioning (Grebe et al., 2002). GNOM function is required during endocytic recycling of PIN auxin efflux carrier proteins, thereby facilitating their localisation at polar plasma membrane domains (Geldner et al., 2001; Geldner et al.,

2003; Kleine-Vehn et al., 2008). Consistently, PIN2 was found to be mislocalised in cortical cells of weak *gnom* mutants, and *PIN2* mutation was not able to alter planar polarity in any multiple mutant combination where *GNOM* function was reduced (Ikeda et al., 2009), supporting the idea that GNOM modulates planar polarity via its role in endocytic recycling of PIN proteins. In addition to being required for cellular PIN distribution, GNOM may indirectly be involved in the regulation of PIN activity at the plasma membrane via regulation of the subcellular distribution of a kinase acting on PINs. The D6 PROTEIN KINASE (D6PK) belongs to the family of AGCVIII kinases, which are related to the mammalian protein kinases A, G and C (Pearce et al., 2010). It is a basal plasma membrane marker in the *Arabidopsis* root, which phosphorylates PINs *in vitro* and *in vivo* (Zourelidou et al., 2009; Willige et al., 2013; Barbosa et al., 2014). The subcellular distribution of D6PK between the basal plasma membrane and endosomes depends on fully functional GNOM (Barbosa et al., 2014). Intriguingly, the subcellular distribution of D6PK responds faster and is more sensitive to BFA treatment than PIN protein distribution. Low concentrations of BFA, which affect the plasma membrane localisation of D6PK but not PINs, interferes with PIN phosphorylation status and auxin distribution in the *Arabidopsis* root. This suggests that the transport of auxin via PINs is not only depending on the subcellular localisation of the efflux carriers, but also on their activity, which may be regulated via phosphorylation by AGCVIII kinases including D6PK. Since the *in vivo* phosphorylation of PINs is suggested to depend on the plasma membrane localisation of D6PK, it is possible that GNOM indirectly regulates PIN activity via control of subcellular D6PK localisation (Barbosa et al., 2014). However, a role for D6PK during planar polarity formation remains to be investigated. Also, the relatively strong mutant phenotype of hypomorphic *gnom* mutants compared to *pin1;pin2;pin3;pin7* quadruple mutants further suggests that GNOM has a function in planar polarity in addition to acting on the control of PIN protein localisation (Fischer et al., 2006).

Further support for a role of endocytic recycling in planar polarity formation, potentially via modification of the polar accumulation of auxin transporters in the epidermis, came from analyses of a mutant defective in CLATHRIN LIGHT CHAIN2 (CLC2) and CLC3, which is defective in clathrin-mediated endocytosis (Wang et al., 2013a). These seedlings revealed an apical shift in root hair positioning, which was suggested to be at least partially due to an interference with auxin transport and signalling (Wang et al., 2013a). While this still remains to be addressed experimentally, the results clearly

demonstrate that clathrin coat components are required for planar polarity formation.

Taken together, the central components for establishing an auxin concentration gradient which instructs planar polarity in the root epidermis, are the tip-localised biosynthesis and polar transport of auxin (Figure 6). At the molecular level this includes the action of components of the ethylene signalling pathway, specifically expressed and polarly localised auxin transporters and actors involved in the endocytic trafficking of these auxin transporters (Figure 6).

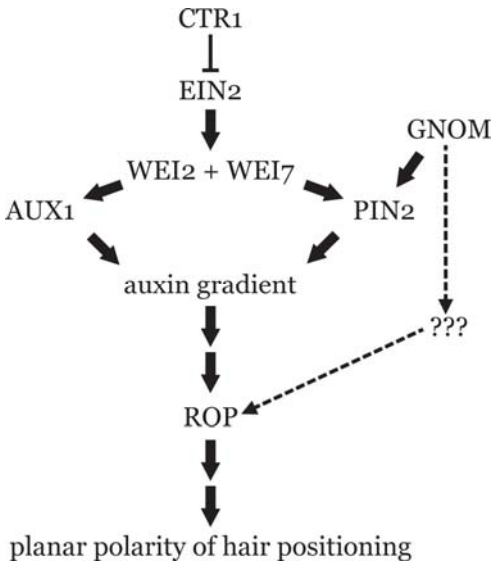

Figure 6: Genetic framework of planar polarity formation in the root epidermis. Ethylene signalling via CTR1 and EIN2 affects auxin biosynthesis in the root tip via regulation of auxin precursor-synthetic genes WEI2 and WEI7. Auxin transport via AUX1 and PIN2 contributes to an auxin concentration gradient, which leads to the polar positioning of ROPs and root hairs. PIN2 and ROP polarity requires GNOM. Diagram is based on work described in Fischer et al., 2006 and Ikeda et al., 2009.

Structural components involved in planar polarity formation

Besides the central actors required for auxin gradient formation, there are several other components involved in mediating polar root hair placement. One of the structural components is the membrane sterol composition. Mutants defective in *STEROL METHYLTRANSFERASE1* (*SMT1*), which have an altered membrane sterol composition, display a more randomised root hair positioning (Willemsen et al., 2003). The phenotype may in part be due to the

requirement of sterol-dependent endocytosis in the polarisation of auxin transporters. Indeed, the post-cytokinetic localisation of PIN2 to the apical membrane of epidermal cells depends on correct membrane sterol composition, as revealed by the mispolarisation of PIN2 in *cyclopropylsterol isomerase1* (*cpi1*) mutants which display an almost exclusive abberant accumulation of cyclopropylsterols (Men et al., 2008). Furthermore, application of the sterol-complexing dye filipin induces intracellular aggregation of early endosomal markers, suggesting that sterol distribution and/or abundance can modulate endocytic trafficking in general. The specific enrichment of fluorescent filipin-sterol complexes at the polar membrane domain prior to bulge appearance (Ovecka et al., 2010), further suggests a role for sterols during hair site selection. Together, the data reveal the localisation of and the requirement for sterols during planar polarity formation.

Another lipid component likely to be involved in planar polarity formation is phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2). Mutations in the *PHOSPHATIDYLINOSITOL 4-PHOSPHATE (PtdIns(4)P) 5-KINASE* (*PIP5K*) genes *PIP5K1* and *PIP5K2*, encoding enzymes which catalyse the conversion of PtdIns(4)P into PtdIns(4,5)P_2, induce PIN polarity defects and a failure of seedlings to establish distinct auxin concentration maxima in the embryo and in the root tip (Tejos et al., 2014). Considering the role of a local auxin concentration maximum in the root tip for planar polarity in the root epidermis, it is likely that phosphoinisitide signalling contributes to polar hair positioning. Also, a functional PIP5K3 fluorescent fusion localises to hair initiation sites during bulge formation, and remains localised to this membrane domain during root hair elongation (Kusano et al., 2008), further supporting a role for PtdIns(4,5)P_2 during hair formation.

Another structural component involved in planar polarity formation is the actin cytoskeleton. Mutants defective in *ACTIN2* (*ACT2*), an actin isoform expressed mainly in differentiating cells of the root (Kandasamy et al., 2009), display an apical shift in polar root hair positioning (Ringli et al., 2002). Furthermore, local actin cytoskeletal rearrangements were described to occur shortly after bulge outgrowth (Baluska et al., 2000), supporting a role for the actin cytoskeleton in hair site selection and root hair formation. In addition, interference with cell wall structure in the *procuste 1* (*prc1*) mutant defective in *CELLULOSE SYNTHASE6* (*CESA6*) causes a more random distribution of root hair positions on trichoblasts, revealing a role for CESA6-dependent cell wall biosynthesis in planar polarity formation (Singh et al., 2008). The dependence of the

motility of CESA-containing Golgi and of CESA6 plasma membrane distribution on the actin cytoskeleton (Crowell et al., 2009; Gutierrez et al., 2009; Sampathkumar et al., 2013) indicates that CESA trafficking depends on the actin cytoskeleton, but as to whether an effect of actin on planar polarity occurs via modulation of cell wall organization remains to be determined. Additionally, the microtubule (MT) cytoskeleton is required for correct localisation of CESA6 at the plasma membrane (Crowell et al., 2009; Gutierrez et al., 2009). Again, how this may influence root hair positioning remains to be addressed, but antisense-induced downregulation of α-tubulin6 (TUA6) has previously been shown to affect the number of hair initiation sites formed from root hair cells, suggesting a requirement of tubulin function during site selection (Bao et al., 2001).

Together, these data reveal a role for membrane sterols, clathrin-mediated endocytosis and recycling, phosphoinositide-signalling, actin, tubulin and potentially cell wall morphology in planar polarity formation. Their mechanistic contributions and interactions, however, largely remain to be addressed.

Cellular read-out of an auxin concentration gradient

The earliest molecular polarity read-out that relies on the auxin concentration gradient during planar polarity formation is the polar accumulation of small Rho-of-plant GTPases (ROPs) at early hair initiation sites (Molendijk et al., 2001; Jones et al., 2002). ROPs are molecular switches belonging to the Rac/Rho-type family of small GTPases which are found in all eukaryotes (Craddock et al., 2012). In the GTP-bound active state, ROPs associate with membranes via their lipid anchors, whereas GDP-bound inactive ROPs localise to the cytosol. ROP activation commonly occurs in close association with membranes (Gu et al., 2003; Xu et al., 2010), and active ROPs in trichoblasts are early markers of the membrane sites where hairs will form (Molendijk et al., 2001; Jones et al., 2002). Despite the increasing knowledge about the molecular regulation of ROP activity in diverse model systems, very little is known about how ROPs are polarly recruited to and maintained at early hair initiation sites during planar polarity formation. While simple Turing-type models help to explain small GTPase-patch formation at random or earmarked sites in a single cell system, they are insufficient to describe and motivate the uniform placement of these patches close to basal ends of trichoblasts in the epidermal tissue context (Payne and Grierson, 2009). A more advanced mathematical model, which incorporates an apical-basal gradient of ROP activation/inactivation

as a factor to describe ROP patch positioning, at the single-cell level faithfully reproduces the root hair phenotypes observed in diverse hair positioning mutants (Payne and Grierson, 2009). This gradient in ROP activation/inactivation was found to be directionally biased by an auxin concentration gradient in the model, suggesting that cellular scale inhomogeneities in auxin distribution could act to direct polar localisation of ROPs (Payne and Grierson, 2009). This favors a spatially tight link between auxin perception and ROP activation, which assumably is envisaged in close proximity to activated ROPs at the plasma membrane. Indeed, although not analysed during planar polarity formation in the root epidermis, a membrane-associated complex including a likely auxin receptor, AUXIN BINDING PROTEIN1 (ABP1), and TRANSMEMBRANE KINASE1 (TMK1) has been described to bind auxin and activate ROPs in the *Arabidopsis* leaf epidermis (Xu et al., 2010; Xu et al., 2014). While these data generally support the above-mentioned model, its validity in the root tissue context *in planta* remains to be addressed.

The above-mentioned data suggest a function for ROP signalling mainly during downstream events of polar hair positioning. Indications that ROPs may be decisive in selection of the hair initiation site come from the observation that overexpression of wild type ROP2 or expression of a constitutively active (CA) ROP2 induced the formation of multiple hairs on single trichoblasts, which also appeared in apically shifted positions (Jones et al., 2002). A similar phenotype was observed in mutants of *SUPERCENTIPEDE1* (*SCN1*) (Parker et al., 2000), encoding for a RhoGDP dissociation inhibitor (Rho-GDI) (Carol et al., 2005), adding further support for a possible function of ROPs during the selection of hair initiation sites. Interestingly, *spike1* (*spk1*) mutants defective in a gene coding for a ROP-GEF revealed an altered subcellular distribution of PIN2 (Lin et al., 2012), but effects of *spk1* mutations on root hair initiation have not been reported. SPK1 is required for ROP6 activation in protoplasts and both *SPK1* as well as *ROP6* are required for the auxin-dependent inhibition of BFA-induced PIN2 internalisation in the root epidermis. This suggests that ROP signalling downstream of SPK1 regulates endocytic trafficking of auxin transporters (Lin et al., 2012). This could potentially be achieved via ROP-INTERACTIVE CRIB-MOTIVE CONTAINING PROTEINs (RICs), which modulate the actin and tubulin cytoskeleton during diverse developmental processes (Fu et al., 2005; Lin et al., 2012). Accordingly, expression of CA-ROP2 or a dominant negative (DN) construct of ROP2 altered actin cytoskeleton organisation in developing hairs (Jones et al., 2002), supporting a role for ROPs in modulating actin dynamics in the root

epidermis as well. While these data suggest a role for ROP signalling prior to hair site selection, genetic loss-of-function data for ROPs or ROP activator (e.g. Rho-GEF function) function in planar polarity is still lacking.

Functions of ROP and actin in directional cell expansion and other components involved in polar membrane domain organisation

Tip-growing root hairs and pollen tubes, as well as the interdigitating growth of leaf epidermal cells, are model systems to study the function of ROPs and their interaction with the cytoskeleton during spatially restricted cell expansion (Rounds and Bezanilla, 2013; Nakamura et al., 2012). While root hair tip growth and leaf epidermal cell morphogenesis offer the possibility to study directional cell expansion in the context of tissue development, which includes phytohormone signalling via ethylene and auxin (Schiefelbein, 2000; Nakamura et al., 2012), pollen tubes are a more easily accessible model system to investigate the molecular components acting during polar growth (Rounds and Bezanilla, 2013). Hence, studies on pollen tube growth have provided substantial mechanistic insight into the regulation of and the events occurring at a single polar membrane domain. Many parallels exist between root hair and pollen tube tip growth as well as organisation and the knowledge accumulated in either system might contribute to a better understanding of the other. Combination of the research on polar domain organisation and planar polarity formation will ultimately lead to a better understanding of how cell polarity is established in the tissue context.

The pollen tube model system

Tip growth in pollen tubes shares many parallels with tip growth in root hairs. Pollen tubes are fast-growing cells that extend directionally towards the ovary along a gradient of chemoattractants (Palanivelu et al., 2003). Similar to root hair tip growth, cell expansion in pollen tubes occurs via polarised oscillatory growth at the apex (Hepler et al., 2001), including oscillations in ROP GTPase activity as well as intracellular Ca^{2+} fluxes, filamentaous actin (F-actin) dynamics and reactive oxygen species (ROS) production (Hwang et al., 2005; Messerli et al., 2000; Fu et al., 2001; Lassig et al., 2014; Kaya et al., 2014). The cell apex of tip growing cells is characterised by the polar accumulation of specific plasma membrane

proteins, as well as a cytoplasmic region enriched in Golgi stacks, endoplasmic reticulum (ERs) and vesicles, generally refered to as the 'clear zone' (Figure 7a) (Rounds and Bezanilla, 2013). Behind this clear zone, in the subapical region of the tip, actin filaments are rather loosely organised in highly dynamic networks, thought to be required for the delivery of vesicular cargo to the apex in a process called reverse-fountain streaming (Figure 7b). This streaming further depends on long-distance transport of vesicles mediated by thick F-actin bundles in the shank, where nucleus and vacuole localise.

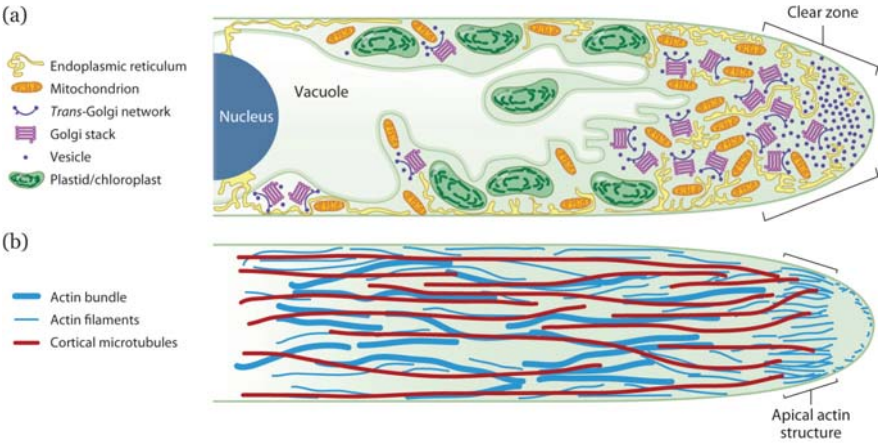

Figure 7: Organellar and cytoskeletal organisation of a tip-growing cell. (a) Distribution of organelles in a typical tip-growing cell. Note that chloroplasts are absent in developing root hairs. (b) Cytoskeletal organisation in a tip-growing cell. Image adapted from Rounds and Bezanilla, 2013 with permission of the publisher.

At the core of polar cell expansion – controlling ROP activity

Pollen tube tip growth requires ROP1 (Kost et al., 1999; Li et al., 1999), the oscillatory activation of which, at the cell apex, precedes and polarly directs oscillatory growth (Hwang et al., 2005). By activating the two downstream components RIC3 and RIC4, ROP1 signalling modulates a tip-focused Ca^{2+} concentration gradient and the assembly of tip-localised filamentaous actin (F-actin), respectively (Gu et al., 2005). While RIC4 overexpression induces a dense network of F-actin at the apex, RIC3 overexpression causes the loss of fine F-actin in this region, potentially by inducing Ca^{2+}-activated actin disassembly factors (Kovar et al., 2000; Huang et al., 2004). The observation that simultaneous overexpression of the two RICs neutralises the growth defects detected in single RIC overexpressing lines suggests that a check and balance mechanism between the two

RIC pathways acting to control tip-localised actin dynamics is required for polar growth (Gu et al., 2005).

The activity of small GTPases can be modulated by GTPase-activating proteins (GAPs) and GEFs. ROP ENHANCER1 (REN1), a RhoGAP, is delivered to the plasma membrane via exocytic vesicles (Hwang et al., 2008). Interestingly, exocytosis at the apex depends on F-actin (Lee et al., 2008), opening the possibility that actin may be involved in the regulation of ROP activity. Similarly, apical membrane localisation of overexpressed POLLEN RECEPTOR KINASE2 (PRK2), a transmembrane protein required for the polar recruitment of ROP1-activating RopGEF12, is negatively modulated by actin polymerisation (Zhao et al., 2013), further suggesting a role for actin in the control of ROP1 activity. Since both actin filament-stabilising as well as actin filament-destabilising drugs disrupt the transport of secretory vesicles (Zhang et al., 2010b), the precise role of F-actin at the apex remains unclear. Further complexity to the role of actin is derived from the observation that ARA6- and ARA7-labelled endosomes respond differentially to interference with actin cytoskeleton organisation during pollen tube growth (Zhang et al., 2010b). Thus, actin may regulate the delivery of cargo to the plasma membrane in different ways, depending on the nature of the vesicle. Although the mechanisms remain elusive, it is possible that actin may contribute to a feedback modulation of ROP1 activity at the apex, potentially including both positive and negative regulatory functions.

Transforming ROP signalling into polar cell expansion

To facilitate cell expansion directionally determined by ROP1, the cell wall has to be continuously modified, which requires the continuous delivery of membrane and cell wall materials to the apex (McKenna et al., 2009). This exocytic delivery presumably depends on the octameric tethering complex called the exocyst. Mutants defective in the exocyst subunits SEC5, SEC6, SEC8 and SEC15A are defective in polar pollen tube growth and investigation of SEC6 and SEC8 in tobacco pollen tubes revealed their localisation to be at the apex (Hala et al., 2008). Among the cell wall materials secreted at the tip are pectins, including homogalacturonan (HG). While HG is exocytosed in the methoxylated form, it becomes gradually demethoxylated by PECTIN METHYL ESTERASE (PME) (Winship et al., 2010), which in tobacco pollen tubes was shown to localise to secretory vesicles that depend on apical F-actin for exocytic targeting (McKenna et al., 2009; Wang et al., 2013b). Once demethoxylated, HG can bind Ca^{2+}, which promotes cross-linkage and stiffening of the

gel-like pectin composition (Vincent and Williams, 2009). Since methoxylated HG is continuously secreted at the tip, this mechanism could facilitate further extension of the pollen tube in this region, due to a more loosely organised cell wall.

Besides Ca^{2+}, ROS are modulators of cell wall organisation and are required for pollen tube growth (Schopfer, 2001; Potocky et al., 2007). One ROS that is thought to be required for loosening of the cell wall is the hydroxyl radical (Cosgrove, 2005). It can be formed by copper ions or peroxidases that use hydrogen peroxide produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases. All plant NADPH oxidases, commonly refered to as RESPIRATORY BURST OXIDATIVE HOMOLOG (RBOH) proteins, possess two Ca^{2+}-binding EF hand motifs in the N-terminal intracellular domain, six transmembrane helices and a flavin adenine dinucleotide (FAD)/NADPH-binding C-terminal intracellular domain (Kaya et al., 2014). Mutants of pollen-specific *RBOHH* and *RBOHJ* display reduced ROS production and defects in pollen tube growth (Lassig et al., 2014; Kaya et al., 2014). Interestingly, it appears that RBOHH and RBOHJ are required to provide negative feedback during tip growth in order to dampen the growth oscillations (Lassig et al., 2014). The observation that the cell walls of *rbohh;rbohj* tubes often ruptured and the tubes burst after low amplitude/high frequency growth phases, which show the highest rates of exocytosis and growth, suggest that ROS are required to arrest growth upon phases of increased exocytosis, when the deposition of new cell wall components temporarily soften the cell wall and make it unstable (Lassig et al., 2014). This idea is further supported by the observation that ANXUR1 (ANX1)-overexpressing pollen tubes, which over-activate exocytosis and over-accumulate secreted cell wall materials, do not burst, but arrest growth and form invaginations of the plasma membrane, most likely due to the excess amount of membrane material (Boisson-Dernier et al., 2013). This growth arrest requires the function of RBOHH and RBOHJ, revealing their downstream function in ANX1-mediated signalling (Boisson-Dernier et al., 2013). ANX1 and its close homolog ANX2 are receptor-like kinases (RLK) and are homologous to FERONIA, which is required for ROS production via RopGEF4 and RopGEF10 during root hair growth (Duan et al., 2010; Huang et al., 2013). While such a direct connection with ROP activation has not been described for ANX1 or ANX2, yet, it is conceivable that ROS and ROP signalling mutually regulate each other to control pollen tube growth. Another example revealing a direct genetic link between ROS and ROP signalling during root hair formation is provided by the interaction of *RHD2*

and *SCN1* (Carol et al., 2005). *RHD2* encodes RBOHC which is preferentially expressed in the root epidermis (Foreman et al., 2003), whereas *SCN1* encodes a Rho-GDI which is required for spatial restriction of root hair formation (Carol et al., 2005). The *rhd2* mutant displays reduced root hair length and *scn1* mutant trichoblasts often form multiple bulges (Foreman et al., 2003; Carol et al., 2005). In the double mutant, however, the formation of multiple bulges is suppressed, revealing epistasy of *RHD2* over *SCN1* (Carol et al., 2005).

Control mechanisms that link ROS and ROP signalling could potentially involve Ca^{2+} distribution between cytoplasm and apoplast, since RIC3 signalling as well as RBOHH and RBOHJ activity depend on Ca^{2+} (Gu et al., 2005; Kaya et al., 2014) and the availability of Ca^{2+} in the apoplast regulates the cross-linkage of cell wall components such as HG (Vincent and Williams, 2009). The communication between ROS and Ca^{2+} may act via ROS-dependent activation of Ca^{2+}-permeable transporters such as ANNEXIN1, which is required for hydroxyl radical mediated Ca^{2+} transport in the root (Foreman et al., 2003; Laohavisit et al., 2012). In support of this, *rbohh;rbohj* mutants were found to be defective in Ca^{2+} homeostasis during pollen tube growth (Boisson-Dernier et al., 2013).

The function of lipids in polar membrane domain organisation

Interestingly, not only the organisation of the cytoplasm and the cell wall are important for tip growth, but also the membrane composition at the apical domain. Labelling membrane sterols using filipin or employing the lipid-order probe di-4-ANEPPDHQ revealed that sterol-enriched microdomains polarise to the tip of pollen tubes in *Picea meyeri* and to the tip of elongating root hairs in *Arabidopsis* (Liu et al., 2009; Ovecka et al., 2010). By complexing with sterols, filipin interferes with membrane organisation (Papanikolaou et al., 2005). This is likely the cause for the decrease in root hair tip growth in filipin-treated *Arabidopsis* seedlings (Ovecka et al., 2010), or the dissipation of a tip-localised Ca^{2+} gradient and arrest of growth in filipin-treated pollen tubes (Liu et al., 2009). Mechanistically, the Ca^{2+} gradient dissipation in might be due to reduced ROS signalling. This idea is supported by the fact that ROS regulate plant cell expansion through the activation of Ca^{2+} channels in the root (Foreman et al., 2003) and that filipin application inhibits superoxide production by RBOH *in vitro* and furthermore interferes with RBOH localisation in pollen tubes. The observation that RBOH localisation is sensitive to filipin is consistent with the enrichment of RBOHs in

detergent insoluble membranes (DIMs) (Mongrand et al., 2004), which are biochemically isolated, sterol-, sphingolipid- and specific phospholipid-enriched membrane fractions resistant to solubilisation by non-ionic detergents.

Besides sterols, phospholipids including phosphatidic acid (PA) and PtdIns(4,5)P_2 are involved in the organisation of the polar membrane domain. PIP5K4, PIP5K5 and PIP5K6 are three PIP5K homologs that are highly expressed in pollen and *Arabidopsis pip5k4* mutants are defective in pollen tube growth and polarity (Sousa et al., 2008). *In vitro*, the *pip5k4* phenotypes are rescued by application of PtdIns(4,5)P_2, suggesting that PIP5K4 modulates pollen tube growth via its PtdIns(4,5)P_2-biosynthetic activity. Interestingly, while in tobacco pollen tubes transiently expressed PIP5K4 localised to the subapical membrane region, overexpressed PIP5K4 mislocalised to the very apex, concomitant with a perturbation of pollen tube growth, thus providing evidence that spatially restricted phospholipid signalling, depending on a proper balance of different phosphoinositides, is important during polar cell expansion. This is supported by the observation that PIP5K6 overexpression-induced plasma membrane abnormalities are not only suppressed by co-overexpression of PHOSPHOLIPASE C2 (PLC2), which decreases PtdIns(4,5)P_2 levels, but also by co-overexpression of PHOSPHATIDYLINOSITOL 4-KINASE β1 (PI4Kβ1), which increases the PtdIns(4)P levels (Zhao et al., 2010). The differential localisation of different phospholipids in the pollen tube tip further supports a spatially specified function of certain phospholipid subspecies in pollen tube growth. While PtdIns(4,5)P_2 is stably enriched at the apical membrane (Kost et al., 1999; Helling et al., 2006), PA is only observed in the apical domain when growth is arrested, whereas it localises to the subapical plasma membrane region in growing tobacco pollen tubes (Potocky et al., 2014). One possible mechanism by which phospholipids may modulate tip growth is via activation of RBOHs. In addition to being activated by Ca^{2+} and sterols, RBOH activity purified from tobacco pollen tubes was found to be positively regulated by PA and PtdIns(4,5)P_2, but not by phosphatidyl monophosphates, diacylglycerol (DAG), phosphatidylcholine, or phosphatidylethanolamine (Potocky et al., 2012). This further suggests that different subspecies of phospholipids at the pollen tube membrane can differentially modulate RBOH activity, depending on their spatiotemporal distribution. Another possible mechanism by which phospholipids may contribute to tip growth is via modulation of endocytic trafficking or the actin cytoskeleton. Mutant alleles of *PIP5K4* and *PIP5K6* revealed defects in endocytosis (Sousa et al.,

2008; Zhao et al., 2010), which for the latter were suggested to be due to interference with clathrin-mediated endocytosis.

Taken together, studies using pollen tube tip growth as a model system have revealed several components involved in the organisation of a polar domain and highlighted the importance of mutual control between the components and the pathways they act through. Considering the conserved function of homologues and the mechanistic parallels shared with root hair tip growth, this knowledge may contribute to a better understanding of polar domain generation and organisation also in the root epidermis.

Failure to maintain a single polar membrane domain

A peculiar phenotype observed in some root hair defective mutants, is that the hairs form branches or the trichoblasts initiate multiple hairs. This suggests that the genes and the mechanisms which they modulate are required for the organisation of a polar domain, or that they are required for the suppression of second-site domain formation. Similar to the situation in pollen tubes, phospholipids have been shown to be required for directional cell expansion in root hairs, since mutants of *PIP5K3* or *ROOT HAIR DEFECTIVE4* (*RHD4*), encoding a phosphatidylinositol-4-phosphate phosphatase, display shorter hairs (Kusano et al., 2008; Thole et al., 2008). Interestingly, mutants defective in *CAN OF WORMS1* (*COW1*), a phosphatidylinositol transfer protein required for normal root hair growth, form multiple branched hairs (Grierson et al., 1997; Yoo et al., 2012), furthermore suggesting that phosphoinositide distribution in the hair is important for the maintenance and spatial restriction of a polar domain. Potentially, this involves the function of phospholipids in regulation of endocytic trafficking. Mutants of *ARF-GAP DOMAIN1* (*AGD1*), which encodes a class I ARF-GAP required for the polar targeting of ROP2 to the apical plasma membrane in growing root hairs, show similar defects in root hair growth (Vernoud et al., 2003; Yoo et al., 2012). ARF-GAPs interact with ARF GTPases to modulate membrane trafficking (Chavrier and Goud, 1999). Consistent with this idea, transiently expressed fluorescent fusions of AGD1 co-localise with the endocytic tracer FM4-64 in tobacco pollen tubes (Yoo et al., 2008). AGD1 contains a pleckstrin homology (PH) domain, which mediates the association of proteins with phosphoinositides of biological membranes (Lemmon et al., 2002) and was shown to bind to PtdIns(3)P, PtdIns(4)P and PtdIns(5) in protein-lipid binding assays (Yoo et al., 2012). Furthermore, *RHD4* mutation behaved epistatic to mutation of *AGD1* (Yoo et al., 2012;

Yoo and Blancaflor, 2013), supporting the idea that phospholipids modulate polar domain organisation via regulation of endocytic trafficking.

Another component involved in polar domain maintenance is the ARMADILLO REPEAT-CONTAINING KINESIN1 (ARK1), revealed by the branching phenotype of root hair growth in *ark1* mutants (Yang et al., 2007; Sakai et al., 2008; Yoo et al., 2008). Since active ROP GTPases are the part of the growth machinery which predicts the direction of growth, the branching of *ark1* root hairs is most likely due to the failure to spatially restrict ROP2 to the apical domain in the mutant (Yang et al., 2007; Yoo and Blancaflor, 2013). While the mechanism by which ARK1 modulates polar ROP2 localisation is unknown, one possibility for this may be through an interaction with the cytoskeleton. Similar mutant phenotypes as observed for *ark1* mutants are detected in root hairs of seedlings treated with the MT-destabilising drug oryzalin (Bibikova et al., 1999) and *ark1* mutants display an increase in endoplasmic MTs as well as protrusion of actin bundles into the apical region of the root hair (Sakai et al., 2008; Yoo et al., 2008). Furthermore, when transiently expressed in tobacco leaf epidermal cells, ARK1 localisation to filamentous structures is disrupted upon oryzalin treatment (Yoo and Blancaflor, 2013), together suggesting that ARK1 can bind to and modulate MTs. Interestingly, a fragment of ARK1 containing the ARM-domain binds F-actin *in vitro*, and *ark1* mutants are hypersensitive to treatment with the actin filament destabilising drug latrunculin B (LatB) (Yang et al., 2007). Consistently, actin is required for the polar localisation of RABA4b (Preuss et al., 2004), which is mislocalised in *ark1* mutants (Yoo and Blancaflor, 2013), supporting the idea that ARK1 acts in polar domain organisation via control of the MT and of the actin cytoskeleton.

The actin cytoskeleton – an evolutionarily conserved structure and its interactors.

Actin is evolutionarily conserved in all eukaryotes and forms part of the cytoskeleton. It serves a multitude of cellular functions, which include the establishment and maintenance of cell shape and polarity, tip growth, cell division and organisation of the cytoplasm by mediating cytoplasmic streaming and organellar movement and positioning (Hussey et al., 2006). Furthermore the actin cytoskeleton is involved in the control of exo- and endocytosis (Hussey et al., 2006; Henty-Ridilla et al., 2013). To match these diverse cellular demands, the actin cytoskeleton needs to be able to dynamically reshape its

organisation. Thus, it requires a high degree of structural flexibility, which preconditions a subset of biochemical properties of actin itself as well as of its interactors.

Monomeric globular actin (G-actin) is an ATPase that can polymerise into filaments, called F-actin for filamentous actin. These filaments are highly dynamic polar structures that display a left-handed helical organisation. One full repeat of the helix requires 13 subunits, is 72 nm long and has a thickness of up to 9 nm (Holmes et al., 1990). Addition of G-actin subunits in their ATP-bound state occurs preferentially at the plus (barbed) end, while disassembly of actin monomers upon hydrolysis of ATP into ADP and phosphate occurs preferentially at the minus (pointed) end, in a process termed treadmilling. The equilibrium between polymerisation and depolymerisation is highly dependent on pH, salt concentration and the presence of ATP in the cell (Coluccio and Tilney, 1983; Wang et al., 1989) and under physiological conditions is in favour of polymerisation (Henty-Ridilla et al., 2013). While treadmilling was long thought to be the main mechanism regulating actin filament turnover, filament internal severing events were recently highlighted to be required for sufficient F-actin dynamics (Staiger et al., 2009). This regulatory mechanism, where bigger fragments of filaments can be cut off and reannealed to existing filaments, is termed stochastic dynamics (Staiger et al., 2009). The reorganisation of the actin cytoskeleton via treadmilling and stochastic dynamics mechanisms is orchestrated and tightly controlled by actin-binding proteins (ABPs) (Blanchoin et al., 2010). They can act to nucleate, crosslink, bundle, sever or cap filaments. In the following chapters their molecular and developmental functions, especially with respect to root hair tip growth, will be described. First, however, the actin gene family of *Arabidopsis* will be introduced.

Actin in Arabidopsis thaliana

In *Arabidopsis* actin is encoded by ten genes, of which eight are expressed and two are pseudogenes (McDowell et al., 1996b; McKinney and Meagher, 1998). Based on phylogeny and preferential expression pattern the eight expressed genes can be simplistically divided into a vegetative and a reproductive class of actins. Among the three vegetative isoforms, ACT7 is most abundant in the root apical meristem and young root tissue, while ACT2 and ACT8 are expressed mainly during later stages of root development (McDowell et al., 1996a; Kandasamy et al., 2009). Consistently, *act7* mutants show a more severe root growth defect with strongly altered root

architecture and shorter roots, whereas *act2* and *act8* mutants show stronger defects in root hair elongation (Kandasamy et al., 2009). Interestingly, ACT2 and ACT8 complement not only the root hair growth defects of *act2* mutants, but also the root growth defects of *act7* mutants when expressed from the ACT2 promoter. ACT7, expressed from the same regulatory sequence, only complements the root growth phenotype of *act7*, but not the hair elongation phenotype of *act2* mutants. Thus, despite being 90% identical at the protein level, ACT2 and ACT7 have evolved biochemical properties that specify their activity *in planta*. This most likely includes interaction specificities with ABPs. Consistent with this idea, class-specific interactions between actins and ABPs are thought to be essential for the regulation of plant growth and development (Kandasamy et al., 2007). To better understand these specific interactions, research describing the activity and developmental function of ABPs is of high interest.

Actin nucleation

The *de novo* formation of an actin filament is an energetically unfavorable process and requires the activity of actin-nucleating factors to overcome the initial hurdle (Pollard and Borisy, 2003). The ARP2/3 complex, which consists of seven subunits including ACTIN-RELATED PROTEIN2 (ARP2) and ARP3, posseses actin filament-nucleating activity (Rodal et al., 2005). In the activated state, the subunits ARP2 and ARP3 mimic the surface of an actin filament, allowing the addition of G-actin to generate a filament. Beyond initiating novel filaments, ARP2/3 can branch filaments by initiating a new filament on the side of an existing one (Higgs and Pollard, 2001) and is thereby involved in the generation of fine actin networks. Interestingly, ARP3 localises to the apical membrane during root hair tip growth in maize (Van Gestel et al., 2003) and *Arabidopsis* mutants of the smallest ARP2/3 subunit ARPC5 frequently form multiple hairs on one trichoblast (Mathur et al., 2003b). This suggests that ARP2/3 is involved in the establishment of a polar membrane domain during root hair site selection. The root hair growth defects in single ARP2/3 complex mutants are only moderate (Mathur et al., 2003a; Mathur et al., 2003b), but a mutant defective in ARP2 and CYCLASE-ASSOCIATED PROTEIN1 (CAP1), which catalyses the ADP to ATP exchange in actin (Chaudhry et al., 2007), reveals an additive effect of the mutations in the process (Deeks et al., 2007). Thus suggesting a role of ARP2/3 during root hair tip growth, presumably by modifying actin filament dynamics. The activity of

ARP2/3 in plants is likely to be regulated by a complex containing Wiskott-Aldrich syndrome protein (WASP) family verprolin homologous (WAVE) and SUPPRESSOR OF cAMP RECEPTOR (SCAR) (Szymanski, 2005), referred to as WAVE/SCAR regulatory complex (W/SRC). *Arabidopsis* SCAR2 interacts with ARPC3 in yeast and activates the actin-polymerising activity of ARP2/3 *in vitro* (Basu et al., 2005; Uhrig et al., 2007). Furthermore the trichome growth defects in *scar2* and *scar2;scar4* resemble those in *arp2/3*, supporting their interaction *in planta* (Hülskamp et al., 1994; Le et al., 2003; El-Din El-Assal et al., 2004; Uhrig et al., 2007). Interestingly, subunits of W/SRC interact with SPK1, potentially linking actin organisation to sites of active ROP signalling (Uhrig et al., 2007).

Actin filaments can further be nucleated by FORMIN HOMOLOGY (FH) proteins, which are present as a large family in *Arabidopsis* (Yi et al., 2005; Cheung et al., 2010). Class 1 FHs often contain a transmembrane domain in the N-terminal part, while class 2 are more variable and sometimes contain a phosphatase and tensin homologue (PTEN) domain in the N-terminal part which can bind to PtdIns(4,5)P_2 (van Gisbergen and Bezanilla, 2013). Interestingly, a fluorescent fusion of FH5, which belongs to the class 1 FHs, localises to the apex of growing pollen tubes, with a slight enrichment at the apical flank and promotes actin nucleation and accumulation of vesicles in the apical dome (Cheung et al., 2010). The increase in tube growth in fluorescent FH5-fusion expressing lines was concomitant with an increase in dense subapical actin filament network, thus implicating the importance of the actin cytoskeleton and its regulation by FH5 during tip growth.

Another FH thought to nucleate filaments during polar cell expansion is FH8, also belonging to the class I FHs (Yi et al., 2005). Overexpression of FH8 induces the formation of branches on growing root hairs, more explicitly suggesting that FH8 is important for the maintenance of a polar domain during tip growth (Yi et al., 2005). This may require an actin-nucleating activity of FH8. While a soluble FH8 fragment in *in vitro* studies mainly displayed a filament capping activity when incubated with actin alone, it strongly promoted filament nucleation and elongation when PROFILIN (PFN) was present (Yi et al., 2005). PFNs are highly abundant in cells and bind G-actin to form a 1:1 complex, thereby acting to prevent spontaneous filament nucleation and polymerisation and allowing higher concentrations of G-actin (Valenta et al., 1993; Kovar et al., 2000; Ketelaar, 2013). Considering the role of FH8 in polar cell expansion and the direct binding of FHs such as *Arabidopsis* FH8 to PFNs (Yi et

al., 2005; Deeks et al., 2005) it can be assumed that PFNs are involved in root hair tip growth as well. Consistently, overexpression of PFN1 in *Arabidopsis* leads to longer root hairs (Ramachandran et al., 2000), while loss of PFN function in the moss *Physcomitrella patens* leads to a complete loss of tip growth of protonemal cells (Vidali et al., 2007). Furthermore, PFNs localise to the bulge and apex of emerging root hairs in maize and maize PFN5 binds to tip-enriched phospholipids such as PtdIns(4,5)P_2 *in vitro* (Braun et al., 1999; Baluska et al., 2000; Kovar et al., 2001). Together these data support the idea that PFNs modulate actin filament dynamics during polar cell expansion, potentially via an actin-nucleating function together with FHs.

Actin filament depolymerisation

The most prominent filament-destabilising proteins belong to the family of ACTIN-DEPOLYMERISING FACTORs (ADFs), which act to sever filaments internally and enhance the depolymerisation from minus ends (Maciver and Hussey, 2002). It has proven difficult to investigate the function of ADFs in plant development, likely due to the large number of ADF isoforms of which the *Arabidopsis thaliana* genome harbours eleven (Kandasamy et al., 2007). However, maize ADF3 was found to be regulated via a Ca^{2+}-DEPENDENT PROTEIN KINASE (CDPK) (Smertenko et al., 1998; Allwood et al., 2001), suggesting that ADF activity is linked to Ca^{2+} signalling. Furthermore, ADF3 binds to and is inhibited by PtdIns(4,5)P_2 (Gungabissoon et al., 1998). Together with its regulation via Ca^{2+}, it constitutes a potential link between control of actin organisation and signalling at polar membrane domains such as the pollen tube or the root hair tip, where PtdIns(4,5)P_2 and Ca^{2+} gradients act to control tip growth. The shortening of root hairs and irregular actin organisation observed in *Arabidopsis* seedlings ectopically overexpressing ADF1 support this idea (Dong et al., 2001).

While ADFs can depolymerise filaments by themselves, *in vitro* studies on lily pollen-specific ADF1 and *in vivo* studies on moss ADF revealed a synergistic effect with ACTIN-INTERACTING PROTEIN1 (AIP1) (Allwood et al., 2002; Augustine et al., 2011). A homolog of AIP1 was first identified in yeast (Amberg et al., 1995), where it interacts physically as well as genetically with ADF and ACTIN and depolymerises filaments (Rodal et al., 1999). In moss, AIP1 promotes F-actin dynamics and requires ADF for this function (Augustine et al., 2011). Consistently, lily ADF1 displays only moderate filament depolymerising activity *in vitro*, while addition of *Arabidopsis* pollen-

specific AIP1-1 to the reaction synergistically enhanced depolymerisation (Allwood et al., 2002). In *Arabidopsis*, simultaneous knock-down of both AIP1 isoforms, the reproductive AIP1-1 and the vegetative AIP1-2, by RNA interference induces the assembly of filaments into bundles, which reach into the very apex of growing root hairs and it causes an overall reduction of root hair elongation rates (Ketelaar et al., 2004). Moreover, overexpression of AIP1-1 leads to the formation of thick actin bundles and swelling of root hairs (Ketelaar et al., 2007), supporting a role for one and/or the other AIP1 in the regulation of fine actin dynamics. While overexpression of a filament-destabilising protein is expected to decrease the amount of filamentous actin structures, the phenotypes induced by AIP1-1 overexpression in the root epidermis may be due to subclass-specific interactions between ABPs and actin. Consistent with this idea, the defects created in vegetative plant growth by ectopic expression of reproductive ACT1 are suppressed only by coexpression of reproductive ABPs such as PFN4 and ADF7 but not by the constitutively expressed PFN1 and ADF9 (Kandasamy et al., 2007). Hence, the precise role of AIP1 in vegetative plant development still needs to be investigated.

Since severing of filaments generates new plus ends where polymerisation can reinitiate, capping of these ends is important for the regulation of filament stability (Staiger et al., 2010). In *Arabidopsis*, the heterodimeric CAPPING PROTEIN (CP) fulfills this function, as revealed by the increase in filament elongation and filament-filament annealing on free filament ends in *cp* mutants (Huang et al., 2003; Li et al., 2012). Interestingly, the mutants display an increase in cortical fine actin, which is phenocopied by application of PA to the seedlings (Li et al., 2012). Considering the finding that, *in vitro*, CP binds to PtdIns(4,5)P_2 and PA and is inhibited by PA (Huang et al., 2003; Huang et al., 2006), CP is a prime candidate to mediate the translation from phospholipid signalling into actin cytoskeleton organisation.

A recently identified ABP with depolymerising function is MICROTUBULE-DESTABILISING PROTEIN25 (MDP25) (Qin et al., 2014). MDP25 binds directly to and severs filaments *in vitro*, and this activity is strongly enhanced by application of Ca^{2+}. Interestingly, the Ca^{2+}-binding ability is required for proper MDP25 localisation in the pollen tube tip and *mdp25* pollen tubes display an enhanced elongation (Qin et al., 2014). In addition, MDP25 binds to and destabilises cortical microtubules in elongating hypocotyl cells (Li et al., 2011). Etiolated hypocotyls of *mdp25* mutants are longer and overexpression of MDP25 produces the shortening of hypocotyl cells,

revealing a negative regulatory function during hypocotyl elongation which is likely due to the effect of MDP25 on microtubule organisation (Li et al., 2011). The microtubule-destabilising function of MDP25 is further suggested to depend on its dissociation from the plasma membrane to the cytosol and this relocalisation is induced by increased levels of Ca^{2+} (Ide et al., 2007; Li et al., 2011). Thus, it is likely that MDP25 links tip-localised Ca^{2+}-dependent signalling with actin and microtubule organisation. This may further include phospholipid signalling, since MDP25 has a phosphatidylinositol di- and triphosphate binding activity *in vitro*, which is negatively influenced by increasing concentrations of Ca^{2+} (Nagasaki et al., 2008).

Actin bundling

The actin cytoskeleton can adopt different shapes, depending on its subcellular localisation. In the shank of root hairs and pollen tubes, ABPs like VILLINs (VLNs) assemble filaments into thick bundles (Zhang et al., 2010a; Zhang et al., 2011). Mutation in *VLN4* interferes with bundle formation and cytoplasmic streaming in root hairs, concomitantly with the formation of short root hairs, revealing its function in polar cell expansion (Zhang et al., 2011). Interestingly, besides binding to and bundling filaments *in vitro*, VLN4 also severs and destabilises actin filaments in a Ca^{2+}-dependent manner, suggesting a dual effect of VLNs in actin regulation. The increase in fine F-actin abundance and stability in the apex of pollen tubes where VLN2 and VLN5 are downregulated support an actin-destabilising activity of VLNs *in vivo* (Qu et al., 2013). Furthermore, the observation that the activity of all *Arabidopsis* VLNs except VLN1 are modifiable by Ca^{2+}, suggests that the activity of VLNs *in planta* is generally regulated via Ca^{2+}-dependent signalling (REF). Considering the requirement for VLNs in tip growth, they could potentially couple actin cytoskeleton organisation to Ca^{2+} gradients occurring in the apex of tip-growing cells.

Actin filament tracking

The actin cytoskeleton serves as a track for the movement of organelles including Golgi and secretory vesicles. Their transport is facilitated by MYOSIN (MYO) motor proteins, which in *Arabidopsis* are present in a large copy number (Peremyslov et al., 2008; Reddy and Day, 2001). Disruption of several *MYOs* interferes with organelle trafficking and cytoplasmic streaming and in root hairs leads to a

reduced growth rate (Peremyslov et al., 2008; Prokhnevsky et al., 2008; Ojangu et al., 2007). Moreover, multiple *myo* mutants display an aberrant organisation of actin bundles in midvein epidermal cells and elongating root hairs (Peremyslov et al., 2010), suggesting that MYOs can affect polar tip growth not only by controlling organellar transport, but also by directly modifying the actin cytoskeleton. The activity of class XI MYOs, which are the most abundant in *Arabidopsis*, is modulated by Ca^{2+} (Tominaga et al., 2012). Together with the observation that in multiple *myo* mutants root hairs branch more frequently (Peremyslov et al., 2010), this suggests that MYOs are involved in polar tip growth not only via transport of cargo to the polar domain, but more directly in the maintenance of the polar domain via organisation of the actin cytoskeleton and that this activity may be regulated by Ca^{2+} signalling.

Actin and microtubule cytoskeleton interactions

Highly dynamic processes such as polar tip growth depend on fine actin networks and rapid actin filament turnover. Furthermore, the MT cytoskeleton is also involved in polar cell expansion, for instance in planar morphogenesis of leaf pavement cells as well as in the polar tip growth of pollen tubes and root hairs (Nakamura et al., 2012; Rounds and Bezanilla, 2013). Visualisation of F-actin and MT organisation in mutants defective in one or the other cytoskeletal component and co-visualisation of the two further suggest that the two cytoskeletal components are mechanistically linked and may sometimes share a functional pathway (Schwab et al., 2003; Saedler et al., 2004; Sampathkumar et al., 2011; Rosero et al., 2013).

While interference with MT and F-actin stability during later stages of root hair growth have different effects on hair development (Bibikova et al., 1999), both are required for initiation of root hair growth and reorganise at hair initiation sites during early stages (Takahashi et al., 2003; Baluska et al., 2000; Van Bruaene et al., 2004). This indicates that they are involved in root hair initiation and that they may potentially interact. In hypocotyl cells, F-actin requires MTs for reorganisation and motility, and the two cytoskeletal components are thought to interact during dynamic cytoskeleton restructuring (Sampathkumar et al., 2011). It may be assumed that this could also be the case for polar root hair positioning, but remains to be determined. Common targets for MT and F-actin during planar polarity in the root epidermis are CESAs. CESAs require both MTs and F-actin for their correct localisation at the membrane (Gutierrez et al., 2009; Sampathkumar et al., 2013) and mutants defective in

CESA6, such as *prc1*, display a defect not only in cell wall structure, but also in planar polarity of root hair and ROP positioning (Singh et al., 2008). Together, these data suggest that an interaction between the F-actin and the MT cytoskeleton in the root epidermis may be important for spatially controlled CESA activity contributing to robust planar polarity formation.

Actin and microtubule cytoskeletal rearrangements are important already at earlier stages of root epidermal cell differentiation and there is evidence suggesting their interaction during cytokinesis in the root meristem, where they together orchestrate the organised division of cells. The first apparent cytoskeletal reorganisation during cell division is the formation of a preprophase band (PPB), which is an MT belt surrounding the nucleus and which is formed by the selective depolymerisation of surrounding cortical MTs (Dhonukshe and Gadella, 2003). Once the MT PPB has formed, F-actin assembles around it in an MT-dependent manner (Vanstraelen et al., 2006) and presumably acts to further narrow the PPB into a region referred to as the cortical division zone (CDZ) (Eleftheriou and Palevitz, 1992; McMichael and Bednarek, 2013). The PPB, by marking the cortical division site (CDS), predicts cell division plane orientation (Pickett-Heaps and Northcote, 1966). While the position of the CDS in symmetrically dividing cells, such as root epidermal cells, is determined by cell geometry, its position can be altered by external stress (Besson and Dumais, 2011; Lintilhac and Vesecky, 1981). Interestingly, actin has been suggested to be required for stress-induced reorientation of the cell division plane (Rasmussen et al., 2013), suggesting that actin is involved also in earlier stages of PPB formation. Consistent with a function in CDS determination, mutants defective in *ACT7*, the main actin in young roots (Kandasamy et al., 2009), display defects in cell division plane orientation (Gilliland et al., 2003), as do mutants defective in the MT interactor CYTOPLASMIC LINKER ASSOCIATED PROTEIN (CLASP) (Ambrose et al., 2007). A candidate to mediate an interaction between F-actin and MT during cell division is KINESIN CDKA;1-ASSOCIATED1 (KCA1) (Vanstraelen et al., 2006). Similar to actin, which is depleted from the central PPB region, also referred to as the actin-depleted zone (ADZ), KCA1 is lacking from the CDS. KCA1 is required for the movement of chloroplasts along actin filaments, suggesting an interaction with actin, and it has further been found to interact with KATANIN (KTN), an MT-severing protein required for the response of shoot meristematic cells to external stress (Uyttewaal et al., 2012). It is hence conceivable that KTN and KCA1 act to modulate and coordinate the actin and microtubule cytoskeleton

during cell division, providing a potential link between the two components.

Since F-actin and MT cytoskeleton function are tightly controlled and intimately linked with each other, it is likely that they share a subset of common interactors. These components may act to modify the cell shape and organisation during fundamental developmental processes such as cell division and cell or tissue polarity formation. Research aiming at identifying common pathways and potential common interactors is therefore of high interest.

Aim of the study

The main aim of this study was to characterise functions of the actin cytoskeleton during establishment of planar polarity in *Arabidopsis* and to analyse its potential interaction with AIP1-2 during this process. The study started off with analysing the roles of of ACT2, ACT7 and ACT8, their interaction with AIP1-2 when expressed in yeast and during polar root hair positioning. It was subsequently focused on ACT7, when it became clear this is the main actin required during this process. The study further aimed at elucidating the expression pattern and subcellular localisation of AIP1-2 during planar polarity formation, which led us to investigate its expression and/or function in relation to CTR1 and WER-mediated patterning. With the upcoming isolation of additional players in planar polarity formation throughout the project, the relationship between the subcellular localisation of the actin and the microtubule cytoskeleton and SABRE was investigated during cell division orientation and planar polarity formation. Moreover, the contribution of the actin cytoskeleton to planar polarity establishment and its function in the formation of a polar membrane domain, specified by local accumulation of ROPs, DRPs, PIP5K3, D6PK and sterols, was investigated.

Results and Discussion

The analysis of root hair positioning in *Arabidopsis* provides a robust and powerful tool to characterise components involved in planar polarity establishment. It has facilitated the formulation of a genetic framework which includes the core components and mechanisms involved in planar polarity formation in plants identified to date (Figure 6). The upstream events, leading to the alignment of a polar axis in the tissue layer via the establishment of an auxin concentration gradient, are rather well understood. However, relatively little is known about how single cells contained within the tissue layer perceive polarity cues and reorganise along this polar axis. This reorganisation is likely to involve the actin cytoskeleton and indeed *ACT2* is required for planar polarity in the *Arabidopsis* root (Ringli et al., 2002). How this requirement relates to the other components of planar polarity formation, however, remains unanswered.

The actin cytoskeleton is required for planar polarity downstream of *CTR1*

CTR1 is one of the components of the ethylene signalling pathway that acts upstream in the genetic framework of planar polarity. Downregulation of CTR1 function leads to an over-activation of the planar polarity pathway and a hyper-polar positioning of root hairs at the very basal ends of cells. Since the planar polarity pathway is over-activated in *ctr1* knock down mutants, mutant alleles defective in positive modulators that act downstream in the process are expected to partially suppress the hyper-polar hair positioning of *ctr1* mutants. On the other hand *CTR1* is expected to behave epistatically over negative modulators of planar polarity. Hence, multiple mutant analyses including *ctr1* alleles provide a useful method to characterise planar polarity modulators.

ACT7 and *ACT2 are required for planar polarity of root hair positioning downstream of CTR1*

In order to investigate the function of the actin cytoskeleton during planar polarity establishment, we obtained mutant alleles of *ACT2*, *ACT7* and *ACT8*, which are three actin isoforms that are highly expressed in the young root (An et al., 1996; McDowell et al., 1996a). In *act2-3* we observed a slight apical shift of root hair position

confirming a function of *ACT2* in the process (Ringli et al., 2002), while hairs were shifted both apically and basally in *act7* alleles (Manuscript (MS) I, Figure 1A-C,G and supplementary Figure 1A-B), revealing an even stronger contribution of *ACT7* to the process. Mutants defective in *ACT8* did not display aberrant root hair positioning (MS I, supplementary Figure 1D), suggesting that ACT8 is not primarily required for planar polarity formation. Compared to either of the single mutants, the *act2-3;act7-6* double mutant displayed a stronger defect in polar hair positioning with a wide spreading of hair initiation sites along the outer lateral membrane, thereby revealing a synergistic effect of *ACT2* and *ACT7* (MS I, Figure 1B-D,H). Both mutation of *ACT2* and *ACT7* partially suppressed the hyper-polar positioning of $ctr1^{btk}$ (MS I, supplementary Figure 1E-F). Furthermore, in the *act2-3;act7-6;*$ctr1^{btk}$ triple mutant, the $ctr1^{btk}$ phenotype was fully suppressed, revealing a function of the actin cytoskeleton downstream of *CTR1*. $ctr1^{btk}$ mutants display increased auxin biosynthesis in the root tip (Ikeda et al., 2009) and auxin has been reported to induce *ACT7* expression (Kandasamy et al., 2001). Hence, the interaction between ethylene signalling and the actin cytoskeleton may further depend on auxin biosynthesis, transport and signalling. We frequently observed localisation of actin to future hair initiation sites prior to hair outgrowth (MS I, Figure 1K and supplementary Figure 1G-J), consistent with the idea that actin could be involved in the cellular read-out of an auxin concentration gradient. Furthermore, this local abundance of the actin cytoskeleton suggested that dynamic actin reorganisation is important for polar root hair positioning, which may involve actin-binding proteins.

AIP1-2 interacts with ACTINs in yeast and in vitro

To identify actin-binding proteins with a potential role in polar root hair positioning, we had a commercial yeast two-hybrid screen conducted at Hybrigenics (France) using ACT7, the actin isoform with the major contribution to planar polarity, as a bait. This screen revealed AIP1-2 as the only interacting candidate. We confirmed the interaction in yeast using AIP1-2 as a bait as well as a prey in the LexA yeast two-hybrid system and further included the pollen-specific AIP1-1 and ACT1, as well as ACT2 and ACT8, known to be involved in root development. When used as either bait or prey, the two AIP1 homologues and ACT7 interacted with all actins tested and ACT7 further interacted with both AIP1-1 and AIP1-2 (MS I, Figure 2A and supplementary Figure 2A-B). Thus the results suggest that there is no specificity of interaction between any actin or AIP1 isoform in yeast.

However, since yeast is a eukaryotic organism and possesses an actin cytoskeleton itself, it is possible that other yeast actin-binding proteins may facilitate this interaction. This could potentially include COFILIN1, which is an interactor of ACT1 and AIP1 in yeast (Rodal et al., 1999).

To confirm and further investigate the interaction between AIP1-2 with ACT2 and ACT7, we conducted *in vitro* pull down assays using GST-AIP1-2 as a bait. GST-AIP1-2 precipitated actins from protein extracts obtained from a Col-0 root cell suspension culture (MS I, Figure 2B and supplementary Figure 2D). To specify whether GST-AIP1-2 could bind to ACT2 and ACT7, we expressed these two proteins as 6xHis-tagged fusion proteins in *Escherichia coli* and used them in pull down experiments. GST-AIP1-2 precipitated both actin isoforms from bacterial protein extracts (MS I, Figure 2C and supplementary Figure 2E-F), suggesting that AIP1-2 can directly interact with these two actins.

AIP1-2 genetically interacts with ACTINs

To investigate whether AIP1-2 function and the observed interaction between AIP1-2 and ACT7 in yeast and *in vitro* could be of relevance for *Arabidopsis* development and for planar polarity, we isolated T-DNA insertion mutant alleles of *AIP1-2* which we named *aip1.2-1* and *aip1.2-2* and crossed them with *act7* mutants. In *act7;aip1-2* double mutants we observed a strong reduction in the seed germination rate and a synthetic lethality (MS I, Figure 3C and supplementary Figure 3A), revealing a synergistic effect of the two mutations *in planta*. The interaction was not fully penetrant, however, allowing the further phenotypic characterisation of the small non-lethal part of the population displaying the weakest phenotype and hence the weakest genetic interaction. When compared with the respective single mutants or with the wild type, these plants showed alterations in shoot morphology with twisted stems and abnormally oriented siliques (MS I, Figure 3D-G and supplementary Figure 3B-F), further supporting the importance of the *AIP1-2* and *ACT7* interaction *in vivo*.

AIP1-2 is required for planar polarity formation

To investigate whether *AIP1-2* is required for planar polarity formation in the root epidermis, we analysed hair positions in the isolated mutant alleles. Both *aip1.2-1* and *aip1.2-2* displayed a basal shift of root hair positioning (MS I, Figure 4A-D and supplementary

Figure 4A), suggesting a negative contribution of AIP1-2 to the process. This phenotype in *aip1.2-1* was restored to wild-type levels by expression of different AIP1-2-fusions under control of genomic *AIP1-2* sequences (MS I, Figure 4A-D and supplementary Figure 4B). Thus showing that the basal shift in hair positioning was the result of reduced *AIP1-2* function.

Simultaneous knock-down of *AIP1-1* and *AIP1-2* using RNAi induces the stabilisation of actin filaments into bundles (Ketelaar et al., 2004) and mutation of *ACT2* leads to a destabilisation of actin filaments (Nishimura et al., 2003; Kandasamy et al., 2009). The more randomised hair positioning in *act7* mutants with a basal as well as an apical shift and the apical shift observed in *act2-3* may hence be explained by a destabilisation of actin filaments. Accordingly, the basal shift observed in *aip1-2* mutants could be explained by a stabilisation of filamentous actin structures. In root epidermal *aip1-2* cells, however, we did not observe an increase in actin bundling, which may be due to the weak phenotypic defects of the single mutants that do not allow to distinguish subtle differences in actin organisation.

To further investigate whether interaction between AIP1-2 and ACT2, ACT7 or ACT8 is relevant for planar polarity formation, we measured the hair positions in the respective double mutants. In *act7;aip1-2* seedlings where the genetic interaction was not fully penetrant, we observed epistasy of *act7* over *aip1-2* (MS I, Figure 3 and supplementary Figure 3). A similar result was observed in *act2-3;aip1.2-2* mutants (Figure 8A), together revealing a genetic interaction between *AIP1-2* and *ACT2* as well as *ACT7* during polar hair positioning. In *act8;aip1-2* mutants, we observed a hair positioning phenotype intermediate to the respective single mutant phenotypes, with hairs being slightly basally shifted compared to the *act8-2* single mutant but not as far shifted as in the respective *aip1-2* mutant (Figure 8B-C). Thus, together these results show that *AIP1-2* is a modulator of polar hair positioning, which genetically interacts with *ACT2*, *ACT7* and *ACT8* during the process. The epistasy of *act7* and *act2* over *aip1-2* further suggests that the corresponding interactions have a major function during planar polarity formation, consistent with the more pronounced effect of *ACT7* and *ACT2* mutations on polar hair positioning when compared to mutations of *ACT8*.

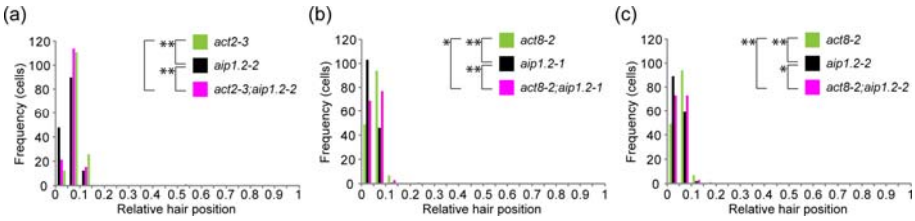

Figure 8: *AIP1-2* modulates planar polarity together with *ACT2* and *ACT8*. Quantitative analysis of relative root hair position of (a) *act2-3* and *aip1.2-2* compared with *act2-3;aip1.2-2*, (b) *act8-2* and *aip1.2-1* compared with *act8-2;aip1.2-1* and (c) *act8-2* and *aip1.2-2* compared with *act8-2;aip1.2-2*. Bars represent the number of cells (frequency) with relative hair positions in classes from 0 (basal most) to 1 (apical most). n = 150 cells from 30 roots per genotype. Significances *P* were determined by non-parametric, two-sample Kolmogorov-Smirnov test. (a) $^{**}P$ = 0.000 *act2-3* vs. *aip1.2-2*; *P* = 0.128 *act2-3* vs. *act2-3;aip1.2-2*; $^{**}P$ = 0.000 *aip1.2-2* vs. *act2-3;aip1.2-2*. (b) $^{**}P$ = 0.000 *act8-2* vs. *aip1.2-1*; $^{*}P$ = 0.019 *act8-2* vs. *act8-2;aip1.2-1*; $^{**}P$ = 0.000 *aip1.2-1* vs. *act8-2;aip1.2-1*. (c) $^{**}P$ = 0.000 *act8-2* vs. *aip1.2-2*; $^{**}P$ = 0.009 *act8-2* vs. *act8-2;aip1.2-2*; $^{*}P$ = 0.013 *aip1.2-2* vs. *act8-2;aip1.2-2*. For experimental procedures, see MS I.

To gain further insight into the cellular function of AIP1-2 we localised it using a functional AIP1-2-mCherry fusion. AIP1-2-mCherry was detected in the root epidermis and showed a patterned expression in root hair files from the elongation zone onwards (MS I, Figure 4F). At the sub-cellular level AIP1-2-mCherry localised to the cytosol, thereby being abundant at hair initiation sites prior to morphological hair outgrowth together with actin (MS I, Figure 4F-H, Figure 1K and supplementary Figure 1G-J). This cytosolic co-localisation in elongating and differentiating trichoblasts, together with the genetic data mentioned above, supports the idea that the interaction between AIP1-2 and actins observed in yeast, *in vitro* and *in planta* can principally also occur *in vivo* in the root epidermis. Potentially this interaction could act to modulate actin dynamics at the polar hair initiation site, thereby contributing to the positioning and generation of a polar membrane domain and/or to the physical execution of hair outgrowth downstream of ROP signalling at this domain.

ACT7 and AIP1-2 are required for planar polarity upstream of ROP positioning

To further investigate the role of the actin cytoskeleton during planar polarity formation, we localised ROPs at polar hair initiation sites in wild type, *act7-6*, *aip1.2-1* and *act7-6;aip1.2-1* mutants. Compared to the wild type the polar ROP positioning in *aip1.2-1* was

shifted basally (MS I, Figure 4A-B,E), while in *act7-6* ROP positions were shifted both basally and apically (MS I, Figure 4A,C,F). ROP positioning was significantly different in *act7-6;aip1.2-1* when compared to *aip1.2-1* but not when compared to *act7-6* (MS I, Figure 4B-D,G), revealing epistasy of *act7-6* over *aip1.2-1*. Together, these results show that actin and its interactor AIP1-2 are required for polar ROP positioning. Thus, while ROP signalling in diverse cellular processes has been shown to modify actin organisation via its effectors (Fu et al., 2005; Nagawa et al., 2012; Lin et al., 2012), our data reveal a function of the actin cytoskeleton upstream of ROPs in the establishment of tissue polarity. Interestingly, short-term treatment with F-actin-destablising drugs does not interfere with ROP positioning at hair initiation sites (Molendijk et al., 2001). The shifts in ROP positions observed in the actin cytoskeleton mutants are hence unlikely to be the result of a direct requirement of actin reorganisation in the polar membrane association of ROPs. Instead, the actin cytoskeleton may affect polar ROP localisation in an indirect manner. This could potentially include a function of actin reorganisation in polar auxin transport, or in the generation and maintenance of a polar membrane domain where ROP activity becomes enriched. In support of the first idea, treatment of seedlings with the actin filament-destabilising drug latrunculin B (LatB) affects the BFA-induced internalisation of AUX1 and PIN1 in the root (Geldner et al., 2001) and the subcellular distribution and polarity of AUX1 and PIN1 in protophloem cells (Kleine-Vehn et al., 2006). In support of the second idea, root hair tip growth, which depends on ROP signalling (Molendijk et al., 2001; Jones et al., 2002), requires a normal cellular distribution of sterols (Ovecka et al., 2010). Since endocytic trafficking of sterols in root epidermal cells is dependent on actin (Grebe et al., 2003), actin may modify tip growth and other ROP signalling dependent processes via control of sterol-dependent endocytosis. Further investigations on the contribution of the actin cytoskeleton to the establishment and maintenance of a polar membrane domain are part of the work contributing to MS III, which will be discussed below.

AIP1-2 expression is dependent on WER-patterning and ethylene signalling downstream of CTR1

The patterning of root hair fate in the *Arabidopsis* root is dependent on WER signalling (Lee and Schiefelbein, 1999). To investigate whether the trichoblast-specific expression of AIP1-2 also depends on this pathway, we analysed the localisation of AIP1-2-

mCherry in the *wer-1;aip1.2-1* background. AIP1-2-mCherry in these roots was detected in all epidermal files, those in hair as well as those in non-hair positions (MS I, Figure 6A-C). This result shows that the expression of AIP1-2 is spatially restricted by *WER* signalling, indicating a connection between fate patterning and planar polarity establishment. To investigate whether *WER* has a function in polar root hair positioning, potentially via transcriptional control of *AIP1-2*, we quantified hair positions in *wer-1* and compared it with wild type. We did not observe any effect on planar polarity formation in *wer-1*, neither in NH nor in RH files (Figure 10), suggesting that *WER* is not required for normal positioning of root hairs. However, we cannot exclude compensatory mechanisms acting to counteract *WER* loss of function. Future analyses including the *wer-1;myb23-1* double mutant, defective also in *MYB23* which can act in part redundantly with *WER* (Kang et al., 2009), may help to further address a potential role of the patterning pathway in planar polarity establishment.

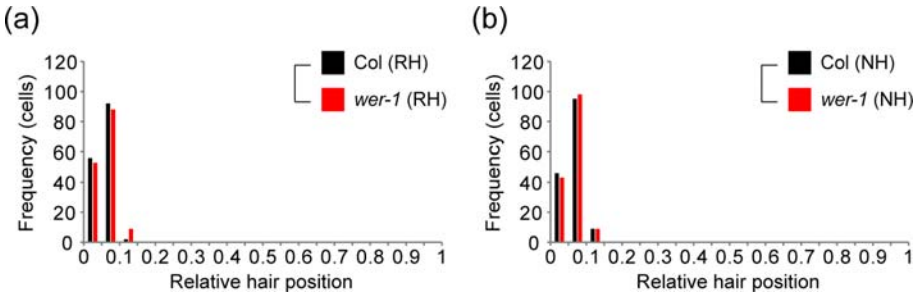

Figure 10: Root hair positioning in *wer-1*. Quantitative analysis of relative root hair position of Col and *wer-1* in (a) RH files and (b) NH files. n = 150 cells per genotype. Significances *P* were determined by non-parametric, two-sample Kolmogorov-Smirnov test. (a) *P* = 0.097 Col (RH) vs. *wer-1* (RH). (b) *P* = 0.882 Col (NH) vs. *wer-1* (NH).

CTR1-dependent ethylene signalling as well as auxin signalling affect root hair cell differentiation downstream of the fate patterning pathway and interference with ethylene or auxin levels leads to an increase in the formation hairs in NH positions, also referred to as ectopic hair positions (Dolan et al., 1994; Tanimoto et al., 1995; Masucci and Schiefelbein, 1996). We therefore investigated whether the two hormones may be involved in the control of AIP1-2 expression as well. In *ctr1^{btk};aip1.2-1* we observed an increase in ectopic AIP1-2-mCherry expression in NH cells forming a hair, which was also observed in *aip1.2-1* seedlings grown on medium supplemented with the ethylene biosynthetic precursor ACC (MS I; Figure 6C-E). These results show that the patterned AIP1-2

expression is dependent on *CTR1* function and that it is sensitive to changes in ethylene precursor concentration, which strongly suggests that ethylene signalling downstream of *CTR1* can modulate AIP1-2 expression. Since ethylene signalling induces the biosynthesis of auxin in the root tip, we next investigated whether alteration of auxin concentration can affect AIP1-2 expression. In *aip1.2-1* seedlings grown on medium supplemented with the synthetic auxins 2,4-Dichlorophenoxyacetic acid (2,4-D) or 1-Naphthaleneacetic acid (1-NAA), there was an increase in ectopic AIP1-2 expression, indicating that trichoblast-restricted AIP1-2 expression is sensitive to changes in auxin levels.

The strong defects in RH-restricted AIP1-2-mCherry enrichment in the *wer-1* background suggested that the WER patterning system is central to the control of patterned AIP1-2 expression. Hormonal signalling via the ethylene and auxin pathways may modulate further downstream events of WER signalling, thereby affecting the *WER*-dependent control of AIP1-2 expression. A potential target for this interference of auxin with the WER pathway is ROOT HAIR DEFECTIVE 6-LIKE4 (RSL4), a transcription factor acting downstream of WER and which is induced by auxin (Yi et al., 2010). However, AIP1-2 was not found to be transcriptionally upregulated by RSL4, suggesting that there are other components or mechanisms involved in the crosstalk between auxin and WER signalling.

CTR1 is epistatic over AIP1-2 during polar hair positioning

To further investigate the function of AIP1-2 during planar polarity formation, we compared the hair positioning in an *aip1.2-1;ctr1^{btk}* double mutant with the ones in the respective single mutants. While the hair positioning phenotype was significantly different between *aip1.2-1* and *aip1.2-1;ctr1^{btk}*, we did not observe any significant difference between *ctr1^{btk}* and *aip1.2-1;ctr1^{btk}*, neither in hair positioning nor in any other assessed phenotypes (MS I, Figure 7). Thus, the results revealed epistasy of *CTR1* over *AIP1-2* during early root devlopment, which may suggest that AIP1-2, a negative modulator of planar polarity, acts downstream of CTR1 function.

Together with the requirement of *ACT2* and *ACT7* downstream of *CTR1* and the interaction between the two actins and AIP1-2 during polar hair positioning, these data place functions of the actin cytoskeleton downstream of CTR1 in planar polarity formation. Furthermore, the altered ROP distribution in *act7* and *aip1-2* mutants place a function of the actin cytoskeleton upstream of polar ROP positioning. Together, these results provide the foundation for future

more detailed studies on the role of the actin cytoskeleton and the dynamic regulation of its organisation during planar polarity formation. In addition, the requirement of WER function for the control of cell file-restricted AIP1-2 expression provides a starting point for further investigations addressing the molecular crosstalk between pattern formation and planar polarity in more detail.

SABRE is required for planar polarity formation in the *Arabidopsis* root epidermis

During the course of this study on actin and AIP1-2 function, the *kreuz und quer* (*kuq*) [german for criss-cross] mutant was isolated in a forward genetic screen for mutants defective in root hair positioning in our laboratory and shown to be a mutant allele of the *SABRE* (*SAB*) gene (MS II, Figure 2). *SAB* encodes a 2607 amino acid protein with a predicted molecular weight of 290 kDa. Depending on the protein topology algorithm employed, SAB is furthermore predicted to contain one to three transmembrane domains. Despite the presence of sequence-related proteins in all eukaryotic organisms (a subset of which are displayed in MS II, Figure 4), so far no molecular function of SAB or any SAB-like protein has been described. Developmentally, however, *KINKY POLLEN* (*KIP*) and *ABERRANT POLLEN TRANSMISSION1* (*APT1*), which are homologs of *SAB* in *Arabidopsis* and maize, respectively, are required for normal pollen tube growth (Procissi et al., 2003; Xu and Dooner, 2006). In addition to being required for pollen tube tip growth, KIP is also required for root hair tip growth as *kip* mutants form shorter and thicker root hairs (Procissi et al., 2003). Similar defects in root hair elongation have been described in mutants defective in the actin cytoskeleton (Baluska et al., 2000; Ringli et al., 2002; Kandasamy et al., 2009). While the morphology of *sab* pollen tubes or root hairs does not differ from wild type, *sab* enhances the tip growth defects of *kip*, suggesting that *SAB* may also be involved in root hair elongation (Procissi et al., 2003).

After the isolation of the sab^{kuq} allele we quantified its hair positioning phenotype and further looked at the polar ROP localisation. In sab^{kuq}, both hair and ROP positions were shifted basally as well as apically (MS II, Figure 1A-G), revealing *SABRE* as a novel component of the planar polarity system acting upstream of polar ROP positioning.

The resemblance of the *sab* with the *clasp* mutant phenotype in trichome positioning (Kirik et al., 2007; MS II, Figure 1H-J) prompted the investigation of a potential interaction between the

respective genes. While we observed a synergistic effect of *SAB* and *CLASP* mutation in the branching of trichomes in the leaf epidermis (MS II, Figure 5A), we observed epistasy of *SAB* over *CLASP* during ROP positioning (MS II, Figure 5G,I-K) and cell division plane orientation (MS II, Figure 6A-Q). Interestingly, defects in cell division plane orientation have not only been described for mutants defective in MT organisation such as *clasp* (Ambrose et al., 2007) but also in *act7* mutants (Gilliland et al., 2003), supporting the idea that SAB may interact with MTs and/or F-actin.

Analysis of the MT cytoskeleton in *sab-5* revealed a role for *SAB* in MT organisation. In the *sab-5* mutant, cortical MT arrays oriented more randomly in early elongating trichoblasts and the reorientation from a transversal to a bipolar organisation in late elongating cells was less prominent compared to the wild type (MS II, Figure 7A-G, supplementary Figure 4A-C and supplementary Movie 5). In addition, a radial MT arrangement often observed at root hair initiation sites in wild type was absent in *sab-5* (MS II, Figure 7H-L), further suggesting a function of SAB in the reorganisation of MTs.

In five-day-old *sab-5;act7-6* seedlings growth was delayed and roots were much shorter and radially swollen compared to either of the parental mutant lines (Figure 10A-B in Pietra, 2014). The distribution of ROP positions displayed a basal as well as an apical shift and this phenotype was significantly different when compared with any of the single mutants (Figure 10C in Pietra, 2014). The results show that *ACT7* and *SAB* act together during planar polarity formation, further supporting the possible existence of a functional connection between SAB and the actin cytoskeleton.

SABRE co-localises with ACTIN and associates with MTs in root epidermal cells

To gain insight into the molecular role of SAB, we localised a functional C-terminal fusion of 3xYpet with SAB in *sab-5* mutant roots. Consistent with the prediction of one to three transmembrane domains in SAB, the co-localisation of SAB-3xYpet with the plasma membrane marker NPSN12-mCherry and the retraction of SAB-3xYpet from the cell wall upon plasmolysis (MS II, Figure 9C-E) suggested that SAB is an integral plasma membrane protein. In elongating cells about to form a bulge or just after bulge formation, SAB was present at hair initiation sites, although not specifically enriched (MS II, Figure 9V-W). Since the SAB-3xYpet signal in differentiating trichoblasts was very weak, we focused on analysing SAB localisation during earlier phases of epidermis development. We

observed an association of SAB-3xYpet with different MT structures labelled by mCherry-TUA5 in cells undergoing mitosis: In the mitotic prophase, SAB-3xYpet localised at the nuclear envelope together with MTs and furthermore closely associated with PPBs (MS II, Figure 9I). In metaphase and anaphase cells, patches of SAB-3xYpet associated with spindle MTs (MS II, Figure 9J-K). Interestingly, SAB-3xYpet not only associated with MTs during mitosis, but also with F-actin labelled by rhodamine phalloidin: In pro-, meta- and anaphase there was a high degree of overlap between SAB-3xYpet and F-actin at the 'actin cage', which is an accumulation of F-actin present along the cell periphery that clenches the mitotic cell (MS II, Figure 9O-Q). Furthermore, in late telophase and cytokinetic cells SAB-3xYpet and F-actin co-labelled at the centre of phragmoplasts and at forming cell plates, which were framed by MTs (MS II, Figure 9L-M,R-S). The co-labelling between SAB-3xYpet and F-actin at the apical and basal membrane of daughter cells also remained after cytokinesis was completed (MS II, Figure 9T).

While the intracellular co-association of SAB-3xYpet with MT structures could not be located to a specific subcellular compartment (MS II, Figure 9F-H), the prediction of one to three transmembrane domains and the co-localisation with F-actin at existing or forming plasma membranes suggest that SAB may be required for mediating an interaction between the cytoskeleton and the plasma membrane. However, further experiments that address the molecular function of SAB and localisation studies at the electron microscopic level will be required to address this in more detail.

Potential functions of SABRE during planar polarity formation

The phenotypic defects of *sab* mutants together with the co-localisation data suggest a molecular connection between SAB and both the MT as well as the F-actin cytoskeleton during diverse developmental processes. During planar polarity establishment, the defects of the *sab-5* mutant very much resembled those of *act7* mutants and *SAB* and *ACT7* acted together in polar ROP positioning. This may suggest that *SAB* genetically interacts closely with the actin cytoskeleton during planar polarity formation. Consistent with this idea, MT disorganisation was previously shown to be required for hair outgrowth (Van Bruaene et al., 2004), but besides *sab* and *clasp* no mutant defective in MT organisation has so far been reported to have defects in planar polarity of hair positioning. The radial reorganisation of MTs at hair initiation sites could potentially involve a function of SAB during hair formation downstream of the polar

positioning of the hair initiation site, including a role in cell expansion. To further investigate this, it remains interesting to analyse whether the defects in polar hair positioning in *sab* are related to defects in actin and/or MT cytoskeleton organisation, or possibly even related to a defect in the crosstalk between the two.

While we observed a high degree of co-localisation between SAB-3xYpet and F-actin in different stages of mitosis and the *act7* and *sab* hair position phenotypes are very reminiscent of each other, the *act7;sab* planar polarity phenotype so far does not allow to make a statement on how the respective genes or proteins relate to each other during this process except for that they somehow interact genetically. This may potentially be due to functional redundancy between *ACT7* and *ACT2*, which may partially mask the effect that loss of one actin isoform has on the process. Hence, it would be interesting to analyse the root hair or ROP positioning in an *act2;act7;sab* triple mutant. In addition, visualisation of F-actin in elongating *sab* trichoblasts could provide further insight into a mechanistic contribution of SAB to polar hair positioning.

Characterisation of actin cytoskeletal functions during the positioning and establishment of a polar membrane domain

Our results had shown that the precise polar placement of ROPs at the hair initiation sites requires a functional actin cytoskeleton, which contributes to planar polarity downstream of *CTR1*. We next aimed at gaining further insight into the establishment of planar polarity and how it is translated into a cell polarity. In the case of hair positioning, cell polarity is first manifested by the recruitment of ROPs to a polar membrane domain, which marks the future hair position. Other components known to localise at this membrane site are the PtdIns(4,5)P_2-biosynthetic enzyme PHOSPHATIDYLINOSITOL 4-PHOSPHATE 5-KINASE3 (PIP5K3) and membrane sterols (Kusano et al., 2008; Ovecka et al., 2010). However, little is known about how this polar membrane domain is positioned and which components and mechanisms are involved in the establishment or maintenance of it, including the role of the actin cytoskeleton.

Identification of polar membrane domain markers

To gain further insight into the organisation of the polar membrane domain marking the hair initiation site, we co-visualised membrane

sterols complexed by filipin with some candidate proteins likely to be involved in the organisation of the hair initiation site. By using an optimised protocol for the *in situ* visualisation of sterols in *Arabidopsis* roots (Boutte et al., 2011), we confirmed the enrichment of filipin-sterol complexes at hair initiation sites (Ovecka et al., 2010; MS III, Figure 1A,D,G,J,M). Furthermore, these filipin-sterol complexes co-localised with fluorescent ROP2-, ROP6- and PIP5K3-fusion proteins when these were expressed under control of their native promoters (MS III, Figure 1A-I). PIP5K activity has been suggested to contribute to polar cell expansion via modulation of clathrin-mediated endocytosis during pollen tube tip growth (Sousa et al., 2008; Zhao et al., 2010). Furthermore, function of the GNOM ARF GEF required for endocytic recycling (Steinmann et al., 1999; Geldner et al., 2001) and endocytosis (Naramoto et al., 2010) is required for planar polarity of root hair positioning (Fischer et al., 2006), which prompted us to further investigate a potential contribution of the endocytic machinery to the organisation of polar hair initiation sites. Dynamins are large GTPases that commonly act to tubulate and to fission membranes (Praefcke and McMahon, 2004). Thereby they mediate endocytosis of clathrin-coated vesicles, as this has also been suggested for the DYNAMIN-RELATED PROTEINs (DRPs) DRP1A and DRP2B in *Arabidopsis* (Fujimoto et al., 2010). Fluorescent fusions of DRP1A and DRP2B expressed from their native promoters co-localised with filipin-sterol complexes at polar membrane domains (MS III, Figure 1J-P), supporting the idea that clathrin-mediated endocytosis is involved in the organisation of this site.

Sterols, DRPs and PIP5K3 are required for planar polarity of a polar membrane domain

In order to investigate a potential contribution of the components detected at the early hair initiation site to its polar positioning, we analysed the positions of ROPs and root hairs in seedlings defective in cellular sterol distribution or defective in genes coding for hair initiation site-enriched proteins. *CYCLOPROPYLSTEROL ISOMERASE1* (*CPI1*) codes for an enzyme required for normal sterol biosynthesis and its mutation leads to an altered cellular sterol composition (Men et al., 2008). In the *cpi1-1* mutant we observed a basal as well as an apical shift in ROP and root hair positioning (MS III, Figure 2A-D, supplementary Figure 2A-E,G), while pharmacological interference with sterol biosynthesis via supplementation of sterol-biosynthesis inhibitors fenpropimorph or

lovastatin to the growth medium induced only an apical shift (MS III, Figure 2E-I, supplementary Figure 2I-M). Together, these data highlighted the requirement of a normal sterol composition for the positioning of a polar membrane domain during hair initiation.

In *drp1a* mutants, we observed a basal as well as an apical shift in ROP and root hair positioning (MS III, Figure 2J-L, supplementary Figure 3A-G), while *drp2b* and *pip5k3* mutants displayed only a basal shift (MS III, Figure 2M-P, supplementary Figure 3H-T). Together, these results revealed a role for *DRP1A*, *DRP2B* and *PIP5K3* in planar polarity formation.

Strikingly, these analyses showed that the hair initiation site markers do not only localise to the polar membrane domain, but that they furthermore are required for its coordinate polar positioning underlying planar polarity.

Since the positioning of the markers is already polarly organised, it is conceivable (1) that they have independent functions in the establishment of planar polarity and at the hair initiation site, or (2) that the proteo-lipid composition of the hair initiation site contributes to the polar positioning of it. A possible role in planar polarity establishment which is independent of the function at the hair initiation site could include a contribution of the polar markers to polar auxin transport and the generation of an auxin concentration gradient. Consistent with this idea PIN2 mispolarises in post-cytokinetic cells in the *cpi1-1* mutant (Men et al., 2008) and the subcellular distribution and polar localisation of AUX1 and PIN1 is altered in a mutant defective in the sterol biosynthetic enzyme STEROL METHYLTRANSFERASE1 (SMT1) (Willemsem et al., 2003; Kleine-Vehn et al., 2006). In support of the second hypothesis, the *cpi1-1* and *drp1a* mutants display a basal and an apical shift in ROP positioning. This suggests that their contribution to planar polarity is not linear, as would be expected for a component that acts on the auxin concentration gradient. The proposed hypotheses are not the only possible, however, and the contribution of the different hair initiation site markers to the polar positioning of this domain may be more complex. To further investigate this, it would be interesting to see whether *drp1a* and *cpi1* mutants, which both display basal and apical shifts in hair site positioning, show defects in the organisation of the polar membrane domain which are distinguishable from wild type or *drp2b* and *pip5k3* mutants.

D6PK is a polar membrane domain marker and is required for planar polarity

While our study had identified the contributions of several endocytosis components as well as sterols and phospholipid signalling components to the polar placement of the hair initiation site "microdomain" at the plasma membrane, we had not yet identified signalling components that might rely on such domain organisation. The AGCVIII kinase D6 protein kinase (D6PK) has been reported to localise to the basal membrane in root epidermal cells and to phosphorylate several PIN proteins *in vitro* and *in vivo* (Zourelidou et al., 2009; Willige et al., 2013; Barbosa et al., 2014). Strikingly, we observed that YFP-D6PK localisation switches from basal to hair-initiation-site polarity late during epidermal cell elongation both when expressed from the 35S-promoter or as a functional fusion expressed under control of its endogenous promoter in its mutant background ((MS III, Figure 3A-C)). These findings revealed a signalling component involved in root hair initiation. We therefore investigated whether D6PK does actually function in planar polarity establishment. In a D6PK overexpressing line both ROP and hair positioning displayed an apical shift (MS III, Figure 3D-E,H,K-L,N), while quadruple mutants with reduced activity of D6PK and its three closest homologues revealed basal shifts compared to wild type (MS III, Figure 3D,F,J-K,M-N). These results revealed a regulatory contribution of D6PK to planar polarity, suggesting that it can act upstream of polar ROP positioning. Furthermore it may be assumed that D6PK acts downstream of GNOM function, since subcellular D6PK localisation at basal plasma membranes is highly sensitive to BFA treatments (Barbosa et al., 2014). Interestingly, the switch in subcellular localisation of YFP-D6PK from basal to hair-initiation-site polarity occurred relatively late, when compared to the early localisation of ROPs at hair initiation sites. Yet, modification of D6PK function did alter polar ROP positioning. There are at least two scenarios how this may be explained. Either D6PK, when still localised at the basal plasma membrane, has an early function that contributes to polar ROP localisation and differs from a direct function at the hair initiation site, or when D6PK becomes localised to the hair initiation site both ROP and D6PK become interdependent on each other for the maintenaince of the polar site in the right position. At present, we cannot distinguish between these two scenarios and they may also coexist next to each other.

D6PK polarity is dependent on sterols, PIP5K3 and DRP1A

We next investigated whether D6PK localisation at hair initiation sites depends on sterols, *PIP5K3* and *DRP1A*. YFP-D6PK co-localised with filipin-sterol complexes at hair initiation sites (MS III, Figure 4A-C). Strikingly, however, shortly after addition of filipin to the analysed seedlings, YFP-D6PK fluorescence intensity decreased (MS III, Figure 4D-F). This decrease was most strongly pronounced at hair initiation sites, suggesting that a normal sterol distribution is required for D6PK localisation at this domain. Consistently, YFP-D6PK was less enriched at hair initiation sites in the *cpi1-1* mutant when compared to wild type (MS III, Figure 4G-H), and lovastatin or fenpropimorph induced a loss or decrease of YFP-D6PK enrichment at hair initiation sites, respectively (MS III, Figure 4I-K). Furthermore, the hair initiation site-specific enrichment of YFP-D6PK was lost in *drp1a* and *pip5k3* seedlings (MS III, Figure 4L-M), showing that the localisation of YFP-D6PK at this domain required *DRP1A* and *PIP5K3* function as well. This suggests that endocytosis involving DRP1A and phosphoinositide signalling involving PIP5K3 contribute to YFP-D6PK polarity at hair initiation sites.

D6PK polarity is dependent on actin

To specify the function of the actin cytoskeleton during planar polarity formation, we investigated whether actin is required for the polar positioning of D6PK at hair initiation sites. In the *act7-6* mutant, YFP-D6PK often displayed an equal distribution along the outer lateral membrane (MS III, Figure 5A-B), revealing a role for *ACT7* in planar polarity of D6PK positioning. Furthermore, treatment with the actin cytoskeleton destabilising drug latrunculin B depolarised the YFP-D6PK distribution in bulging trichoblasts, as well as in trichoblasts prior to bulging (MS III, Figure 5C-D). This further revealed a role of the actin cytoskeleton in the establishment and/or maintenance of a polar membrane domain during hair initiation.

Our results suggest that the actin cytoskeleton may have two functionally distinct roles in planar polarity formation. On the one hand, it is required for the polar positioning of ROPs. Since *act2* mutants induce only an apical shift and *aip1-2* mutants only a basal shift in hair site positioning (MS I, Figure 1G and Figure 4D), the actin cytoskeleton is likely to have a linear contribution to the process which acts upstream of ROP polarity. On the other hand, actin is required for the organisation of the polar membrane domain. The

apical as well as basal shifting of ROP positions in *act7-6* (MS I, Figure 5F) and the partial loss of D6PK polarity in seedlings with an altered actin cytoskeleton (MS III, Figure 5B,D), suggest that the actin cytoskeleton also has a non-linear contribution to planar polarity. Since polar positioning of ROPs at hair initiation sites is insensitive to short-term treatments with actin-destabilising drugs and ROPs still localised to distinct and spatially restricted patches in *act* mutants (Molendijk et al., 2001; MS I, Figure 5C-D), the contribution of actin to polar domain organisation is likely to be downstream of ROPs during hair initiation. Consistent with this notion, ROPs have been suggested to act upstream of actin to modulate endocytic trafficking of membrane proteins in the root epidermis (Lin et al., 2012). This would further imply that actin is actively reorganised at hair initiation sites. Consistent with this idea, filamentous actin networks are enriched in bulges during early hair initiation together with actin-binding proteins (Baluska et al., 2000) and we often observed a dense actin network at hair initiation sites prior to bulge formation (MS I, Figure 1K and supplementary Figure 1G-J).

Membrane sterols and actin localise at polar membrane domains independently of each other

The effect of *ACT7* mutation on the distribution of filipin-sterol complexes was analysed to further investigate the contribution of actin to the organisation of the hair initiation site. While the position of filipin-sterol-enriched membrane domains was often apically shifted in *act7-6* compared to wild type, the complexes still localised at these domains in a spatially restricted manner (MS III, Figure 5E-H). This suggests that actin is required for planar polarity of hair initiation site positioning, but not for the spatial restriction of sterols to this domain. Vice versa, in *cpi1-1* mutant trichoblasts the actin cytoskeleton was present at hair initiation sites, also when these occurred at an apically shifted position (MS III, Figure 5P-Q). Hence, these results suggest that a reorganisation of actin at hair initiation sites does not require the presence of a specific sterol distribution.

Together, these results indicate that the actin cytoskeleton and membrane sterols have overlapping functions during planar polarity formation that may be partially independent from each other. However, further experiments aimed at unravelling the processes that contribute to the positioning and establishment of the polar hair initiation site in more mechanistic detail are required.

To gain further insight into the role of sterols and the actin cytoskeleton and how the two structures relate to each other during

planar polarity formation, it would be interesting to analyse ROP and root hair positioning in an *act7;cpi1* double mutant. Furthermore, it would be interesting to investigate not only the position of polar domains along the outer lateral membrane, but also their size. This may reveal minor defects in the organisation of these domains, which may not be as striking as the defects in YFP-D6PK polarity observed in *act7-6* or *cpi1-1*. For instance, root hair initiation sites in *act7-6* often appeared slightly broadened compared to wild type. This suggests that ACT7 may be involved in the spatial restriction of other components than D6PK at this site and that this could potentially include ROP and PIP5K activity, clathrin-dependent endocytic trafficking and sterol distribution.

Conclusions and Perspectives

Over a decade ago, a role of *ACT2* in polar hair positioning was described (Ringli et al., 2002), suggesting the requirement of a functional actin cytoskeleton for planar polarity formation. However, so far it remained unclear what the function of actin during the process is. Here, we show that the actin cytoskeleton is required for the establishment of planar polarity downstream of CTR1-dependent ethylene signalling and that it contributes both to the positioning as well as to the maintenance of a polar membrane domain at hair initiation sites.

Interference with the actin cytoskeleton in an *act2;act7;ctr1* mutant fully suppressed the *ctr1* root hair positioning phenotype, revealing a function of the actin cytoskeleton downstream of *CTR1*. Since ethylene signalling downstream of CTR1 positively regulates auxin biosynthesis in the root tip (Ikeda et al., 2009) and *ACT7* is induced by auxin (Kandasamy et al., 2001), it is likely that actin has functions downstream of auxin signalling in planar polarity formation. However, it is also possible that interference with the actin cytoskeleton affects the establishment of an auxin concentration gradient in the root. Hence, further more detailed studies investigating the relationship between auxin signalling, auxin transport and the actin cytoskeleton are required.

We identify AIP1-2, which together with its homolog AIP1-1 is involved in actin cytoskeleton organisation (Ketelaar et al., 2004), as an interactor of actin and a negative modulator of planar polarity formation. The dependence of *AIP1-2* on *CTR1* and on balanced ethylene levels reveals a molecular link between ethylene signalling and actin reorganisation. While ethylene has so far mainly been implicated in the control of microtubule dynamics (Steen and Chadwick, 1981; Lang et al., 1982), these results open up for future studies aimed at investigating a mechanistic link between ethylene signalling and control of actin cytoskeletal dynamics. Furthermore, our data show the dependence of *AIP1-2* expression on *WER* function, revealing a connection between the cell fate patterning pathway and planar polarity formation. While a role of actin and AIP1 in planar polarity formation has also been described in *Drosophila* and hence appears to be evolutionary conserved (Ren et al., 2007), our data reveal that expression of planar polarity modulators is under control of a cell fate patterning system in *Arabidopsis*. Our findings thus pave the way for further investigations on the functional

importance of control of *AIP1* and actin organisation via a cell fate specification system.

We furthermore identify *SAB* as a component acting in the planar polarity of root hair positioning. While the molecular function of SAB during cell division or planar polarity formation still remains to be resolved, our data strongly support a role for SAB in the organisation of the cytoskeleton. While we failed to identify interactors of SAB in diverse yeast two-hybrid screens when using different SAB bait constructs (Pietra & Grebe, unpublished results), co-immunoprecipitation may prove more successful in elucidating the functions and interactions of SAB. Furthermore, it will be interesting to investigate the actin cytoskeleton organisation in *sab* mutants and to localise functional SAB fusions in an *act7* background. By providing all the required materials for these analyses, our work paves the way for future analyses on the function of SAB and the actin cytoskeleton during cell division and planar polarity formation.

The requirement of the actin cytoskeleton in the establishment and maintenance of a polar membrane domain has so far mainly been studied in 'unicellular' model systems, including yeast and elongating pollen tubes or root hairs (Dyer et al., 2013; Hussey et al., 2006). However, how such a polar domain is organised during the establishment of planar polarity and whether it contributes to the establishment of planar polarity itself has not been addressed to date. We further characterise the polar membrane domain which marks the hair initiation site and show that it is enriched in sterols, ROP2, ROP6, PIP5K3, DRP1A, DRP2B and D6PK. The observation that membrane sterols, PIP5K3, DRP1A, DRP2B and D6PK are involved in the positioning of the hair initiation site reveals a function of these potential downstream components in the establishment of planar polarity of root hair positioning. Whether this contribution is due to a dual function of the components during planar polarity formation or whether solely a specific proteo-lipid composition at the hair initiation site is required to control the polar positioning of the domain remains to be investigated. Nevertheless, our data shows that the signalling molecule D6PK strongly relies on correct sterol and phospholipid function at this site, suggesting that specific enrichment of sterol and phospholipids at this domain could be required for the recruitment of proteins involved in the maintenance of this polar site as well as in downstream promotion of hair outgrowth. It would hence be interesting to investigate whether some of the proteins involved in hair positioning and formation such as D6PK display binding specificities towards one or the other lipid component. Also, it would be interesting to investigate the organisation of the hair

initiation site in more microscopic detail employing super-resolution microscopy techniques. This could help to determine as to whether the components that localised at this site are uniformly distributed or whether they show a sub-domain specific localisation.

Taken together, our findings provide a deeper insight into the function of actin and its interactions during planar polarity formation in *Arabidopsis*. We identify novel components with distinct contributions to polar positioning of hair initiation sites and the organisation of these domains. Our results provide the base for further investigations on the mechanisms underlying planar polarity formation in more molecular detail.

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References

Ahringer, J. (2003). Control of cell polarity and mitotic spindle positioning in animal cells. *Curr. Opin. Cell Biol.* **15**, 73-81.

Allwood, E. G., Anthony, R. G., Smertenko, A. P., Reichelt, S., Drobak, B. K., Doonan, J. H., Weeds, A. G. and Hussey, P. J. (2002). Regulation of the pollen-specific actin-depolymerizing factor LlADF1. *Plant Cell* **14**, 2915-2927.

Allwood, E. G., Smertenko, A. P. and Hussey, P. J. (2001). Phosphorylation of plant actin-depolymerising factor by calmodulin-like domain protein kinase. *FEBS Lett.* **499**, 97-100.

Alonso, J. M., Hirayama, T., Roman, G., Nourizadeh, S. and Ecker, J. R. (1999). EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. *Science* **284**, 2148-2152.

Altschuler, S. J., Angenent, S. B., Wang, Y. and Wu, L. F. (2008). On the spontaneous emergence of cell polarity. *Nature* **454**, 886-889.

Amberg, D. C., Basart, E. and Botstein, D. (1995). Defining protein interactions with yeast actin in vivo. *Nat. Struct. Biol.* **2**, 28-35.

Ambrose, J. C., Shoji, T., Kotzer, A. M., Pighin, J. A. and Wasteneys, G. O. (2007). The *Arabidopsis CLASP* Gene Encodes a Microtubule-Associated Protein Involved in Cell Expansion and Division. *Plant Cell* **19**, 2763-2775.

An, Y. Q., McDowell, J. M., Huang, S., McKinney, E. C., Chambliss, S. and Meagher, R. B. (1996). Strong, constitutive expression of the Arabidopsis ACT2/ACT8 actin subclass in vegetative tissues. *Plant J.* **10**, 107-121.

Augustine, R. C., Pattavina, K. A., Tuzel, E., Vidali, L. and Bezanilla, M. (2011). Actin interacting protein1 and actin depolymerizing factor drive rapid actin dynamics in Physcomitrella patens. *Plant Cell* **23**, 3696-3710.

Baluska, F., Salaj, J., Mathur, J., Braun, M., Jasper, F., Samaj, J., Chua, N. H., Barlow, P. W. and Volkmann, D. (2000). Root hair formation: F-actin-dependent tip growth is initiated by local assembly of profilin-supported F-actin meshworks accumulated within expansin-enriched bulges. *Dev. Biol.* **227**, 618-632.

Bao, Y., Kost, B. and Chua, N. H. (2001). Reduced expression of alpha-tubulin genes in *Arabidopsis thaliana* specifically affects root growth and morphology, root hair development and root gravitropism. *Plant J.* **28**, 145-157.

Barbosa, I. C., Zourelidou, M., Willige, B. C., Weller, B. and Schwechheimer, C. (2014). D6 PROTEIN KINASE Activates Auxin Transport-Dependent Growth and PIN-FORMED Phosphorylation at the Plasma Membrane. *Dev. Cell* **29**, 674-685.

Basu, D., Le, J., El-Essal Sel, D., Huang, S., Zhang, C., Mallery, E. L., Koliantz, G., Staiger, C. J. and Szymanski, D. B. (2005). DISTORTED3/SCAR2 is a putative arabidopsis WAVE complex subunit that activates the Arp2/3 complex and is required for epidermal morphogenesis. *Plant Cell* **17**, 502-524.

Benfey, P. N. and Scheres, B. (2000). Root development. *Curr. Biol.* **10**, R813-815.

Berger, F., Haseloff, J., Schiefelbein, J. and Dolan, L. (1998). Positional information in root epidermis is defined during embryogenesis and acts in domains with strict boundaries. *Curr. Biol.* **8**, 421-430.

Bernhardt, C., Lee, M. M., Gonzalez, A., Zhang, F., Lloyd, A. and Schiefelbein, J. (2003). The bHLH genes GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) specify epidermal cell fate in the Arabidopsis root. *Development* **130**, 6431-6439.

Bernhardt, C., Zhao, M., Gonzalez, A., Lloyd, A. and Schiefelbein, J. (2005). The bHLH genes GL3 and EGL3 participate in an intercellular regulatory circuit that controls cell patterning in the Arabidopsis root epidermis. *Development* **132**, 291-298.

Besson, S. and Dumais, J. (2011). Universal rule for the symmetric division of plant cells. *Proc. Natl. Acad. Sci. USA* **108**, 6294-6299.

Bibikova, T. N., Blancaflor, E. B. and Gilroy, S. (1999). Microtubules regulate tip growth and orientation in root hairs of Arabidopsis thaliana. *Plant J.* **17**, 657-665.

Blanchoin, L., Boujemaa-Paterski, R., Henty, J. L., Khurana, P. and Staiger, C. J. (2010). Actin dynamics in plant cells: a team effort from multiple proteins orchestrates this very fast-paced game. *Curr. Opin. Plant Biol.* **13**, 714-723.

Boisson-Dernier, A., Lituiev, D. S., Nestorova, A., Franck, C. M., Thirugnanarajah, S. and Grossniklaus, U. (2013). ANXUR receptor-like kinases coordinate cell wall integrity with growth at the pollen tube tip via NADPH oxidases. *PLoS Biol.* **11**, e1001719.

Boutte, Y., Men, S. and Grebe, M. (2011). Fluorescent in situ visualization of sterols in Arabidopsis roots. *Nat. Prot.* **6**, 446-456.

Bramsiepe, J., Wester, K., Weinl, C., Roodbarkelari, F., Kasili, R., Larkin, J. C., Hulskamp, M. and Schnittger, A. (2010). Endoreplication controls cell fate maintenance. *PLoS Gen.* **6**, e1000996.

Braun, M., Baluska, F., von Witsch, M. and Menzel, D. (1999). Redistribution of actin, profilin and phosphatidylinositol-4, 5-bisphosphate in growing and maturing root hairs. *Planta* **209**, 435-443.

Brown, P. J., Kysela, D. T. and Brun, Y. V. (2011). Polarity and the diversity of growth mechanisms in bacteria. *Sem. Cell & Dev. Biol.* **22**, 790-798.

Bruex, A., Kainkaryam, R. M., Wieckowski, Y., Kang, Y. H., Bernhardt, C., Xia, Y., Zheng, X., Wang, J. Y., Lee, M. M., Benfey, P., et al. (2012). A gene regulatory network for root epidermis cell differentiation in Arabidopsis. *PLoS Gen.* **8**, e1002446.

Brunoud, G., Wells, D. M., Oliva, M., Larrieu, A., Mirabet, V., Burrow, A. H., Beeckman, T., Kepinski, S., Traas, J., Bennett, M. J., et al. (2012). A novel sensor to map auxin response and distribution at high spatio-temporal resolution. *Nature* **482**, 103-106.

Bryant, D. M. and Mostov, K. E. (2008). From cells to organs: building polarized tissue. *Nat. Rev. Mol. Cell Biol.* **9**, 887-901.

Carol, R. J., Takeda, S., Linstead, P., Durrant, M. C., Kakesova, H., Derbyshire, P., Drea, S., Zarsky, V. and Dolan, L. (2005). A RhoGDP dissociation inhibitor spatially regulates growth in root hair cells. *Nature* **438**, 1013-1016.

Chant, J. (1999). Cell polarity in yeast. *Ann. Rev. Cell Dev. Biol.* **15**, 365-391.

Chant, J. and Stowers, L. (1995). GTPase cascades choreographing cellular behavior: movement, morphogenesis, and more. *Cell* **81**, 1-4.

Chaudhry, F., Guerin, C., von Witsch, M., Blanchoin, L. and Staiger, C. J. (2007). Identification of Arabidopsis cyclase-associated protein 1 as the first nucleotide exchange factor for plant actin. *Mol. Biol. Cell* **18**, 3002-3014.

Chavrier, P. and Goud, B. (1999). The role of ARF and Rab GTPases in membrane transport. *Curr. Opin. Cell Biol.* **11**, 466-475.

Cheung, A. Y., Niroomand, S., Zou, Y. and Wu, H. M. (2010). A transmembrane formin nucleates subapical actin assembly and controls tip-focused growth in pollen tubes. *Proc. Natl. Acad. Sci. USA* **107**, 16390-16395.

Chin, M. L. and Mlodzik, M. (2013). The Drosophila selectin furrowed mediates intercellular planar cell polarity interactions via frizzled stabilization. *Dev. Cell* **26**, 455-468.
Coluccio, L. M. and Tilney, L. G. (1983). Under physiological conditions actin disassembles slowly from the nonpreferred end of an actin filament. *J. Cell Biol.* **97**, 1629-1634.
Cormack, R. G. H. (1962). Development of root hairs in angiosperms. II. *The Bot. Rev.* **28**, 446-464.
Cosgrove, D. J. (2005). Growth of the plant cell wall. *Nat. Rev. Mol. Cell Biol.* **6**, 850-861.
Cove, D. J. (2000). The generation and modification of cell polarity. *J. Exp. Bot.* **51**, 831-838.
Craddock, C., Lavagi, I. and Yang, Z. (2012). New insights into Rho signaling from plant ROP/Rac GTPases. *Trends Cell Biol.* **22**, 492-501.
Crowell, E. F., Bischoff, V., Desprez, T., Rolland, A., Stierhof, Y. D., Schumacher, K., Gonneau, M., Hofte, H. and Vernhettes, S. (2009). Pausing of Golgi bodies on microtubules regulates secretion of cellulose synthase complexes in Arabidopsis. *Plant Cell* **21**, 1141-1154.
Das, M., Drake, T., Wiley, D. J., Buchwald, P., Vavylonis, D. and Verde, F. (2012). Oscillatory dynamics of Cdc42 GTPase in the control of polarized growth. *Science* **337**, 239-243.
Deal, R. B. and Henikoff, S. (2010). A simple method for gene expression and chromatin profiling of individual cell types within a tissue. *Dev. Cell* **18**, 1030-1040.
Deeks, M. J., Cvrckova, F., Machesky, L. M., Mikitova, V., Ketelaar, T., Zarsky, V., Davies, B. and Hussey, P. J. (2005). Arabidopsis group Ie formins localize to specific cell membrane domains, interact with actin-binding proteins and cause defects in cell expansion upon aberrant expression. *New Phyt.* **168**, 529-540.
Deeks, M. J., Rodrigues, C., Dimmock, S., Ketelaar, T., Maciver, S. K., Malho, R. and Hussey, P. J. (2007). Arabidopsis CAP1 - a key regulator of actin organisation and development. *J. Cell Sci.* **120**, 2609-2618.
Dhonukshe, P. and Gadella, T. W., Jr. (2003). Alteration of microtubule dynamic instability during preprophase band formation revealed by yellow fluorescent protein-CLIP170 microtubule plus-end labeling. *Plant Cell* **15**, 597-611.
Dolan, L., Duckett, C. M., Grierson, C., Linstead, P., Schneider, K., Lawson, E., Dean, C., Poethig, S. and Roberts, K. (1994). Clonal relationships and cell patterning in the root epidermis of Arabidopsis. *Development* **120**, 2465-2474.
Dolan, L. and Scheres, B. (1998). Root pattern: shooting in the dark? *Sem. Cell & Dev. Biol.* **9**, 201-206.
Dong, C. H., Xia, G. X., Hong, Y., Ramachandran, S., Kost, B. and Chua, N. H. (2001). ADF proteins are involved in the control of flowering and regulate F-actin organization, cell expansion, and organ growth in Arabidopsis. *Plant Cell* **13**, 1333-1346.
Drubin, D. G. and Nelson, W. J. (1996). Origins of cell polarity. *Cell* **84**, 335-344.
Duan, Q., Kita, D., Li, C., Cheung, A. Y. and Wu, H. M. (2010). FERONIA receptor-like kinase regulates RHO GTPase signaling of root hair development. *Proc. Natl. Acad. Sci. USA* **107**, 17821-17826.
Dyer, J. M., Savage, N. S., Jin, M., Zyla, T. R., Elston, T. C. and Lew, D. J. (2013). Tracking shallow chemical gradients by actin-driven wandering of the polarization site. *Curr. Biol.* **23**, 32-41.
El-Din El-Assal, S., Le, J., Basu, D., Mallery, E. L. and Szymanski, D. B. (2004). DISTORTED2 encodes an ARPC2 subunit of the putative Arabidopsis ARP2/3 complex. *Plant J.* **38**, 526-538.

Eleftheriou, E. P. and Palevitz, B. A. (1992). The effect of cytochalasin D on preprophase band organization in root tip cells of *Allium*. *J. Cell Science* **103**, 989-998.

Ezan, J. and Montcouquiol, M. (2013). Revisiting planar cell polarity in the inner ear. *Sem. Cell & Dev. Biol.* **24**, 499-506.

Fischer, U., Ikeda, Y. and Grebe, M. (2007). Planar polarity of root hair positioning in Arabidopsis. *Biochem. Soc. Trans.* **35**, 149-151.

Fischer, U., Ikeda, Y., Ljung, K., Serralbo, O., Singh, M., Heidstra, R., Palme, K., Scheres, B. and Grebe, M. (2006). Vectorial Information for *Arabidopsis* Planar Polarity Is Mediated by Combined *AUX1*, *EIN2*, and *GNOM* Activity. *Curr. Biol.* **16**, 2143-2149.

Foreman, J., Demidchik, V., Bothwell, J. H., Mylona, P., Miedema, H., Torres, M. A., Linstead, P., Costa, S., Brownlee, C., Jones, J. D., et al. (2003). Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature* **422**, 442-446.

Fu, Y., Gu, Y., Zheng, Z., Wasteneys, G. and Yang, Z. (2005). Arabidopsis interdigitating cell growth requires two antagonistic pathways with opposing action on cell morphogenesis. *Cell* **120**, 687-700.

Fu, Y., Wu, G. and Yang, Z. (2001). Rop GTPase-dependent dynamics of tip-localized F-actin controls tip growth in pollen tubes. *J. Cell Biol.* **152**, 1019-1032.

Fujimoto, M., Arimura, S., Ueda, T., Takanashi, H., Hayashi, Y., Nakano, A. and Tsutsumi, N. (2010). Arabidopsis dynamin-related proteins DRP2B and DRP1A participate together in clathrin-coated vesicle formation during endocytosis. *Proc. Natl. Acad. Sci. USA* **107**, 6094-6099.

Galway, M. E., Masucci, J. D., Lloyd, A. M., Walbot, V., Davis, R. W. and Schiefelbein, J. W. (1994). The TTG gene is required to specify epidermal cell fate and cell patterning in the Arabidopsis root. *Dev. Biol.* **166**, 740-754.

Geldner, N., Anders, N., Wolters, H., Keicher, J., Kornberger, W., Muller, P., Delbarre, A., Ueda, T., Nakano, A. and Jurgens, G. (2003). The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. *Cell* **112**, 219-230.

Geldner, N., Friml, J., Stierhof, Y. D., Jurgens, G. and Palme, K. (2001). Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature* **413**, 425-428.

Gilliland, L. U., Pawloski, L. C., Kandasamy, M. K. and Meagher, R. B. (2003). Arabidopsis actin gene ACT7 plays an essential role in germination and root growth. *Plant J.* **33**, 319-328.

Goodrich, L. V. and Strutt, D. (2011). Principles of planar polarity in animal development. *Development* **138**, 1877-1892.

Goryachev, A. B. and Pokhilko, A. V. (2008). Dynamics of Cdc42 network embodies a Turing-type mechanism of yeast cell polarity. *FEBS Lett.* **582**, 1437-1443.

Grebe, M. (2004). Ups and downs of tissue and planar polarity in plants. *BioEssays* **26**, 719-729.

Grebe, M. (2012). The patterning of epidermal hairs in Arabidopsis--updated. *Curr. Opin. Plant Biol.* **15**, 31-37.

Grebe, M., Friml, J., Swarup, R., Ljung, K., Sandberg, G., Terlou, M., Palme, K., Bennett, M. J. and Scheres, B. (2002). Cell polarity signaling in Arabidopsis involves a BFA-sensitive auxin influx pathway. *Curr. Biol.* **12**, 329-334.

Grebe, M., Xu, J., Mobius, W., Ueda, T., Nakano, A., Geuze, H. J., Rook, M. B. and Scheres, B. (2003). Arabidopsis sterol endocytosis involves actin-mediated trafficking via ARA6-positive early endosomes. *Curr. Biol.* **13**, 1378-1387.

Grierson, C. and Schiefelbein, J. (2002). Root hairs. *The Arabidopsis Book / Amer. Soc. Plant Biol.* **1**, e0060.

Grierson, C. S., Roberts, K., Feldmann, K. A. and Dolan, L. (1997). The COW1 locus of arabidopsis acts after RHD2, and in parallel with RHD3 and TIP1, to determine the shape, rate of elongation, and number of root hairs produced from each site of hair formation. *Plant Physiol.* **115**, 981-990.

Gu, Y., Fu, Y., Dowd, P., Li, S., Vernoud, V., Gilroy, S. and Yang, Z. (2005). A Rho family GTPase controls actin dynamics and tip growth via two counteracting downstream pathways in pollen tubes. *J. Cell Biol.* **169**, 127-138.

Gu, Y., Vernoud, V., Fu, Y. and Yang, Z. (2003). ROP GTPase regulation of pollen tube growth through the dynamics of tip-localized F-actin. *J. Exp. Bot.* **54**, 93-101.

Gungabissoon, R. A., Jiang, C. J., Drobak, B. K., Maciver, S. K. and Hussey, P. J. (1998). Interaction of maize actin-depolymerising factor with actin and phosphoinositides and its inhibition of plant phospholipase C. *Plant J.* **16**, 689-696.

Gutierrez, R., Lindeboom, J. J., Paredez, A. R., Emons, A. M. and Ehrhardt, D. W. (2009). *Arabidopsis* cortical microtubules position cellulose synthase delivery to the plasma membrane and interact with cellulose synthase trafficking compartments. *Nat. Cell Biol.* **11**, 797-806.

Hala, M., Cole, R., Synek, L., Drdova, E., Pecenkova, T., Nordheim, A., Lamkemeyer, T., Madlung, J., Hochholdinger, F., Fowler, J. E., et al. (2008). An exocyst complex functions in plant cell growth in Arabidopsis and tobacco. *Plant Cell* **20**, 1330-1345.

Hassan, H., Scheres, B. and Blilou, I. (2010). JACKDAW controls epidermal patterning in the Arabidopsis root meristem through a non-cell-autonomous mechanism. *Development* **137**, 1523-1529.

Helling, D., Possart, A., Cottier, S., Klahre, U. and Kost, B. (2006). Pollen tube tip growth depends on plasma membrane polarization mediated by tobacco PLC3 activity and endocytic membrane recycling. *Plant Cell* **18**, 3519-3534.

Henty-Ridilla, J. L., Li, J., Blanchoin, L. and Staiger, C. J. (2013). Actin dynamics in the cortical array of plant cells. *Curr. Opin. Plant Biol.* **16**, 678-687.

Hepler, P. K., Vidali, L. and Cheung, A. Y. (2001). Polarized cell growth in higher plants. *Ann. Rev. Cell Dev Biol.* **17**, 159-187.

Higgs, H. N. and Pollard, T. D. (2001). Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. *Ann. Rev. Biochem.* **70**, 649-676.

Holmes, K. C., Popp, D., Gebhard, W. and Kabsch, W. (1990). Atomic model of the actin filament. *Nature* **347**, 44-49.

Howell, A. S., Jin, M., Wu, C. F., Zyla, T. R., Elston, T. C. and Lew, D. J. (2012). Negative feedback enhances robustness in the yeast polarity establishment circuit. *Cell* **149**, 322-333.

Howell, A. S., Savage, N. S., Johnson, S. A., Bose, I., Wagner, A. W., Zyla, T. R., Nijhout, H. F., Reed, M. C., Goryachev, A. B. and Lew, D. J. (2009). Singularity in polarization: rewiring yeast cells to make two buds. *Cell* **139**, 731-743.

Huang, G. Q., Li, E., Ge, F. R., Li, S., Wang, Q., Zhang, C. Q. and Zhang, Y. (2013). Arabidopsis RopGEF4 and RopGEF10 are important for FERONIA-mediated developmental but not environmental regulation of root hair growth. *New Phytol.* **200**, 1089-1101.

Huang, S., Blanchoin, L., Chaudhry, F., Franklin-Tong, V. E. and Staiger, C. J. (2004). A gelsolin-like protein from Papaver rhoeas pollen (PrABP80) stimulates calcium-regulated severing and depolymerization of actin filaments. *J. Biol. Chem.* **279**, 23364-23375.

Huang, S., Blanchoin, L., Kovar, D. R. and Staiger, C. J. (2003). Arabidopsis capping protein (AtCP) is a heterodimer that regulates assembly at the barbed ends of actin filaments. *J. Biol. Chem.* **278**, 44832-44842.

Huang, S., Gao, L., Blanchoin, L. and Staiger, C. J. (2006). Heterodimeric capping protein from Arabidopsis is regulated by phosphatidic acid. *Mol. Biol. Cell* **17**, 1946-1958.

Hussey, P. J., Ketelaar, T. and Deeks, M. J. (2006). Control of the actin cytoskeleton in plant cell growth. *Ann. Rev. Plant Biol.* **57**, 109-125.

Hwang, J. U., Gu, Y., Lee, Y. J. and Yang, Z. (2005). Oscillatory ROP GTPase activation leads the oscillatory polarized growth of pollen tubes. *Mol. Biol. Cell* **16**, 5385-5399.

Hwang, J. U., Vernoud, V., Szumlanski, A., Nielsen, E. and Yang, Z. (2008). A tip-localized RhoGAP controls cell polarity by globally inhibiting Rho GTPase at the cell apex. *Curr. Biol.* **18**, 1907-1916.

Hülskamp, M., Misra, S. and Jürgens, G. (1994). Genetic dissection of trichome cell development in Arabidopsis. *Cell* **76**, 555-566.

Ide, Y., Nagasaki, N., Tomioka, R., Suito, M., Kamiya, T. and Maeshima, M. (2007). Molecular properties of a novel, hydrophilic cation-binding protein associated with the plasma membrane. *J. Exp. Bot.* **58**, 1173-1183.

Ikeda, Y., Men, S., Fischer, U., Stepanova, A. N., Alonso, J. M., Ljung, K. and Grebe, M. (2009). Local auxin biosynthesis modulates gradient-directed planar polarity in *Arabidopsis*. *Nat. Cell Biol.* **11**, 731-738.

Ishida, T., Kurata, T., Okada, K. and Wada, T. (2008). A genetic regulatory network in the development of trichomes and root hairs. *Ann. Rev. Plant Biol.* **59**, 365-386.

Johnson, D. I. (1999). Cdc42: An essential Rho-type GTPase controlling eukaryotic cell polarity. *Microbiol. Mol. Biol. Rev.* **63**, 54-105.

Johnson, J. M., Jin, M. and Lew, D. J. (2011). Symmetry breaking and the establishment of cell polarity in budding yeast. *Curr. Opin. Gen. Dev.* **21**, 740-746.

Jones, A. R., Kramer, E. M., Knox, K., Swarup, R., Bennett, M. J., Lazarus, C. M., Leyser, H. M. and Grierson, C. S. (2009). Auxin transport through non-hair cells sustains root-hair development. *Nat. Cell Biol.* **11**, 78-84.

Jones, M. A., Shen, J. J., Fu, Y., Li, H., Yang, Z. and Grierson, C. S. (2002). The Arabidopsis Rop2 GTPase Is a Positive Regulator of Both Root Hair Initiation and Tip Growth. *Plant Cell* **14**, 763-776.

Jones, V. A. and Dolan, L. (2012). The evolution of root hairs and rhizoids. *Ann. Bot.* **110**, 205-212.

Ju, C., Yoon, G. M., Shemansky, J. M., Lin, D. Y., Ying, Z. I., Chang, J., Garrett, W. M., Kessenbrock, M., Groth, G., Tucker, M. L., et al. (2012). CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **109**, 19486-19491.

Kandasamy, M. K., Burgos-Rivera, B., McKinney, E. C., Ruzicka, D. R. and Meagher, R. B. (2007). Class-specific interaction of profilin and ADF isovariants with actin in the regulation of plant development. *Plant Cell* **19**, 3111-3126.

Kandasamy, M. K., Gilliland, L. U., McKinney, E. C. and Meagher, R. B. (2001). One plant actin isovariant, ACT7, is induced by auxin and required for normal callus formation. *Plant Cell* **13**, 1541-1554.

Kandasamy, M. K., McKinney, E. C. and Meagher, R. B. (2009). A single vegetative actin isovariant overexpressed under the control of multiple regulatory sequences is sufficient for normal Arabidopsis development. *Plant Cell* **21**, 701-718.

Kang, Y. H., Kirik, V., Hulskamp, M., Nam, K. H., Hagely, K., Lee, M. M. and Schiefelbein, J. (2009). The MYB23 gene provides a positive feedback loop for cell fate specification in the Arabidopsis root epidermis. *Plant Cell* **21**, 1080-1094.

Kaya, H., Nakajima, R., Iwano, M., Kanaoka, M. M., Kimura, S., Takeda, S., Kawarazaki, T., Senzaki, E., Hamamura, Y., Higashiyama, T., et al. (2014). Ca2+-Activated Reactive Oxygen Species Production by Arabidopsis RbohH and RbohJ Is Essential for Proper Pollen Tube Tip Growth. *Plant Cell* **26**, 1069-1080.

Ketelaar, T. (2013). The actin cytoskeleton in root hairs: all is fine at the tip. *Curr. Opin. Plant Biol.* **16**, 749-756.

Ketelaar, T., Allwood, E. G., Anthony, R., Voigt, B., Menzel, D. and Hussey, P. J. (2004). The actin-interacting protein AIP1 is essential for actin organization and plant development. *Curr. Biol.* **14**, 145-149.

Ketelaar, T., Allwood, E. G. and Hussey, P. J. (2007). Actin organization and root hair development are disrupted by ethanol-induced overexpression of Arabidopsis actin interacting protein 1 (AIP1). *New Phytol.* **174**, 57-62.

Kirik, V., Herrmann, U., Parupalli, C., Sedbrook, J. C., Ehrhardt, D. W. and Hulskamp, M. (2007). CLASP localizes in two discrete patterns on cortical microtubules and is required for cell morphogenesis and cell division in *Arabidopsis*. *J. Cell Sci.* **120**, 4416-4425.

Kirik, V., Lee, M. M., Wester, K., Herrmann, U., Zheng, Z., Oppenheimer, D., Schiefelbein, J. and Hulskamp, M. (2005). Functional diversification of MYB23 and GL1 genes in trichome morphogenesis and initiation. *Development* **132**, 1477-1485.

Kleine-Vehn, J., Dhonukshe, P., Sauer, M., Brewer, P. B., Wisniewska, J., Paciorek, T., Benkova, E. and Friml, J. (2008). ARF GEF-dependent transcytosis and polar delivery of PIN auxin carriers in Arabidopsis. *Curr. Biol.* **18**, 526-531.

Kleine-Vehn, J., Dhonukshe, P., Swarup, R., Bennett, M. and Friml, J. (2006). Subcellular trafficking of the Arabidopsis auxin influx carrier AUX1 uses a novel pathway distinct from PIN1. *Plant Cell* **18**, 3171-3181.

Knoll, A. H., Niklas, K. J., Gensel, P. G. and Tiffney, B. H. (1984). Character diversification and patterns of evolution in early vascular plants. *Paleobiology* **10**, 34-47.

Kost, B., Lemichez, E., Spielhofer, P., Hong, Y., Tolias, K., Carpenter, C. and Chua, N. H. (1999). Rac homologues and compartmentalized phosphatidylinositol 4, 5-bisphosphate act in a common pathway to regulate polar pollen tube growth. *J. Cell Biol.* **145**, 317-330.

Kovar, D. R., Drobak, B. K., Collings, D. A. and Staiger, C. J. (2001). The characterization of ligand-specific maize (Zea mays) profilin mutants. *Biochem. J.* **358**, 49-57.

Kovar, D. R., Drobak, B. K. and Staiger, C. J. (2000). Maize profilin isoforms are functionally distinct. *Plant Cell* **12**, 583-598.

Kuo, C. C., Savage, N. S., Chen, H., Wu, C. F., Zyla, T. R. and Lew, D. J. (2014). Inhibitory GEF phosphorylation provides negative feedback in the yeast polarity circuit. *Curr. Biol.* **24**, 753-759.

Kurata, T., Ishida, T., Kawabata-Awai, C., Noguchi, M., Hattori, S., Sano, R., Nagasaka, R., Tominaga, R., Koshino-Kimura, Y., Kato, T., et al. (2005). Cell-to-cell movement of the CAPRICE protein in Arabidopsis root epidermal cell differentiation. *Development* **132**, 5387-5398.

Kusano, H., Testerink, C., Vermeer, J. E., Tsuge, T., Shimada, H., Oka, A., Munnik, T. and Aoyama, T. (2008). The *Arabidopsis* Phosphatidylinositol Phosphate 5-Kinase PIP5K3 is a Key Regulator of Root Hair Tip Growth. *Plant Cell* **20**, 367-380.

Kwak, S. H. and Schiefelbein, J. (2007). The role of the SCRAMBLED receptor-like kinase in patterning the Arabidopsis root epidermis. *Dev. Biol.* **302**, 118-131.

Kwak, S. H. and Schiefelbein, J. (2008). A feedback mechanism controlling SCRAMBLED receptor accumulation and cell-type pattern in Arabidopsis. *Curr. Biol.* **18**, 1949-1954.

Lang, J. M., Eisinger, W. R. and Green, P. B. (1982). Effects of Ethylene on the Orientation of Microtubules and Cellulose Microfibrils of Pea Epicotyl Cells with Polylamellate Cell Walls. *Protoplasma* **110**, 5-14.

Laohavisit, A., Shang, Z., Rubio, L., Cuin, T. A., Very, A. A., Wang, A., Mortimer, J. C., Macpherson, N., Coxon, K. M., Battey, N. H., et al. (2012). Arabidopsis annexin1 mediates the radical-activated plasma membrane Ca(2)+- and K+-permeable conductance in root cells. *Plant Cell* **24**, 1522-1533.

Lassig, R., Gutermuth, T., Bey, T. D., Konrad, K. R. and Romeis, T. (2014). Pollen tube NAD(P)H oxidases act as a speed control to dampen growth rate oscillations during polarized cell growth. *Plant J.* **78**, 94-106.

Le, J., El-Assal Sel, D., Basu, D., Saad, M. E. and Szymanski, D. B. (2003). Requirements for Arabidopsis ATARP2 and ATARP3 during epidermal development. *Curr. Biol.* **13**, 1341-1347.

Lee, M. M. and Schiefelbein, J. (1999). WEREWOLF, a MYB-related protein in Arabidopsis, is a position-dependent regulator of epidermal cell patterning. *Cell* **99**, 473-483.

Lee, M. M. and Schiefelbein, J. (2002). Cell pattern in the Arabidopsis root epidermis determined by lateral inhibition with feedback. *Plant Cell* **14**, 611-618.

Lee, Y. J., Szumlanski, A., Nielsen, E. and Yang, Z. (2008). Rho-GTPase-dependent filamentous actin dynamics coordinate vesicle targeting and exocytosis during tip growth. *J. Cell Biol.* **181**, 1155-1168.

Li, H., Lin, Y., Heath, R. M., Zhu, M. X. and Yang, Z. (1999). Control of pollen tube tip growth by a Rop GTPase-dependent pathway that leads to tip-localized calcium influx. *Plant Cell* **11**, 1731-1742.

Li, J., Henty-Ridilla, J. L., Huang, S., Wang, X., Blanchoin, L. and Staiger, C. J. (2012). Capping protein modulates the dynamic behavior of actin filaments in response to phosphatidic acid in Arabidopsis. *Plant Cell* **24**, 3742-3754.

Li, J., Wang, X., Qin, T., Zhang, Y., Liu, X., Sun, J., Zhou, Y., Zhu, L., Zhang, Z., Yuan, M., et al. (2011). MDP25, a novel calcium regulatory protein, mediates hypocotyl cell elongation by destabilizing cortical microtubules in Arabidopsis. *Plant Cell* **23**, 4411-4427.

Lin, D., Nagawa, S., Chen, J., Cao, L., Chen, X., Xu, T., Li, H., Dhonukshe, P., Yamamuro, C., Friml, J., et al. (2012). A ROP GTPase-dependent auxin signaling pathway regulates the subcellular distribution of PIN2 in Arabidopsis roots. *Curr. Biol.* **22**, 1319-1325.

Lintilhac, P. M. and Vesecky, T. B. (1981). Mechanical Stress and Cell Wall Orientation in Plants. II. The Application of Controlled Directional Stress to Growing Plants; with a Discussion on the Nature of the Wound Reaction. *Amer. J. Bot.* **68**, 1222-1230.

Liu, P., Li, R. L., Zhang, L., Wang, Q. L., Niehaus, K., Baluska, F., Samaj, J. and Lin, J. X. (2009). Lipid microdomain polarization is required for NADPH oxidase-dependent ROS signaling in Picea meyeri pollen tube tip growth. *Plant J.* **60**, 303-313.

Lybarger, S. R. and Maddock, J. R. (2001). Polarity in action: asymmetric protein localization in bacteria. *J. Bacteriol.* **183**, 3261-3267.

Maciver, S. K. and Hussey, P. J. (2002). The ADF/cofilin family: actin-remodeling proteins. *Gen. Biol.* **3**, reviews3007.

Marchant, A., Kargul, J., May, S. T., Muller, P., Delbarre, A., Perrot-Rechenmann, C. and Bennett, M. J. (1999). AUX1 regulates root gravitropism in Arabidopsis by facilitating auxin uptake within root apical tissues. *EMBO J.* **18**, 2066-2073.

Masucci, J. D., Rerie, W. G., Foreman, D. R., Zhang, M., Galway, M. E., Marks, M. D. and Schiefelbein, J. W. (1996). The homeobox gene GLABRA2 is required for position-dependent cell differentiation in the root epidermis of Arabidopsis thaliana. *Development* **122**, 1253-1260.

Masucci, J. D. and Schiefelbein, J. W. (1994). The *rhd6* Mutation of *Arabidopsis thaliana* Alters Root-Hair Initiation through an Auxin- and Ethylene-Associated Process. *Plant Physiol.* **106**, 1335-1346.

Masucci, J. D. and Schiefelbein, J. W. (1996). Hormones act downstream of TTG and GL2 to promote root hair outgrowth during epidermis development in the Arabidopsis root. *Plant Cell* **8**, 1505-1517.

Mathur, J., Mathur, N., Kernebeck, B. and Hulskamp, M. (2003a). Mutations in actin-related proteins 2 and 3 affect cell shape development in Arabidopsis. *Plant Cell* **15**, 1632-1645.

Mathur, J., Mathur, N., Kirik, V., Kernebeck, B., Srinivas, B. P. and Hulskamp, M. (2003b). Arabidopsis CROOKED encodes for the smallest subunit of the ARP2/3 complex and controls cell shape by region specific fine F-actin formation. *Development* **130**, 3137-3146.

Mauriello, E. M. (2010). Cell polarity/motility in bacteria: closer to eukaryotes than expected? *EMBO J.* **29**, 2258-2259.

McDowell, J. M., An, Y. Q., Huang, S., McKinney, E. C. and Meagher, R. B. (1996a). The arabidopsis ACT7 actin gene is expressed in rapidly developing tissues and responds to several external stimuli. *Plant Physiol.* **111**, 699-711.

McDowell, J. M., Huang, S., McKinney, E. C., An, Y. Q. and Meagher, R. B. (1996b). Structure and evolution of the actin gene family in Arabidopsis thaliana. *Genetics* **142**, 587-602.

McKenna, S. T., Kunkel, J. G., Bosch, M., Rounds, C. M., Vidali, L., Winship, L. J. and Hepler, P. K. (2009). Exocytosis precedes and predicts the increase in growth in oscillating pollen tubes. *Plant Cell* **21**, 3026-3040.

McKinney, E. C. and Meagher, R. B. (1998). Members of the Arabidopsis actin gene family are widely dispersed in the genome. *Genetics* **149**, 663-675.

McMichael, C. M. and Bednarek, S. Y. (2013). Cytoskeletal and membrane dynamics during higher plant cytokinesis. *New Phytol.* **197**, 1039-1057.

Meinhardt, H. and Gierer, A. (1974). Applications of a theory of biological pattern formation based on lateral inhibition. *J. Cell Sci.* **15**, 321-346.

Men, S., Boutte, Y., Ikeda, Y., Li, X., Palme, K., Stierhof, Y. D., Hartmann, M. A., Moritz, T. and Grebe, M. (2008). Sterol-dependent endocytosis mediates post-cytokinetic acquisition of PIN2 auxin efflux carrier polarity. *Nat. Cell Biol.* **10**, 237-244.

Messerli, M. A., Creton, R., Jaffe, L. F. and Robinson, K. R. (2000). Periodic increases in elongation rate precede increases in cytosolic Ca2+ during pollen tube growth. *Dev. Biol.* **222**, 84-98.

Meyerowitz, E. M. (1999). Plants, animals and the logic of development. *Trends Cell Biol.* **9**, M65-68.

Meyerowitz, E. M. (2002). Plants compared to animals: the broadest comparative study of development. *Science* **295**, 1482-1485.

Minorsky, P. V. (2002). The hot and the classic: mutant studies on root hair function. *Plant Physiol.* **129**, 438-439.

Mogilner, A., Allard, J. and Wollman, R. (2012). Cell polarity: quantitative modeling as a tool in cell biology. *Science* **336**, 175-179.

Molendijk, A. J., Bischoff, F., Rajendrakumar, C. S., Friml, J., Braun, M., Gilroy, S. and Palme, K. (2001). *Arabidopsis thaliana* Rop GTPases are localized to tips of root hairs and control polar growth. *EMBO J.* **20**, 2779-2788.

Mongrand, S., Morel, J., Laroche, J., Claverol, S., Carde, J. P., Hartmann, M. A., Bonneu, M., Simon-Plas, F., Lessire, R. and Bessoule, J. J. (2004). Lipid rafts in higher plant cells: purification and characterization of Triton X-100-insoluble microdomains from tobacco plasma membrane. *J. Biol. Chem.* **279**, 36277-36286.

Morohashi, K. and Grotewold, E. (2009). A systems approach reveals regulatory circuitry for Arabidopsis trichome initiation by the GL3 and GL1 selectors. *PLoS Gen.* **5**, e1000396.

Müller, A., Guan, C., Galweiler, L., Tanzler, P., Huijser, P., Marchant, A., Parry, G., Bennett, M., Wisman, E. and Palme, K. (1998). AtPIN2 defines a locus of Arabidopsis for root gravitropism control. *EMBO J.* **17**, 6903-6911.

Nagasaki, N., Tomioka, R. and Maeshima, M. (2008). A hydrophilic cation-binding protein of Arabidopsis thaliana, AtPCaP1, is localized to plasma membrane via N-myristoylation and interacts with calmodulin and the phosphatidylinositol phosphates PtdIns(3,4,5)P(3) and PtdIns(3,5)P(2). *FEBS J.* **275**, 2267-2282.

Nagawa, S., Xu, T., Lin, D., Dhonukshe, P., Zhang, X., Friml, J., Scheres, B., Fu, Y. and Yang, Z. (2012). ROP GTPase-dependent actin microfilaments promote PIN1 polarization by localized inhibition of clathrin-dependent endocytosis. *PLoS Biol.* **10**, e1001299.

Nakamura, M., Kiefer, C. S. and Grebe, M. (2012). Planar polarity, tissue polarity and planar morphogenesis in plants. *Curr. Opin. Plant Biol.* **15**, 593-600.

Naramoto, S., Kleine-Vehn, J., Robert, S., Fujimoto, M., Dainobu, T., Paciorek, T., Ueda, T., Nakano, A., Van Montagu, M. C., Fukuda, H., et al. (2010). ADP-ribosylation factor machinery mediates endocytosis in plant cells. *Proc. Natl. Acad. Sci. USA* **107**, 21890-21895.

Nishimura, T., Yokota, E., Wada, T., Shimmen, T. and Okada, K. (2003). An *Arabidopsis ACT2* Dominant-Negative Mutation, which Disturbs F-actin Polymerization, Reveals its Distinctive Function in Root Development. *Plant Cell Physiol.* **44**, 1131-1140.

Nübler-Jung, K. (1987). Insect epidermis: disturbance of supracellular tissue polarity does not prevent the expression of cell polarity. *Roux's Arch. Dev. Biol.* **196**, 286-289.

Nüsslein-Vollhard, C., Wieschaus, E. and Kluding, H. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster* I. Zygotic loci on the second chromosome. *Roux's Arch. Dev. Biol.* **193**, 267-282.

Ojangu, E. L., Jarve, K., Paves, H. and Truve, E. (2007). Arabidopsis thaliana myosin XIK is involved in root hair as well as trichome morphogenesis on stems and leaves. *Protoplasma* **230**, 193-202.

Oppenheimer, D. G., Herman, P. L., Sivakumaran, S., Esch, J. and Marks, M. D. (1991). A myb gene required for leaf trichome differentiation in Arabidopsis is expressed in stipules. *Cell* **67**, 483-493.

Ovecka, M., Berson, T., Beck, M., Derksen, J., Samaj, J., Baluska, F. and Lichtscheidl, I. K. (2010). Structural sterols are involved in both the initiation and tip growth of root hairs in Arabidopsis thaliana. *Plant Cell* **22**, 2999-3019.

Palanivelu, R., Brass, L., Edlund, A. F. and Preuss, D. (2003). Pollen tube growth and guidance is regulated by POP2, an Arabidopsis gene that controls GABA levels. *Cell* **114**, 47-59.

Papanikolaou, A., Papafotika, A., Murphy, C., Papamarcaki, T., Tsolas, O., Drab, M., Kurzchalia, T. V., Kasper, M. and Christoforidis, S. (2005). Cholesterol-dependent lipid assemblies regulate the activity of the ecto-nucleotidase CD39. *J. Biol. Chem.* **280**, 26406-26414.

Parker, J. S., Cavell, A. C., Dolan, L., Roberts, K. and Grierson, C. S. (2000). Genetic interactions during root hair morphogenesis in Arabidopsis. *Plant Cell* **12**, 1961-1974.

Payne, C. T., Zhang, F. and Lloyd, A. M. (2000). GL3 encodes a bHLH protein that regulates trichome development in arabidopsis through interaction with GL1 and TTG1. *Genetics* **156**, 1349-1362.

Payne, R. J. and Grierson, C. S. (2009). A theoretical model for ROP localisation by auxin in Arabidopsis root hair cells. *PloS One* **4**, e8337.

Pearce, L. R., Komander, D. and Alessi, D. R. (2010). The nuts and bolts of AGC protein kinases. *Nat. Rev. Mol. Cell Biol.* **11**, 9-22.

Peremyslov, V. V., Prokhnevsky, A. I., Avisar, D. and Dolja, V. V. (2008). Two class XI myosins function in organelle trafficking and root hair development in Arabidopsis. *Plant Physiol.* **146**, 1109-1116.

Peremyslov, V. V., Prokhnevsky, A. I. and Dolja, V. V. (2010). Class XI myosins are required for development, cell expansion, and F-Actin organization in Arabidopsis. *Plant Cell* **22**, 1883-1897.

Petersson, S. V., Johansson, A. I., Kowalczyk, M., Makoveychuk, A., Wang, J. Y., Moritz, T., Grebe, M., Benfey, P. N., Sandberg, G. and Ljung, K. (2009). An auxin gradient and maximum in the Arabidopsis root apex shown by high-resolution cell-specific analysis of IAA distribution and synthesis. *Plant Cell* **21**, 1659-1668.

Pickett-Heaps, J. D. and Northcote, D. H. (1966). Organization of microtubules and endoplasmic reticulum during mitosis and cytokinesis in wheat meristems. *J. Cell Sci.* **1**, 109-120.

Pietra, S. (2014). Characterization of New Players in Planar Polarity Establishment in *Arabidopsis*. In *Umeå Plant Science Centre*. Servicecenter KBC: Umeå University. E-version available at http://www.diva-portal.org/smash/get/diva2:711983/FULLTEXT01.pdf.

Pollard, T. D. and Borisy, G. G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. *Cell* **112**, 453-465.

Potocky, M., Jones, M. A., Bezvoda, R., Smirnoff, N. and Zarsky, V. (2007). Reactive oxygen species produced by NADPH oxidase are involved in pollen tube growth. *New Phytol.* **174**, 742-751.

Potocky, M., Pejchar, P., Gutkowska, M., Jimenez-Quesada, M. J., Potocka, A., Alche Jde, D., Kost, B. and Zarsky, V. (2012). NADPH oxidase activity in pollen tubes is affected by calcium ions, signaling phospholipids and Rac/Rop GTPases. *J. Plant Physiol.* **169**, 1654-1663.

Potocky, M., Pleskot, R., Pejchar, P., Vitale, N., Kost, B. and Zarsky, V. (2014). Live-cell imaging of phosphatidic acid dynamics in pollen tubes visualized by Spo20p-derived biosensor. *New Phytol.* **203**, 483-494.

Praefcke, G. J. and McMahon, H. T. (2004). The dynamin superfamily: universal membrane tubulation and fission molecules? *Nat. Rev. Mol. Cell Biol.* **5**, 133-147.

Preuss, M. L., Serna, J., Falbel, T. G., Bednarek, S. Y. and Nielsen, E. (2004). The Arabidopsis Rab GTPase RabA4b localizes to the tips of growing root hair cells. *Plant Cell* **16**, 1589-1603.

Procissi, A., Guyon, A., Pierson, E. S., Giritch, A., Knuiman, B., Grandjean, O., Tonelli, C., Derksen, J., Pelletier, G. and Bonhomme, S. (2003). *KINKY POLLEN* encodes a SABRE-like protein required for tip growth in *Arabidopsis* and conserved among eukaryotes. *Plant J.* **36**, 894-904.

Prokhnevsky, A. I., Peremyslov, V. V. and Dolja, V. V. (2008). Overlapping functions of the four class XI myosins in Arabidopsis growth, root hair elongation, and organelle motility. *Proc. Natl. Acad. Sci. USA* **105**, 19744-19749.

Qin, T., Liu, X., Li, J., Sun, J., Song, L. and Mao, T. (2014). Arabidopsis microtubule-destabilizing protein 25 functions in pollen tube growth by severing actin filaments. *Plant Cell* **26**, 325-339.

Qu, X., Zhang, H., Xie, Y., Wang, J., Chen, N. and Huang, S. (2013). Arabidopsis villins promote actin turnover at pollen tube tips and facilitate the construction of actin collars. *Plant Cell* **25**, 1803-1817.

Ramachandran, S., Christensen, H. E., Ishimaru, Y., Dong, C. H., Chao-Ming, W., Cleary, A. L. and Chua, N. H. (2000). Profilin plays a role in cell elongation, cell shape maintenance, and flowering in Arabidopsis. *Plant Physiol.* **124**, 1637-1647.

Rasmussen, C. G., Wright, A. J. and Muller, S. (2013). The role of the cytoskeleton and associated proteins in determination of the plant cell division plane. *Plant J.* **75**, 258-269.

Raven, J. A. and Edwards, D. (2001). Roots: evolutionary origins and biogeochemical significance. *J. Exp. Bot.* **52**, 381-401.

Reddy, A. S. and Day, I. S. (2001). Analysis of the myosins encoded in the recently completed Arabidopsis thaliana genome sequence. *Gen. Biol.* **2**, RESEARCH0024.

Ren, N., Charlton, J. and Adler, P. N. (2007). The flare gene, which encodes the AIP1 protein of Drosophila, functions to regulate F-actin disassembly in pupal epidermal cells. *Genetics* **176**, 2223-2234.

Ringli, C., Baumberger, N., Diet, A., Frey, B. and Keller, B. (2002). ACTIN2 Is Essential for Bulge Site Selection and Tip Growth during Root Hair Development of Arabidopsis. *Plant Physiol.* **129**, 1464-1472.

Rodal, A. A., Sokolova, O., Robins, D. B., Daugherty, K. M., Hippenmeyer, S., Riezman, H., Grigorieff, N. and Goode, B. L. (2005). Conformational changes in the Arp2/3 complex leading to actin nucleation. *Nat. Struct. Mol. Biol.* **12**, 26-31.

Rodal, A. A., Tetreault, J. W., Lappalainen, P., Drubin, D. G. and Amberg, D. C. (1999). Aip1p interacts with cofilin to disassemble actin filaments. *J. Cell Biol.* **145**, 1251-1264.

Rosero, A., Zarsky, V. and Cvrckova, F. (2013). AtFH1 formin mutation affects actin filament and microtubule dynamics in Arabidopsis thaliana. *J. Exp. Bot.* **64**, 585-597.

Roth, S. and Lynch, J. A. (2009). Symmetry breaking during Drosophila oogenesis. *Cold Spring Harb. Perspect. Biol.* **1**, a001891.

Rounds, C. M. and Bezanilla, M. (2013). Growth mechanisms in tip-growing plant cells. *Ann. Rev. Plant Biol.* **64**, 243-265.

Sachs, T. (1991). The polarization of tissues., pp. 52-69. Pattern Formation in Plant Tissues.: Cambridge University Press.

Saedler, R., Mathur, N., Srinivas, B. P., Kernebeck, B., Hulskamp, M. and Mathur, J. (2004). Actin control over microtubules suggested by DISTORTED2 encoding the Arabidopsis ARPC2 subunit homolog. *Plant Cell Physiol.* **45**, 813-822.

Sakai, T., Honing, H., Nishioka, M., Uehara, Y., Takahashi, M., Fujisawa, N., Saji, K., Seki, M., Shinozaki, K., Jones, M. A., et al. (2008). Armadillo repeat-containing kinesins and a NIMA-related kinase are required for epidermal-cell morphogenesis in Arabidopsis. *Plant J.* **53**, 157-171.

Sampathkumar, A., Gutierrez, R., McFarlane, H., Bringmann, M., Lindeboom, J., Emons, A. M., Samuels, L., Ketelaar, T., Ehrhardt, D. and Persson, S. (2013). Patterning and life-time of plasma membrane localized cellulose synthase is dependent on actin organization in Arabidopsis interphase cells. *Plant Physiol.* **162**, 675-688.

Sampathkumar, A., Lindeboom, J. J., Debolt, S., Gutierrez, R., Ehrhardt, D. W., Ketelaar, T. and Persson, S. (2011). Live Cell Imaging Reveals Structural Associations between the Actin and Microtubule Cytoskeleton in *Arabidopsis*. *Plant Cell* **23**, 2302-2313.

Sauer, M., Balla, J., Luschnig, C., Wisniewska, J., Reinohl, V., Friml, J. and Benkova, E. (2006). Canalization of auxin flow by Aux/IAA-ARF-dependent feedback regulation of PIN polarity. *Gen. Dev.* **20**, 2902-2911.

Schiefelbein, J., Huang, L. and Zheng, X. (2014). Regulation of epidermal cell fate in Arabidopsis roots: the importance of multiple feedback loops. *Frontiers in plant science* **5**, 47.

Schiefelbein, J. W. (2000). Constructing a plant cell. The genetic control of root hair development. *Plant Physiol.* **124**, 1525-1531.

Schopfer, P. (2001). Hydroxyl radical-induced cell-wall loosening in vitro and in vivo: implications for the control of elongation growth. *Plant J.* **28**, 679-688.

Schwab, B., Mathur, J., Saedler, R., Schwarz, H., Frey, B., Scheidegger, C. and Hulskamp, M. (2003). Regulation of cell expansion by the DISTORTED genes in Arabidopsis thaliana: actin controls the spatial organization of microtubules. *Mol. Genet. Genomics* **269**, 350-360.

Singh, S. K., Fischer, U., Singh, M., Grebe, M. and Marchant, A. (2008). Insight into the early steps of root hair formation revealed by the procuste1 cellulose synthase mutant of Arabidopsis thaliana. *BMC Plant Biol.* **8**, 57.

Smertenko, A. P., Jiang, C. J., Simmons, N. J., Weeds, A. G., Davies, D. R. and Hussey, P. J. (1998). Ser6 in the maize actin-depolymerizing factor, ZmADF3, is phosphorylated by a calcium-stimulated protein kinase and is essential for the control of functional activity. *Plant J.* **14**, 187-193.

Sousa, E., Kost, B. and Malho, R. (2008). Arabidopsis phosphatidylinositol-4-monophosphate 5-kinase 4 regulates pollen tube growth and polarity by modulating membrane recycling. *Plant Cell* **20**, 3050-3064.

Staiger, C. J., Poulter, N. S., Henty, J. L., Franklin-Tong, V. E. and Blanchoin, L. (2010). Regulation of actin dynamics by actin-binding proteins in pollen. *J. Exp. Bot.* **61**, 1969-1986.

Staiger, C. J., Sheahan, M. B., Khurana, P., Wang, X., McCurdy, D. W. and Blanchoin, L. (2009). Actin filament dynamics are dominated by rapid growth and severing activity in the Arabidopsis cortical array. *J. Cell Biol.* **184**, 269-280.

Steen, D. A. and Chadwick, A. V. (1981). Ethylene Effects in Pea Stem Tissue : EVIDENCE OF MICROTUBULE MEDIATION. *Plant Physiol.* **67**, 460-466.

Steinmann, T., Geldner, N., Grebe, M., Mangold, S., Jackson, C. L., Paris, S., Galweiler, L., Palme, K. and Jurgens, G. (1999). Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. *Science* **286**, 316-318.

Szymanski, D. B. (2005). Breaking the WAVE complex: the point of Arabidopsis trichomes. *Curr. Opin. Plant Biol.* **8**, 103-112.

Takahashi, H., Hirota, K., Kawahara, A., Hayakawa, E. and Inoue, Y. (2003). Randomization of cortical microtubules in root epidermal cells induces root hair initiation in lettuce (Lactuca sativa L.) seedlings. *Plant Cell Physiol.* **44**, 350-359.

Tanimoto, M., Roberts, K. and Dolan, L. (1995). Ethylene is a positive regulator of root hair development in Arabidopsis thaliana. *Plant J.* **8**, 943-948.

Tejos, R., Sauer, M., Vanneste, S., Palacios-Gomez, M., Li, H., Heilmann, M., van Wijk, R., Vermeer, J. E., Heilmann, I., Munnik, T., et al. (2014). Bipolar Plasma Membrane Distribution of Phosphoinositides and Their Requirement for Auxin-Mediated Cell Polarity and Patterning in Arabidopsis. *Plant Cell* **26**, 2114-2128.

Thole, J. M., Vermeer, J. E., Zhang, Y., Gadella, T. W., Jr. and Nielsen, E. (2008). Root hair defective4 encodes a phosphatidylinositol-4-phosphate phosphatase required for proper root hair development in Arabidopsis thaliana. *Plant Cell* **20**, 381-395.

Tominaga, M., Kojima, H., Yokota, E., Nakamori, R., Anson, M., Shimmen, T. and Oiwa, K. (2012). Calcium-induced mechanical change in the neck domain alters the activity of plant myosin XI. *J. Biol. Chem.* **287**, 30711-30718.

Turing, A. (1952). The chemical basis of morphogenesis. *Philosoph. Trans. Royal Soc. London. Series B, Biol. Sci.* **237**, 37-72.

Uhrig, J. F., Mutondo, M., Zimmermann, I., Deeks, M. J., Machesky, L. M., Thomas, P., Uhrig, S., Rambke, C., Hussey, P. J. and Hulskamp, M. (2007). The role of Arabidopsis SCAR genes in ARP2-ARP3-dependent cell morphogenesis. *Development* **134**, 967-977.

Uyttewaal, M., Burian, A., Alim, K., Landrein, B., Borowska-Wykret, D., Dedieu, A., Peaucelle, A., Ludynia, M., Traas, J., Boudaoud, A., et al. (2012). Mechanical stress acts via katanin to amplify differences in growth rate between adjacent cells in Arabidopsis. *Cell* **149**, 439-451.

Wada, T., Kurata, T., Tominaga, R., Koshino-Kimura, Y., Tachibana, T., Goto, K., Marks, M. D., Shimura, Y. and Okada, K. (2002). Role of a positive regulator of root hair development, CAPRICE, in Arabidopsis root epidermal cell differentiation. *Development* **129**, 5409-5419.

Valenta, R., Ferreira, F., Grote, M., Swoboda, I., Vrtala, S., Duchene, M., Deviller, P., Meagher, R. B., McKinney, E., Heberle-Bors, E., et al. (1993). Identification of profilin as an actin-binding protein in higher plants. *J. Biol. Chem.* **268**, 22777-22781.

Van Bruaene, N., Joss, G. and Van Oostveldt, P. (2004). Reorganization and in vivo dynamics of microtubules during Arabidopsis root hair development. *Plant Physiol.* **136**, 3905-3919.

Van Gestel, K., Slegers, H., Von Witsch, M., Samaj, J., Baluska, F. and Verbelen, J. P. (2003). Immunological evidence for the presence of plant homologues of the actin- related protein Arp3 in tobacco and maize: subcellular localization to actin-enriched pit fields and emerging root hairs. *Protoplasma* **222**, 45-52.

van Gisbergen, P. A. and Bezanilla, M. (2013). Plant formins: membrane anchors for actin polymerization. *Trends Cell Biol.* **23**, 227-233.

Wang, C., Yan, X., Chen, Q., Jiang, N., Fu, W., Ma, B., Liu, J., Li, C., Bednarek, S. Y. and Pan, J. (2013a). Clathrin light chains regulate clathrin-mediated trafficking, auxin signaling, and development in Arabidopsis. *Plant Cell* **25**, 499-516.

Wang, F., Sampogna, R. V. and Ware, B. R. (1989). pH dependence of actin self-assembly. *Biophys. J.* **55**, 293-298.

Wang, H., Zhuang, X., Cai, Y., Cheung, A. Y. and Jiang, L. (2013b). Apical F-actin-regulated exocytic targeting of NtPPME1 is essential for construction and rigidity of the pollen tube cell wall. *Plant J.* **76**, 367-379.

Vanstraelen, M., Van Damme, D., De Rycke, R., Mylle, E., Inze, D. and Geelen, D. (2006). Cell cycle-dependent targeting of a kinesin at the plasma membrane demarcates the division site in plant cells. *Curr. Biol.* **16**, 308-314.

Vernoud, V., Horton, A. C., Yang, Z. and Nielsen, E. (2003). Analysis of the small GTPase gene superfamily of Arabidopsis. *Plant Physiol.* **131**, 1191-1208.

Vidali, L., Augustine, R. C., Kleinman, K. P. and Bezanilla, M. (2007). Profilin is essential for tip growth in the moss Physcomitrella patens. *Plant Cell* **19**, 3705-3722.

Willemsen, V., Friml, J., Grebe, M., van den Toorn, A., Palme, K. and Scheres, B. (2003). Cell polarity and PIN protein positioning in Arabidopsis require STEROL METHYLTRANSFERASE1 function. *Plant Cell* **15**, 612-625.

Willige, B. C., Ahlers, S., Zourelidou, M., Barbosa, I. C., Demarsy, E., Trevisan, M., Davis, P. A., Roelfsema, M. R., Hangarter, R., Fankhauser, C., et al. (2013). D6PK AGCVIII kinases are required for auxin transport and phototropic hypocotyl bending in Arabidopsis. *Plant Cell* **25**, 1674-1688.

Vincent, R. R. and Williams, M. A. (2009). Microrheological investigations give insights into the microstructure and functionality of pectin gels. *Carbohydr. Res.* **344**, 1863-1871.

Winship, L. J., Obermeyer, G., Geitmann, A. and Hepler, P. K. (2010). Under pressure, cell walls set the pace. *Trends Plant Sci.* **15**, 363-369.

Wu, C. F. and Lew, D. J. (2013). Beyond symmetry-breaking: competition and negative feedback in GTPase regulation. *Trends Cell Biol.* **23**, 476-483.

Wu, J., Roman, A. C., Carvajal-Gonzalez, J. M. and Mlodzik, M. (2013). Wg and Wnt4 provide long-range directional input to planar cell polarity orientation in *Drosophila*. *Nat. Cell Biol.* **15**, 1045-1055.

Xu, T., Dai, N., Chen, J., Nagawa, S., Cao, M., Li, H., Zhou, Z., Chen, X., De Rycke, R., Rakusova, H., et al. (2014). Cell surface ABP1-TMK auxin-sensing complex activates ROP GTPase signaling. *Science* **343**, 1025-1028.

Xu, T., Wen, M., Nagawa, S., Fu, Y., Chen, J. G., Wu, M. J., Perrot-Rechenmann, C., Friml, J., Jones, A. M. and Yang, Z. (2010). Cell Surface- and Rho GTPase-Based Auxin Signaling Controls Cellular Interdigitation in *Arabidopsis*. *Cell* **143**, 99-110.

Xu, Z. and Dooner, H. K. (2006). The Maize *aberrant pollen transmission 1* Gene Is a *SABRE/KIP* Homolog Required for Pollen Tube Growth. *Genetics* **172**, 1251-1261.

Yang, G., Gao, P., Zhang, H., Huang, S. and Zheng, Z. L. (2007). A mutation in MRH2 kinesin enhances the root hair tip growth defect caused by constitutively activated ROP2 small GTPase in Arabidopsis. *PloS One* **2**, e1074.

Yang, Z. (2008). Cell polarity signaling in Arabidopsis. *Ann. Rev. Cell Dev. Biol.* **24**, 551-575.

Yi, K., Guo, C., Chen, D., Zhao, B., Yang, B. and Ren, H. (2005). Cloning and functional characterization of a formin-like protein (AtFH8) from Arabidopsis. *Plant Physiol.* **138**, 1071-1082.

Yi, K., Menand, B., Bell, E. and Dolan, L. (2010). A basic helix-loop-helix transcription factor controls cell growth and size in root hairs. *Nat. Genet.* **42**, 264-267.

Yoo, C. M. and Blancaflor, E. B. (2013). Overlapping and divergent signaling pathways for ARK1 and AGD1 in the control of root hair polarity in Arabidopsis thaliana. *Front. Plant Sci* **4**, 528.

Yoo, C. M., Quan, L., Cannon, A. E., Wen, J. and Blancaflor, E. B. (2012). AGD1, a class 1 ARF-GAP, acts in common signaling pathways with phosphoinositide metabolism and the actin cytoskeleton in controlling Arabidopsis root hair polarity. *Plant J.* **69**, 1064-1076.

Yoo, C. M., Wen, J., Motes, C. M., Sparks, J. A. and Blancaflor, E. B. (2008). A class I ADP-ribosylation factor GTPase-activating protein is critical for maintaining directional root hair growth in Arabidopsis. *Plant Physiol.* **147**, 1659-1674.

Zhang, H., Qu, X., Bao, C., Khurana, P., Wang, Q., Xie, Y., Zheng, Y., Chen, N., Blanchoin, L., Staiger, C. J., et al. (2010a). Arabidopsis VILLIN5, an actin filament bundling and severing protein, is necessary for normal pollen tube growth. *Plant Cell* **22**, 2749-2767.

Zhang, Y., He, J., Lee, D. and McCormick, S. (2010b). Interdependence of endomembrane trafficking and actin dynamics during polarized growth of Arabidopsis pollen tubes. *Plant Physiol.* **152**, 2200-2210.

Zhang, Y., Xiao, Y., Du, F., Cao, L., Dong, H. and Ren, H. (2011). Arabidopsis VILLIN4 is involved in root hair growth through regulating actin organization in a Ca2+-dependent manner. *New Phytol.* **190**, 667-682.

Zhang, Z. and Laux, T. (2011). The asymmetric division of the Arabidopsis zygote: from cell polarity to an embryo axis. *Sex. Plant Reprod.* **24**, 161-169.

Zhao, M., Morohashi, K., Hatlestad, G., Grotewold, E. and Lloyd, A. (2008). The TTG1-bHLH-MYB complex controls trichome cell fate and patterning through direct targeting of regulatory loci. *Development* **135**, 1991-1999.

Zhao, X. Y., Wang, Q., Li, S., Ge, F. R., Zhou, L. Z., McCormick, S. and Zhang, Y. (2013). The juxtamembrane and carboxy-terminal domains of Arabidopsis PRK2 are critical for ROP-induced growth in pollen tubes. *J. Exp. Bot.* **64**, 5599-5610.

Zhao, Y., Yan, A., Feijo, J. A., Furutani, M., Takenawa, T., Hwang, I., Fu, Y. and Yang, Z. (2010). Phosphoinositides regulate clathrin-dependent endocytosis at the tip of pollen tubes in Arabidopsis and tobacco. *Plant Cell* **22**, 4031-4044.

Zourelidou, M., Muller, I., Willige, B. C., Nill, C., Jikumaru, Y., Li, H. and Schwechheimer, C. (2009). The polarly localized D6 PROTEIN KINASE is required for efficient auxin transport in Arabidopsis thaliana. *Development* **136**, 627-636.