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3.7. Cytoskeleton and root hair growth

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Abstract

Root hairs are long tubular outgrowths of root epidermis cell that form to increase the root surface in order to assist in the uptake of water and nutrients from soil. Root hair development consists of two distinct processes: root hair initiation and tip growth. During both events, the dynamic organization of the cytoskeleton translates local signaling events into a focused growth response that is critical during root hair growth. Microtubules are primarily important for maintenance of the direction of tip growth. The actin cytoskeleton, on the other hand, is crucial for the selection of the root hair initiation site and maintenance of tip growth. The unique cytoskeletal organization found in growing root hairs controls the polar delivery of membranes to the apex in order to enlarge the cell unidirectionally. Signaling factors, such as calcium and reactive oxygen species, are instrumental in maintaining polarity of the cytoskeleton, vesicle trafficking, and ultimately root hair growth. Interestingly, these regulatory factors are interdependent upon each other, so that an elaborate set of feedback loops forms that results in a stable self-organized cell polarity.

3.7.1. Introduction

Root hairs are highly polarized outgrowths of a subset of root epidermis cells, the so-called trichoblasts. The biological function of root hairs is to increase the surface area of roots in order to facilitate the absorption of water and nutrients from soil. Root hairs are also the site of initial interaction with microorganisms (Geitmann and Emons, 2000). The patterning of root trichoblasts varies between plant species and can also be regulated by environmental factors (Dolan and Costa, 2001). These genetic and environmental regulatory mechanisms have been studied intensively and were reviewed elsewhere (Ishida et al., 2008). Trichoblasts are unique plant cells that first elongate by diffuse growth over their entire surface with the other root cells and subsequently form an outgrowth, the root hair, which elongates only at its very tip. This kind of tip growth also occurs in pollen tubes, which are discussed elsewhere in this volume.

The mechanism of root hair growth can be conceptually divided into two distinct events: root hair initiation, which breaks the symmetry of the root epidermis and results in the formation of a bulge, and unidirectional root hair elongation by tip growth in which all secretion of new cell wall material occurs in a small area at the tip of the hair. Root hairs are not critical for plant growth, so that plants can grow normally on growth media in the lab even with defective root hairs. This permitted studies about the molecular mechanisms underlying root hair development based on mutant screens for defects in root hair growth (Schiefelbein and Somerville, 1990). These mutants showed diverse phenotypes from abnormal length or shape of root hairs to additional root hair initiation. In independent studies, pharmacological analyses using chemicals to disrupt cytoskeletal organization revealed the importance of the cytoskeleton, especially actin filaments, for tip growth (Bibikova et al., 1999; Baluska et al., 2000). More recently, studies with GFP-fused proteins related to root hair growth provided further support for the emerging regulatory network and opened up the additional dimension of temporal dynamics (Hepler et al., 2001; Carol and Dolan, 2002; Cole and Fowler, 2006).

Collectively, these studies have established that three factors are very important for this special type of cell morphogenesis. First, signaling from the trichoblast determination pathway leads to a rearrangement of the cytoskeleton in the root epidermis for bulge formation and ultimately for support of tip growth in the emerging hair (Bibikova et al., 1999; Baluska et al., 2000). Second, polar membrane trafficking is required to provide new plasma membrane and cell

wall components to the growing tip (Ovecka et al., 2005). Finally, there is a unique distribution of regulatory factors, primarily calcium (Felle and Hepler, 1997), reactive oxygen species (Monshausen et al., 2007), and pH (Bibikova et al., 1998) which regulate each other to modulate tip growth (reviewed in Cole and Fowler, 2006). Pollen tubes, the other classical model for tip growth in plants, share a similar growth mechanism that involves similar key regulators (Cole and Fowler, 2006). However, there are also some distinct differences between pollen tubes and root hairs, such as vacuole position and growth rate. It remains to be seen whether a common model can simultaneously explain the specific behavior of root hairs and pollen tubes. However, in this chapter, we will forgo this comparison and focus on the mechanisms of root hair growth.

3.7.2. Root hair Initiation

The first visible step during root hair initiation is that a part of the outer epidermis cell starts swelling in response to local signaling. The position of the initial swelling depends on the plant species. For example, *Arabidopsis* root hairs are initiated on the basal part of the trichoblast, i.e. closest to the root tip (Carol and Dolan 2002), while maize roots form the root hair approximately in the middle of an epidermal cell (Baluska et al., 2000; Fischer et al., 2007). The positioning of root hairs on trichoblasts depends at least partially on hormonal signaling as revealed by mutant analysis. As the first step of root hair initiation, a membrane trafficking effector, ROP-GTPase, and a cell wall loosening enzyme, expansin, accumulate at the initiation site (Molendijk et al., 2001). Both actin and microtubule cytoskeleton then rearrange (Van Bruaene et al., 2004) and local alkalization of the cytosol occurs accompanied by acidification of the cell wall (Bibikova et al., 1998). In addition, the cytosolic calcium concentration increases locally due to a massive uptake of calcium from the environment (Very and Davies, 2000). Finally, actin filaments accumulate and the cell bulges outward and eventually begins tip growth (Ishida et al., 2008).

3.7.2.1. Selection of the bulge site

Auxin and ethylene signaling appears to be a key regulator in determining the position of root hairs, since many root hair defective mutants are directly or indirectly related to auxin or ethylene responses (Guimil and Dunand, 2007). In particular, ethylene signaling mutants showed altered position of root hairs on a trichoblast, suggesting that bulge site selection occurs

downstream of a hormone signaling pathway (Masucci and Schiefelbein, 1994). The auxin transport mutant, *aux1*, also displayed root hairs in an apically shifted position and often carried two root hairs on a cell (Grebe et al., 2002). The apical and basal membrane localization of AUX1 on root epidermis cells appeared to be required for maintaining planar root hair polarity by facilitating the uptake of auxin from more distal cells to maintain a local proximal auxin maximum in the trichoblast (Swarup et al., 2001). Moreover, pharmacological interruption of auxin transport also disrupted proper root hair positioning, supporting the critical role of auxin in determining the root hair position (Grebe, 2004).

How auxin and ethylene signaling affect cytoskeletal reorganization is still not clear. However, ROP-GTPase (Rho-like GTPase of plants) may be part of the signaling cascade. In *Arabidopsis*, immunolocalization of ROP proteins revealed their accumulation under the plasma membrane of the root hair initiation site even before swelling started, and this accumulation was maintained during root hair growth (Fu et al., 2002). This localization, together with evidence that overexpression of ROP-GTPase resulted in multiple root hair formation on a single trichoblast (Yang et al., 2007), further supports the notion that ROP-GTPases are key regulators for root hair initiation. ROP accumulation was not sensitive to cytoskeleton-disrupting drugs suggesting that ROP accumulation is an upstream event of actin and microtubule rearrangement in trichoblasts (Baluska et al., 2000; Molendijk et al., 2001). How localization and activity of ROP-GTPase is functionally regulated by polar auxin transport still remains an open question to be investigated. Recently, a mathematical simulation model hypothesized that the auxin gradient might function to balance the activity of ROPs, so that the root hair can be formed at the proper position of root epidermis cells (Payne and Grierson, 2009). Although there is as of yet little experimental support for this hypothesis, this model is a good starting point to identify the mechanisms that ultimately lead to selection of the bulge site.

3.7.2.2. The cytoskeleton in bulge formation

Root epidermis cells have highly organized transverse cortical microtubule arrays. Once a trichoblast starts root hair formation, this microtubule array becomes irregular and randomized at the site of the future root hair bulge (Baluska et al., 2000). This is correlated with local wall acidification which leads to a change in cell wall formation and ultimately results in wall thinning so that the cell can swell in this region (Bibikova et al., 1998). Remarkably, the apex of

the bulge is devoid of microtubules when the root hair swelling is ready to initiate tip growth (Baluska et al., 2000). At the same time, actin filaments at the future bulge site become arranged parallel to the long axis of the cell (Baluska et al., 2000). G-actin and profilin also accumulate at the tip of the root hair bulge, as observed by immunofluorescence in maize (Braun et al., 1999), and a fine actin meshwork later forms within the bulge (Baluska et al., 2000).

Actin mutants, *act2* and *act8*, showed root hair bulges but no proper tip growth suggesting that the actin cytoskeleton does not have a critical role in the early steps of root hair initiation (Ringli et al., 2002; Kandasamy et al., 2009). However, actin mutants often formed multiple sites of root hair bulges implying that actin might be involved in the process of determining the root hair initiation site on the trichoblast (Kandasamy et al., 2009). Lettuce seedlings that were germinated on media containing 10μM cytochalasin B, an inhibitor of actin polymerization, did not produce any root hairs (Takahashi et al., 2003). This result suggests that longitudinal redistribution of actin filaments on the site of root hair emergence is necessary for root hair initiation. However, cytochalasin D treatment of vetch roots did not show any effects on root hair bulge formation (Miller et al., 1999). Note, however, that cytochalasin variants have different side effects beside actin disruption. Thus, it is conceivable that long-term exposure of lettuce roots to cytochalasin B may have induced additional secondary effects that inhibited root hair development. The actin cytoskeleton is clearly more important in later steps of root hair initiation, namely, during the transition from bulge formation to tip growth since *actin* mutants as well as pharmacological interventions showed that a defect of actin organization prevented the initiation of root hair tip growth (Miller et al., 1999; Baluska et al., 2000; Ringli et al., 2002; Kandasamy et al., 2009).

3.7.3. Root hair tip growth: general considerations and organelle distribution

Once a bulge is established, root hairs continue to increase their surface area only at their tip, away from the root epidermis. *Arabidopsis* root hairs have been reported to be able to grow to a length of about 700μm with the growth rate of 1-2μm/min. However, these parameters can differ significantly depending on growth conditions (Ojangu et al., 2007; Monshausen et al., 2008). Conventionally, root hairs are divided into three zones from the tip toward the root epidermis, apex, subapex, and shank. This subcellular organization of growing root hairs shares some similarity with pollen tubes with some notable differences (compare chapter 3.2 for a

discussion of pollen tube growth). In growing root hairs, the dome-shaped apex, where active growth occurs, is predominantly filled with secretory vesicles. Large organelles are prevented from entering this region, presumably by mesh-like short, randomly distributed actin fragments in the subapex (Baluska et al., 2003). Unlike pollen tubes, apex and subapex of root hairs are often not clearly distinguished; the vesicle-filled apical zone is narrower than in pollen tubes and the actin mesh spreads more broadly. A major difference between root hairs and pollen tubes is the position of the large central vacuole. While the vacuole in pollen tubes is located far behind the tip in the shank, the vacuole in growing root hairs can reach into subapex (Cole and Fowler, 2006).

When root hairs stop growing, the vesicle accumulation in the apex disappears and the vacuole can fill the entire root hair. These characteristic changes in organelle distribution and cell morphology depend strongly on the cytoskeleton which in turn is regulated by a feedback loop involving membrane transport and several signaling molecules. [Figure 1 near here]

3.7.3.1. Actin cytoskeleton in root hair tip growth

Actin filaments are fundamental for root hair growth, similar to what was found in other cell types (reviewed in Smith and Oppenheimer, 2005; Hussey et al. 2006). Visualization of the actin cytoskeleton has been performed with several different probes in fixed or living root hairs. While longitudinal actin bundles along the shank of growing root hairs are relatively consistent in images with different probes, the existence of actin at the subapex and apex is somewhat controversial. Confocal microscopy images of actin visualized with a freeze-shattering technique using actin antibodies showed high actin accumulation at the maize root hair apex (Baluska et al., 2000). Labeling of globular actin (G-actin) by fluorescein isothiocyanate (FITC)-DNase I also showed that G-actin accumulated extensively in the apex in growing root hairs of wheat. This accumulation disappeared and was replaced by thick filamentous actin (F-actin) extending into the tip in fully grown root hairs (He et al., 2006). Co-visualization of G-actin and F-actin by labeling with FITC-DNase I and Tetramethyl Rhodamine Isothiocyanate (TRITC)-Phalloidin, respectively, showed distinct localization of G-actin and F-actin in growing root hairs. While G-actin accumulated at the apex, F-actin was presented in the shank of the growing root hairs and could not penetrate into the apex (He et al., 2006).

GFP-conjugated actin binding proteins also have been used to visualize actin filaments. This technique offers the advantage of revealing F-actin dynamics during root hair growth. However, this has to be balanced with potential artifacts resulting from over-expression of the labels. Initial experiments with GFP-talin transformed *Arabidopsis* displayed the high accumulation of actin at the apex of growing root hairs (Baluska et al., 2000). In subsequent years, it was revealed that over-expression of GFP-talin could cause severe developmental defects in transgenic plants (Ketelaar et al., 2004). Many studies now employ a fimbrin-based marker to visualize actin dynamics in all cells of several species (**Figure 3.7.1.A**). The second actin binding domain of fimbrin fused to the C-terminus of GFP (GFP-FABD2) displayed more fine actin filaments than GFP-talin and did not show any obvious developmental defects (Voigt et al., 2005). In contrast to GFP-talin, GFP-FABD2 (or GFP-FABD2-GFP for brighter signals) did not accumulate in the apex of growing root hairs of transgenic *Arabidopsis* seedlings (Sheahan et al., 2004; Wang et al., 2008). However, FABD2-GFP transformed *Medicago truncatula* displayed accumulation of actin at the apex (Miller et al., 1999; Voigt et al., 2005; Timmers et al., 2007), suggesting that actin organization might differ between the species. Recently, a new actin marker, Lifeact has been introduced which is based on the actin-binding domain of yeast ABP1 (Era et al., 2009). Lifeact-Venus revealed a similar actin cytoskeleton as GFP-FABD2, however, it had a better resolution at the root hair tip, so that an irregular actin mesh in the subapex and highly dynamic fine filaments reaching into the tip of the apex could be observed (Era et al., 2009).

Based on results from a variety of cell types, it is generally assumed that a central function of actin filaments is to deliver membrane compartments to the apex of root hairs in order to provide cell wall components and membrane lipids necessary for growth, as well as several regulatory factors (Baluska and Volkmann, 2002). The contribution of actin dynamics to root hair tip growth has been exposed by treatment with actin disrupting drugs, Latrunculin B (LatB) (Bibikova et al., 1997; Baluska et al., 2000) and cytochalasin D (Miller et al., 1999). The effect of LatB is dosage-dependent. A one hour treatment of as little as 50 nM LatB reduced root hair growth by 25% while concentrations higher than 500 nM led to complete cessation of root hair growth in *Arabidopsis* (Bibikova et al., 1999). Concentrations of more than 1 μM cytochalasin D also caused cytoplasmic streaming to stop within 30 min and 10 μM cytochalasin D could kill root hairs within 15 min in *Medicago* (Miller et al., 1999). Overall, disruption of

actin filaments causes root hairs to stop growing, suggesting that actin organization is critical for tip growth. Genetic studies have corroborated this conclusion. Over decades, numerous root hair mutants of *Arabidopsis* have been isolated in several studies (reviewed in Guimill and Dunand, 2007) and some of them are mutants of either components of actin filaments or regulators of actin dynamics. For example, *der1*, a mutant of *ACTIN2* (*ACT2*), displayed a cessation of root hair growth after bulging (Ringli et al., 2002) with additional pleiotropic phenotypes in different tissues (Gilliland et al., 2002). ACT8, the isoform most similar to ACT2, also contributes to root hair tip growth. *act8* mutants showed around 50% shorter root hairs than wild type and overexpression of *ACT8* could complement the *act2* mutant phenotype (Kandasamy et al., 2009). Mutation of several regulators of actin dynamics also displayed an arrest of tip growth in root hairs; induced overexpression of AIP1, a F-actin capping protein, resulted in short root hairs (Ketelaar et al., 2007), and overexpression of *PFN1*, an *Arabidopsis* profilin isoform, stimulated root hair tip growth and resulted in root hairs that were twice as long as wild type (Ramachandran et al., 2000). Finally, mutation of *AtFH8*, an *Arabidopsis* group Ie formin known to regulate actin dynamics, caused an arrest of root hair tip growth after bulge formation (Deeks et al., 2005).

3.7.3.2. Myosin in root hair tip growth

While the actin cytoskeleton has been recognized as an important factor for tip growth, the involvement of myosins, motor proteins that utilize F-actin as a track, was not clear until recently. Based on evidence from pollen tubes and circumstantial evidence from the importance of the actin cytoskeleton (Tang et al., 1989; Yokota et al., 2000), a contribution of myosins on root hair tip growth has been proposed (Tominaga et al., 2000). Recent studies with truncated class XI myosins fused to GFP variants at their N-terminus showed their localization on various membrane compartments suggesting a function in intracellular vesicle trafficking (Li and Nebenführ, 2007; Avisar et al., 2009). Direct evidence supporting the involvement of myosin in root hair tip growth was provided by the identification of a mutant of *Arabidopsis thaliana*, *xi-k*. *xi-k* plants showed short root hairs and abnormal trichome branching patterns (Ojangu et al., 2007; Peremyslov et al., 2008). *MYA2*, another one of the 13 class XI myosin genes in *Arabidopsis*, also seems to be involved in root hair tip growth based on the fact that *mya2* mutants also displayed short root hairs (Peremyslov et al., 2008). Interestingly, in these mutants,

movements of three organelles, Golgi, peroxisomes, and mitochondria, were dramatically slower than in wild type (Peremyslov et al., 2008). It is still unknown how a single myosin can contribute to the movement of three distinct organelles, particularly, since none of the tested YFP-myosin tail fusions localized to these organelles (Li and Nebenführ, 2007; Reisen and Hanson, 2007; Sparkes et al., 2008). A possible hypothesis is that one of the cargoes of XI-K might be a regulator of cytoskeleton dynamics, thus resulting in defects of movements of many organelles. In support of this contention, recent observation using variable-angle evanescent wave microscopy and spinning disc confocal microscopy showed that actin turnover might be required for myosin-based mitochondrial trafficking (Zheng et al., 2009). Thus, while it is now firmly established that myosins are involved in root hair tip growth, more research is needed to decipher the mechanism by which myosins operate in this process.

3.7.3.3. Microtubules and root hair tip growth

The organization of microtubules has been initially visualized in root hairs using electron microscopy and immunofluorescence with anti-tubulin antibodies (Lloyd and Wells, 1985; Traas et al., 1985). Later, transgenic plants expressing fluorescent proteins fused to α-tubulin 6 (GFP-TUA6) or to the microtubule binding domain of microtubule associated protein 4 (GFP-MBD), were used to reveal dynamic changes of microtubule organization during root hair growth (Bao et al., 2001; Timmers et al., 2007). After the reorganization event during root hair bulging, dense cortical microtubules (CMTs) are arranged longitudinally along the shank of the root hairs. Similar to the organization of F-actin, CMTs have not been detected in the apex of growing root hairs (**Figure 3.7.1.B**). Once root hairs are fully grown and the central vacuole approaches the root hair tip, longer and less dense CMTs are arranged longitudinally or spirally along the root hairs and can reach to the very tip of the root hairs (Van Bruaene et al., 2004; Timmers et al., 2007). Endoplasmic microtubules (EMTs) initially have been observed in CLSM images in the interior of root hairs expressing GFP-MBD in *Medicago truncatula* (Sieberer et al., 2002). EMTs displayed a more irregular arrangement than CMTs and predominantly accumulated around the nucleus as well as in the subapex. This distribution later has also been shown to exist in *Arabidopsis* root hairs (Van Bruaene et al., 2004). EMTs in the subapex of growing root hairs are highly dynamic. While the majority of CMTs array parallel to the shank of root hairs, EMTs at the subapex continuously change their directions and lengths (Van Bruaene et al., 2004).

In contrast to actin filaments, which play a major role in tip growth, microtubules are generally thought of being primarily important for maintaining the direction of root hair growth. Treatment with the microtubule depolymerizing drug, oryzalin, and the microtubule stabilizing drug, taxol, showed a loss of directionality of *Arabidopsis* root hair growth (Bibikova et al., 1999). Both taxol and oryzalin-treated root hairs displayed wavy root hairs as well as branched root hairs. Both drugs showed a similar effect on the angular deviation from straight root hairs, however, taxol was more effective in triggering branching than oryzalin (Bibikova et al., 1999), suggesting that the two drugs can distinguish between two distinct roles of microtubules during root hair growth. Analysis of mutants in α-tubulin (*tua6*) (Bao et al., 2001) and in a microtubule-associated protein (*mor1*) (Whittington et al., 2001) also revealed branched or wavy root hair growth, consistent with the pharmacological analysis. Thus, microtubules appear to be required for maintaining a stable polarity for straight growth.

The growth rates of *Arabidopsis* root hairs did not change during treatment with the microtubule disrupting drugs treatments (Bibikova et al., 1999) although *Medicago truncatula* root hairs had only 60% of root hair growth rate of untreated root hairs (Sieberer et al., 2002). Thus, it is likely that microtubules do not play a direct the role in elongating root hair cell surface. Recent studies of mutants in armadillo repeat-containing kinesins (ARKs or MRH2) of *Arabidopsis* showed abnormal root hair growth but no reduced root hair growth rate, further supporting a microtubule function in restricting the elongation zone of root hair tip growth (Yang et al., 2007; Sakai et al., 2008; Yoo et al., 2008).

Given their co-alignment patterns (Geitmann and Emons, 2000) and evidence from other cell types (Collings et al., 2006), an interaction between microtubules and the actin cytoskeleton has been proposed. Using propyzamide to depolymerize microtubules and cytochalasin B to destabilize actin filaments, the interaction between microtubules and actin cytoskeleton has been studied in detail in the root hairs of *Hydrocharis dubia* (Tominaga et al., 1997). Simultaneous treatment with both inhibitors was sufficient to stop cytoplasmic streaming and growth. Washout of Cytochalasin B in the presence of propyzamide did not allow full recovery of the longitudinal actin cytoskeleton indicating that longitudinal microtubules are required to establish the actin cables normally found in the root hair shank (Tominaga et al., 1997). At the same time, actin filaments appeared to be required for normal MT dynamics since longer treatment of root hairs with LatB permitted the formation of MT bundles in the apical or subapical regions (Timmers et

al., 2007). A possible candidate for this interaction between actin filaments and microtubules is ARK/MRH2 kinesin, since it could be shown that ARK1/MRH2 can bind to actin filaments in vitro (Yang et al., 2007). Further work will be necessary to confirm this hypothesis.

3.7.3.4. Membrane trafficking and tip growth

To increase the cell size, massive secretion at the tip is required to provide membrane and cell wall components. Additionally, active endocytosis occurs at the apex of root hairs to recycle regulators and remove excess membrane (Ovecka et al., 2005). These membrane trafficking activities are reflected in the accumulation of secretory and endocytic vesicles in the apex of growing root hairs. The small GTPase, RabA4b, has been successfully used as a vesicle marker in root hairs (**Figure 3.7.1.C**). In growing root hairs, RabA4b-YFP labeled vesicles accumulated at the apex of root hairs and LatB treatment released this accumulation coincident with a cessation of growth (Preuss et al., 2004).

It is not surprising that many root hair mutants are defective in genes encoding proteins involved in membrane trafficking (Gilliland et al., 2002). Members of the Ras-like small GTPase superfamily have roles on various endomembrane compartments and several mutants of these proteins showed impaired tip growth. A major example is ROP, a Rho-like GTPase that coordinates actin organization and membrane trafficking by stimulating multiple signaling pathways (Yalovsky et al., 2008). Beside its contribution to root hair initiation (see section 3.7.2.2.), ROP-GTPase functions as a critical player in root hair tip growth (Molendijk et al., 2001). ROP-GTPase is normally localized to almost the entire plasma membrane of plant cells but is restricted to the apical plasma membrane of growing root hairs. ROP-GTPase activates phosphatidyl-inositol (PtdIns)-monophosphate kinase (PIPK), a key regulator to maintain tip focused membrane trafficking and actin organization (Yalovsky et al., 2008). It also stimulates NADPH oxidase activity, which leads to the production of reactive oxygen species (Sheahan et al., 2004), a critical factor involved in regulating calcium gradients at the apex of root hairs (Baxter-Burrell et al., 2002). Many regulators of ROP-GTPase activity were also found to be involved in root hair tip growth. An *Arabidopsis* root hair defective mutant, *supercentipede 1* (*scn1*), has a similar phenotype to ROP-GTPase mutants, such that mutants have multiple root hair initiation sites in a cell and root hair tip growth is aborted (Carol et al., 2005). *SCN1* encodes a Rho-GTPase GDP dissociation inhibitor (RhoGDI) that restricts ROP activity to the apex in

order to maintain a polarity for root hair growth. Similarly, ROP localization on the plasma membrane is a prerequisite for its function and S-acylation of ROP-GTPase is necessary for its membrane localization (Yalovsky et al., 2008). *TIP1* encodes a S-acyl transferase (Hemsley et al., 2005) and *Arabidopsis tip1* mutants were initially isolated based on their extremely short, sometimes branched root hair phenotype (Schiefelbein et al., 1993). Since S-acyl transferases tend to modify only specific target proteins, it will be interesting to test whether ROPs are targets for TIP1.

Other regulators of root hair tip growth are specific phosphoinositides that can serve as a recognition landmark for certain cytosolic proteins, thus recruiting them to membrane patches where they then perform their functions. For example, $PI(4,5)P_2$ accumulates in the apical plasma membrane of root hairs and many modifying proteins of this lipids are important for the root hair tip growth mechanism (Cole and Fowler, 2006; Xue et al., 2009). RabA4b interacts with PtdIns-4OH Kinase, PI-4Kβ1 that synthesizes a precursor of $PI(4,5)P_2$ and both proteins colocalize to small vesicles at the apex of growing root hairs (Preuss et al., 2006). A *pi4kβ1/2* double mutant displayed shorter root hairs than wild type, which were often branched or jagged. PI-4Kβ1 also binds to CBL1, a calcium sensor, implicating that activity of PI-4Kβ1 is depending on the calcium gradient present at the root hair apex. Another root hair defective mutant, *rhd4*, showed short and wavy root hairs and RabA4b-YFP accumulation at the apex was altered in the mutants (Thole et al., 2008). *RHD4* encodes PI4P-phophatase and might function to balance PI4P levels to maintain polar tip growth at the root hair apex. RHD4 might also have a function in actin organization since *rhd4* mutants showed more patchy actin organization and thinner filaments in root epidermis cells compared with wild type, however, the detailed mechanism of this interaction is not yet clear.

3.7.3.5. Signaling factors in tip growth

It has long been known that intracellular calcium gradients in the root hair cytoplasm have an important role in root hair growth (Hepler et al., 2001). Besides a role of calcium as a second messenger in signaling cascades, it has been suggested that calcium might restrict the site of membrane trafficking and also modify the cytoskeleton at the root hair tip so that the root hair can grow straight (Wymer et al., 1997). For example, calcium can affect F-actin polymerization by controlling actin-binding protein activity, such as profilin, actin-depolymerizing factor

(ADF), or villin (reviewed in Hussey et al., 2006). In support of this, profilin also accumulates in the root hair tip (Baluska et al., 2000) and can lead to sequestration of G-actin in a calcium dependent manner (Kovar et al., 2000). Calcium may also control tip growth by several other mechanisms in addition to a modulation of F-actin dynamics. For example, G-actin accumulation at the apex of wheat root hairs as visualized with FITC-DNase I could be disrupted by treatment with dibromo-l,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) (He et al., 2006). BAPTA is well known to rapidly dissipate calcium gradients at the growing root hair tip and consequently stops root hair tip growth (Felle and Hepler, 1997). It was also shown that membrane trafficking is regulated by the calcium gradient since treatment with the calcium ionophore A23187 caused the release of the accumulation of RabA4b-YFP from the apex and a stop of root hair growth (Preuss et al., 2006). Despite these insights, the precise mechanism by which calcium regulates tip growth is still unknown. Given the role of calcium as a key regulatory factor for many cellular events, it is likely that the calcium gradient coordinates tip growth by acting through multiple regulatory factors at the same time.

Similar to pollen tubes, the tip-focused calcium gradient in root hairs has been shown to oscillate within a range from 0.2 μM to more than 1.5 μM (Bibikova et al., 1997; Wymer et al., 1997). These calcium oscillations are tightly correlated with the cell growth rate (Monshausen et al., 2008). Recent studies have investigated this connection in more detail by using the ratiometric calcium marker, yellow Cameleon 3.6 (Monshausen et al., 2008). This study demonstrated that root hair growth rate oscillations are typically followed by cytosolic calcium oscillation at the root hair apex with about 5 sec lag time. This observation suggests that a high concentration of cytosolic calcium at the root hair apex restricts root hair growth. This hypothesis was supported by observations that treatment with 200 μM La^{3+}, a blocker of Ca^{2+} channels, led to an acceleration of root hair growth. Similarly, treatment with 10 μM A23187, a calcium ionophore, blocked root hair growth (Monshausen et al., 2008). Cytosolic calcium gradients are accompanied by pH changes and a ROS gradient in the cytosol as well as in the cell wall space. ROS are also considered a critical factor to sustain polar growth, since *rhd2*, a mutant of *Arabidopsis* NADPH oxidase, displayed short root hairs and the typical calcium gradient in root hair tip was absent (Foreman et al., 2003). Given that addition of external ROS can recover the root hair elongation and ROS can activate calcium channels, it is likely that accumulation of ROS at the tip is required to open calcium channels at the plasma membrane of the root hair apex

in order to increase the cytosolic calcium concentration at the tip (Foreman et al., 2003). Interestingly, unlike animal NADPH oxidase, plant NADPH oxidases have two EF hand motifs at their N-terminus suggesting their regulation by calcium (Sagi and Fluhr, 2001). Indeed, RHD2 can be activated by calcium in the growing root hair, resulting in a positive feedback loop (Sagi and Fluhr, 2001; Takeda et al., 2008). This inter-dependence of calcium and ROS signaling leads to alternating oscillations of ROS and Ca^{2+}gradients in the growing root hair tip, which appears to be necessary for maintaining polar root hair growth (Monshausen et al., 2007).

3.7.4. Conclusions

Root hairs are very dynamic cells which can grow relatively fast compared to other plant cells. They are also unique in their asymmetric outgrowth from a part of root epidermis cells. The growth of root hairs is regulated by a highly elaborate mechanism that involves many components, such as the cytoskeleton, membrane trafficking, and signaling factors. Two distinct steps of root hair development, root hair initiation and tip growth, are regulated in different ways by the regulatory factors (**Figure 3.7.2.**). However, there are common rules for this regulatory system. First, signals from genetic and environmental cues are translated into a change of the cytoskeleton. Second, the dynamic cytoskeleton rearrangement results in polar trafficking of membrane compartments to facilitate surface increase at the tip. These processes are coordinated by signaling factors that help to maintain cell polarity and allow growth only at the root hair tip. Importantly, these functional steps are all interdependent, so that they reinforce each other in a series of interlocking feedback loops (**Figure 3.7.3.**).

ROPs are critical for both maintaining cell polarity and growth of root hairs since they determine the site of exocytosis and maintain calcium oscillation by activating ROS production which in turn activates calcium channels. The feedback loop of calcium and ROS oscillation is translated into the distinct organization of actin cytoskeletons in different region of root hairs. High calcium concentration prevents F-actin formation at the apex but allows formation of a dynamic actin mesh in the subapex. Further back in the root hair, F-actin cables parallel to the shank of root hairs deliver organelles to the subapical region. Among these organelles, Golgi stacks provide membrane compartments that contain key regulators to the tip of root hairs. As a result, vesicles accumulate in the apex and exocytosis occurs preferentially at the tip. Exocytosis of calcium channels reinforces the calcium oscillations thereby stabilizing the feedback system.

At the same time, PtdIns 4,5-P_2 (PIP_2), a derived membrane lipid that is deposited at the apex by exocytotic vesicles, activates ROP proteins to stimulate ROS production, while ROP activity is spatially limited by interaction with RhoGDI in the subapex. Remarkably, ROPs activate PIP-kinase to produce PIP_2 in the membrane, resulting in a positive feedback loop that stabilizes ROP activity at the apex. The net result of these interdependent feedback loops is a series of stable oscillation that ensures polar growth over long periods.

Every factor in this regulatory network plays a pivotal role, so that slight imbalances of any of these factors affect the entire system, resulting in failure of normal root hair growth. Recent studies have identified many of the genes responsible for root hair development and their functions, and made big strides in revealing the underlying molecular mechanisms of root hair development. However, it should be emphasized that many details still need to be clarified. For example, the precise role of myosins on root hair development and how the contribution of endocytosis to tip growth still remain in question. Further genetic, cell biological, and biochemical studies to investigate the dynamic interplay of the various factors involved in root hair growth combined with the collection of quantitative data and computational modeling will be necessary to elucidate these and other questions.

3.7.5. References

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FIGURE 1

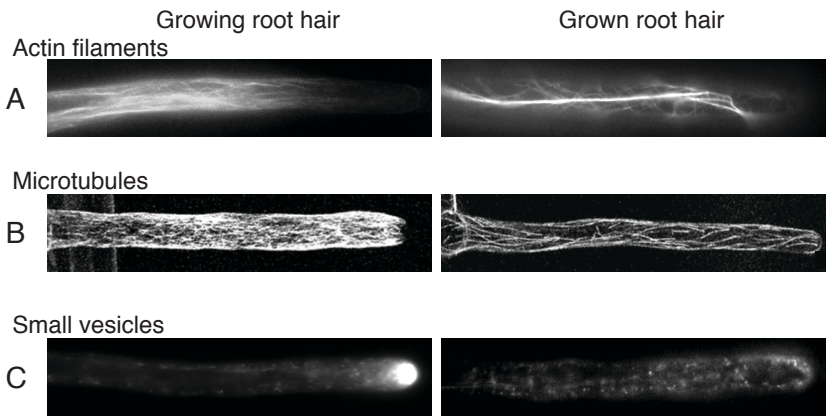

Figure 3.7.1. Distinct distribution of cytoskeletal elements and vesicles during root hair tip growth

(A) Actin cytoskeleton, visualized with FABD2-YFP. In growing root hairs, cortical F-actin filaments array mostly longitudinally along the shank and are absent from subapex and apex of root hairs. In contrast, thick bundles of actin cables reach into the apex in fully-grown root hairs.

(B) Microtubules, visualized by GFP-MBD. Growing root hairs display longitudinal or helical microtubules along the root hair shank. Note that microtubules do not reach the extreme apex of the growing root hair while they do so in growing root hairs (modified after Van Bruaene et al. 2004).

(C) RabA4b-YFP-labeled vesicles accumulate at the tip of growing root hairs. This accumulation is tightly correlated with root hair growth; consequently, fully-grown root hairs lack the accumulation of RabA4b-YFP vesicles at the tip.

FIGURE 2

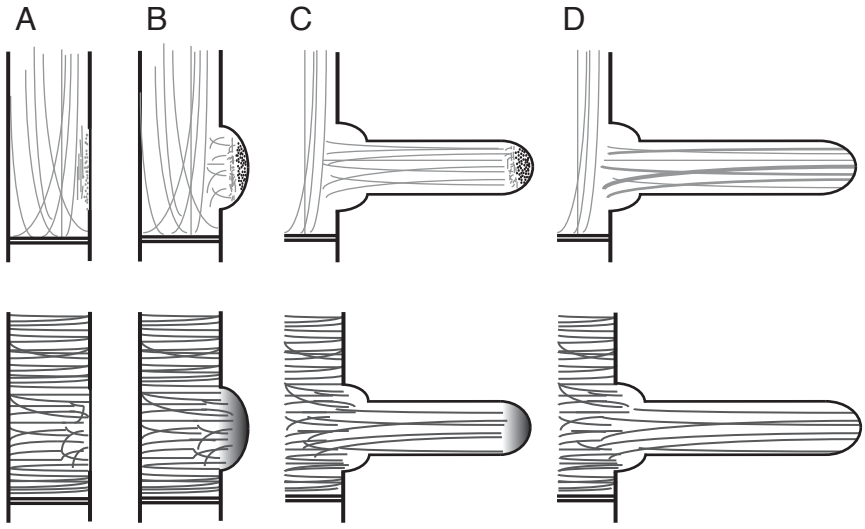

Figure 3.7.2. Cellular architecture during root hair development

Schematic representation of root hairs during four stages of root hair development. Upper panels show actin filaments (grey lines) and secretory vesicles (black dots). Lower panels show microtubules (darker grey lines) and the tip-focused calcium gradient (darker color represents higher calcium concentration).

(A) Early events after bulge site selection. Note the fragmentation of actin filaments and the local loss of microtubule organization.

(B) Initial outgrowth and bulge formation. Actin filaments begin to form a dense mesh behind the tip region where vesicles accumulate and the calcium gradient forms.

(C) Tip growth. Cytoskeletal elements are mostly longitudinal along the shank but do not reach into the apex. Vesicle accumulation and calcium accumulation are maximal at the apex.

(D) Fully grown hair. Vesicle accumulation and calcium gradient at the tip disappear and longitudinal actin filaments and microtubules reach into the apex.

FIGURE 3

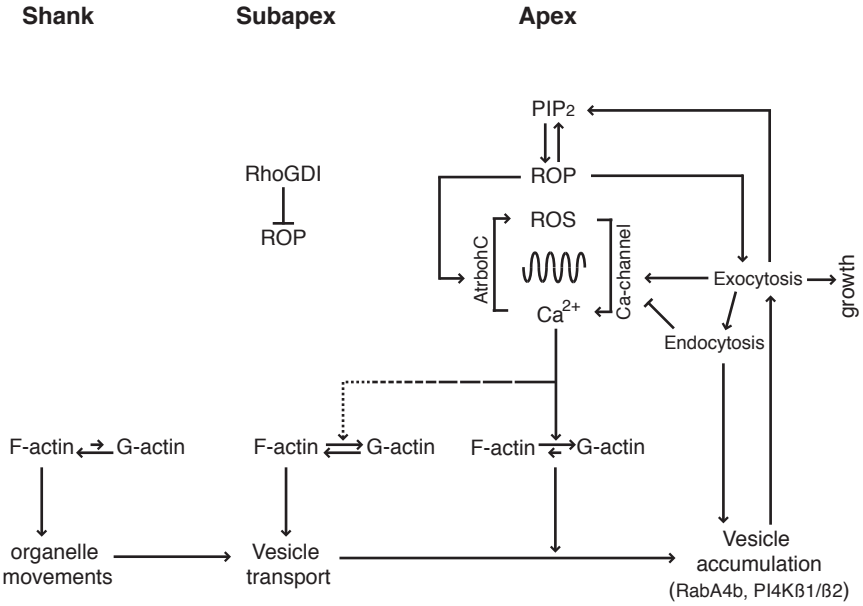

Figure 3.7.3. Self-reinforcing feedback regulation of tip growth

Simplified model of regulatory mechanisms that affect the actin cytoskeleton in different areas of the root hair during tip growth. For details see text.