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REVIEW

Actin organization and regulation during pollen tube growth

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Abstract **Pollen is the male gametophyte of seed plants and its tube growth is essential for successful fertilization. Mounting evidence has demonstrated that actin organization and regulation plays a central role in the process of its germination and polarized growth. The native structures and dynamics of actin are subtly modulated by many factors among which numerous actin binding proteins (ABPs) are the most direct and significant regulators. Upstream signals such as Ca^{2+}, PIP_2 (phosphatidylinositol-4,5-bis-phosphate) and GTPases can also indirectly act on actin organization through several ABPs. Under such elaborate regulation, actin structures show dynamically continuous modulation to adapt to the *in vivo* biologic functions to mediate secretory vesicle transportation and fusion, which lead to normal growth of the pollen tube. Many encouraging progress has been made in the connection between actin regulation and pollen tube growth in recent years. In this review, we summarize different factors that affect actin organization in pollen tube growth and highlight relative research progress.**

Keywords pollen tube, tip growth, actin organization, actin-binding protein, signaling pathway

Introduction

Pollen is the male gametophyte of seed plants. Well-programmed pollen development, germination, and tube growth are essential for successful fertilization. As one of the fastest growing eukaryotic cells (e.g., maize pollen tubes grow at 1 cm per hour) (Bedinger, 1992), pollen tubes elongate within pistil tissues to transport sperm cells to ovules for fertilization (Hepler et al., 2001; Lord and Russell, 2002). Rapid growth is supported by highly active exocytosis and endocytosis at the plasma membrane, which requires coordination between vesicle trafficking dynamics, signaling pathways and the cytoskeleton organization (Franklin-Tong, 1999; Chen et al., 2003; de Graaf et al., 2005; Gu et al., 2005).

Polarized pollen tube growth results from continued transportation and fusion with the plasma membrane by secretary vesicles derived from the Golgi apparatus. Growing pollen tubes apparently contain multiple forms of actin filaments: fine, less abundant but highly dynamic actin filaments in the clear zone, a dense cortical fringe or collar of microfilaments just behind the clear zone, and the abundant longitudinal actin cables in the shank (Hepler et al., 2001; Sagot et al., 2002; Higashida et al., 2004). This multiple forms of actin microfilaments, carefully regulated by numerous actin binding proteins (ABPs), always play significant roles in vesicles trafficking and fusion. Additionally, these processes are modulated by many signaling pathways during germination and tip growth, such as Ca^{2+}, calmodulin, phosphoinositides, protein kinases, cyclic AMP, and GTPases.

In the following review, we summarize studies that have provided insights into actin organization and the regulation of its dynamics in the pollen tube elongation and examine the areas requiring further study.

Actin organization in the process of pollen tube growth

Pollen tubes extend through tip growth and the tip region shows strong zonation. An apical region or clear zone, a sub-apical, organelle rich zone, a nuclear zone, and a distal vacuolated zone or plug region that may extend several centimeters are easily recognized (Mascarenhas, 1993). Elongating pollen tubes show a highly polarized cytoplasmic organization (Steer and Steer, 1989; Derksen et al., 1995; Hepler et al., 2001; Cheung and Wu, 2007, 2008), referred to

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as reverse fountain cytoplasmic streaming: vesicles in cytoplasmic streaming rapidly move to the apex along the cortex and move back in the center of the cell once reaching the tip region. Numerous studies in chemically fixed and living pollen tubes reveal a close relationship between cytoplasm streaming of vesicles and diverse actin structures in pollen tubes. Similarly, careful examination of pollen tubes from a variety of species gives a consensus view of filamentous actin (F-actin) that in the different zones as to apex, sub-apex and shank of pollen tube being arrayed into at least three distinct structures (Kost et al., 1998; Fu et al., 2001; Lovy-Wheeler et al., 2005; Cheung and Wu, 2008; Vidali et al., 2009): fine, less abundant but highly dynamic actin microfilaments in the apical region or clear zone, a dense cortical fringe or collar of microfilaments just behind the clear zone, and the abundant longitudinal actin cables in the shank (Hepler et al., 2001; Sagot et al., 2002; Higashida et al., 2004). The review of Staiger et al. (2010) showed the F-actin in pollen tubes from corn and the field poppy that decorated with rhodamine-phalloidin (see also Gibbon et al., 1999; Geitmannet al., 2000; Snowman et al., 2002; Thomas et al., 2006).

The long actin cables that throughout the shank of the tube, reaching the subapical region but not readily observable within the apical dome, have been proposed to regulate the cytoplasmic streaming by serving as the tracks for vesicle transportation to the apical region of the pollen tube. A prominent actin structure comprising shorter actin cables is consistently observed in the subapical region, but is variably referred to as a ring or a collar (Kost et al., 1998; Gibbon et al., 1999; Fu et al., 2001), a mesh (Geitmann et al., 2000; Chen et al., 2002), a funnelor basket-like structure (Vidali et al., 2001; Hormanseder et al., 2005), and a fringe (Lovy-Wheeler et al., 2005). These seemingly variant structures would seem to suggest a structure that is constantly in flux and highly sensitive to the constantly fluctuating cytoplasmic conditions; or they may reflect a highly fragile structure easily perturbed by fixation or binding by actin reporter proteins, rendering an accurate representation difficult. The role of the actin fringe is not clear, although it is proposed to mediate endocytosis to retrieve excess materials deposited by exocytosis and/or the cytoplasmic organization of the apical region (Lovy-Wheeler et al., 2005; Cárdenas et al., 2008). In the apical region of pollen tubes, fine and subtle structures of actin filaments have been observed. These actin filaments present less abundant but highly dynamic features and is suggested to modulate the apical accumulation of exocytic vesicles and their exocytosis (Sagot et al., 2002; Lee et al., 2008). Besides, both the actin cables and the subapical, cortical fringe also appear to be involved in movements of endoplasmic reticulum (ER) and mitochondria (Lovy-Wheeler et al., 2007).

Although there are still some questions about the true state of the actin organization, it is widely acknowledged that the apical and subapical actin arrays are critical for pollen tube growth. Compelling evidence comes from treatments with the actin-monomer binding drug, Latrunculin B (LatB), (Gibbon et al., 1999; Vidali et al., 2001). Low-dose of LatB perturbs the tip actin organization without markedly altering cytoplasmic streaming or the axial, actin cables, providing indirect evidence for rapid turnover of apical and subapical actin filaments. The dynamic tip actin filament arrays may play a significant role in coordinating secretory vesicle docking and fusion at the apex, as demonstrated recently by Zhenbiao Yang and coworkers (Hwang et al., 2008; Lee et al., 2008). The authors reported that actin polymerization is necessary for secretory vesicles to accumulate in the apical inverted cone and for vesicle docking or fusion at the plasma membrane (Lee et al., 2008). Some alternative models also suggest that actin polymerization contributes directly to pollen tube extension by pushing on the plasma membrane (Mathur, 2005) or permits the pollen protoplast to adhere and 'crawl' along the cell wall analogous to animal cells moving over an extracellular matrix (Lord et al., 1996). However, these models seem rather implausible given that the growth of pollen tubes and plant cells is constrained by a semi-rigid cell wall. In all likelihood, turgor pressure is the likely driving force for growth, with actin contributing via the delivery of new plasma membrane and polysaccharides that expand the cell wall by intercalation of new polymers among old ones (Szymanski and Cosgrove, 2009).

Regulation of the actin organization in the pollen tube

Major pollen actin binding proteins (ABPs) and their functions

To understand how pollen actin turnover is regulated *in vivo*, first, it is necessary to have detailed knowledge about the biochemical properties, cellular abundance, and localization of diverse ABPs in the pollen tube. In eukaryotic cells, more than 70 classes of ABPs have been identified (Kreis and Vale, 1999; Pollard et al., 2000) and an ever expanding subset of these is present in angiosperm pollen (see also Ren and Xiang, 2007; Cheung and Wu, 2008). These factors exert distinct, but often overlapping effects, on actin organization and polymerization. Monomer binding proteins regulate the size and activity of the actin subunit pool. Severing proteins create breaks in the filament backbone, generating new ends for assembly or disassembly. Capping proteins bind with high affinity to filament ends and prevent subunit loss and addition, as well as inhibiting filament–filament annealing. Nucleation factors overcome the rate-limiting step for actin assembly and generate seeds that support subsequent elongation. These ABPs are also reliable sensors and transducers of signaling cascades, as their activities are almost always regulated by Ca^{2+}, pH, and phospholipids. Several excellent reviews deal with ABPs function in plants

and the reader is referred to these for additional information (Hussey et al., 2006; Staiger and Blanchoin, 2006; Thomas et al., 2009). The general properties and pollen-specific characteristics of several central regulators of actin dynamics are summarized here.

Profilin and CAP1

Profilin, the first ABPs identified in angiosperm pollen, was discovered as an allergen from birch trees (Valenta et al., 1991). Profilins are low molecular weight proteins that bind to globular-actin (G-actin) with 1:1 stoichiometry and form moderate affinity profilin–actin complexes (Valenta et al., 1993; Gibbon et al., 1998). Immunocytochemistry and microinjection of fluorescent analogs reveal that profilin is a uniformly-distributed, cytosolic protein (Vidali and Hepler, 1997). In pollen, profilin is present at levels equimolar with total actin and has an estimated cellular concentration of 25–200 μmol/L (Vidali and Hepler, 1997; Gibbon et al., 1999; Snowman et al., 2002). The high concentration of profilin and its affinity ATP–G-actin lead to the prediction that most pollen actin will be present as profilin–actin complex (Gibbon et al., 1999; Snowman et al., 2002; Staiger and Blanchoin, 2006). This complex prevents spontaneous nucleation of new actin filaments and suppresses addition at filament minus-ends. When uncapped actin filaments present, profilin shuttles actin subunits onto filament barbed-ends and contributes to elongation. In contrast, when the barbed-end of filaments is capped, profilin acts like a simple sequestering protein. Several models for actin filament turnover suggest that profilin plays an additional role, as a catalyst for nucleotide exchange on ADP–G-actin that serves to recharge subunits with ATP. Plant profilins do not have this capability, however, even when supplied with actin from a plant source (Perelroizen et al., 1996; Kovar et al., 2001). This might be because nucleotide exchange is not important due to the high endogenous rate of turnover on native pollen actin (Kovar et al., 2001), or because other cellular factors have assumed this role (Chaudhry et al., 2007). Besides binding to actin, profilin also binds to poly-L-proline (PLP), an important motif that existed in some other ABPs such as Formins. Phosphatidylinositol-4,5-bisphosphate (PIP_2), a component of the phosphatidylinositol cycle employed in cell signaling events, also binds to profilin but causes the profilin–actin complex to dissociate. The localization and binding properties of profilin thus suggest that it acts at a critical control point in signaling pathways initiated by events at the plasma membrane and plays an important role in regulating the activity in the microfilament system and intracellular calcium levels.

CAP1 or the adenylate cyclase-associated protein, another abundant monomer binding protein in plants, binds with moderate affinity to G-actin (Chaudhry et al., 2007; Deeks et al., 2007). CAP1 binds with equal affinity to ATP–G- and ADP–G-actin, which contrasts with yeast CAP (Srv2p) that has a marked preference for ADP–G-actin (Chaudhry et al., 2007). Importantly, CAP1 directly enhances nucleotide exchange on actin, by more than 50-fold. It also has a weak ability to shuttle subunits onto the plus-end of filaments. Loss-of-function *cap1* mutant *Arabidopsis* plants have significant defects in pollen germination and tube growth, consistent with a major role in regulating actin dynamics in tip-growing cells (Deeks et al., 2007). However, the nature of actin organization and dynamics in *cap1* mutant pollen, or the subcellular distribution and concentration of CAP1 is not presently known.

Villin/gelsolin/fragmin superfamily proteins

Villin/gelsolin/fragmin superfamily proteins, are identified by sharing three (fragmin, capG) or six (gelsolin,villin) 15 kDa gelsolin-like repeat domains (Way and Weeds, 1988). All members in this family have the conserved gelsolin domains, and some of them have special amino acids sequences in N- (e.g. flightless I) or C- (e.g. villin) terminals, the complicated protein structure leads to the diverse function in some extents. Villin/gelsolin/fragmin superfamily members can sever, cap, nucleate and bundle actin in a Ca^{2+} and/or PIP_2-regulated manners. Severing is a prominent feature responsible for the stochastic dynamics of individual actin filaments in live cells (Vavylonis et al., 2008; Staiger et al., 2009; Okreglak and Drubin, 2010) and it is therefore critical to understand the molecular mechanisms that underpin filament breakage.

Villins were the first actin filament-bundling proteins identified from plants, through the biochemical tour de force of Teruo Shimmen and Etsuo Yokota that used many grams of germinated Easter lily (*Lilium longiflorum*) pollen as starting material (Nakayasu et al., 1998; Yokota et al., 1998; Yokota and Shimmen, 1999). Pollen-135-ABP and P-110-ABP were purified to homogeneity and shown to bundle actin filaments in a calcium- and calmodulin-sensitive manner (Yokota et al., 1998, 2000, 2003; Yokota and Shimmen, 1999). Additional villin-related polypeptides with lower molecular weights have been identified recently from *Lilium davidii* and *L. longiflorum* pollen (Fan et al., 2004; Xiang et al., 2007; Wang et al., 2008). These proteins may be splice variants of villins or proteolytically processed villin isoforms generated from the full-length ABP. Nevertheless, they are able to disrupt actin cable maintenance, tip growth and organization of the tip zone following overexpression by microinjection or bombardment into pollen tubes (Fan et al., 2004; Xiang et al., 2007).

So far as we known, *Arabidopsis* genome has five villins, named *AtVLN 1– AtVLN 5* (Klahre et al., 2000; Staiger and Hussey, 2004). The recombinant protein of AtVLN-GFP and AtVLN headpiece-GFP can bind to actin *in vivo* (Klahre et al., 2000). Surprisingly, unlike other reported plant villins, recombinant AtVLN1 lacks the Ca^{2+}-dependent severing, capping, and nucleating activities *in vitro* while it only has the function of binding to actin and bundling F-actin in a

Ca^{2+}-independent manner. AtVLN1 also could inhibit actin depolymerization by ADF/cofilin *in vitro* (Huang et al., 2005). Another member, *Arabidopsis thaliana* VILLIN5 (VLN5) is highly and preferentially expressed in pollen. Its loss-of-function retarded pollen tube growth and sensitized actin filaments in pollen grains and tubes to LatB. *In vitro* biochemical analyses revealed that VLN5 is a typical member of the villin family and retains a full suite of activities, including barbed-end capping, filament bundling and calcium-dependent severing. A total internal reflection fluorescence microscopy (TIRFM) assay demonstrates the severing activity of VLN5 on individual actin filaments and confirms data from solution-based biochemical assays. Moreover, severing is stimulated by physiologic Ca^{2+} concentrations, implying that it is biologically relevant. VLN5 is a major actin filament stabilizing factor as well as a regulator of actin dynamics that functions in concert with oscillatory Ca^{2+} gradients and regulates pollen tube growth (Zhang et al., 2010). Furthermore, in the presence of physiologic [Ca^{2+}], VLN3 severed actin filaments in the presence or absence of VLN1 *in vitro* (Khurana et al., 2010). Our laboratory find that recombinant AtVLN4 generates long actin bundles at low concentration of Ca^{2+}, shortened the length of actin filaments and generated short bundles through its actin-bundling, -depolymerizing and-capping activities at high concentration of Ca^{2+} *in vitro*. In *atvln4* mutants, we find that AtVLN4 disturbs actin filaments bundling and cytoplasmic streaming in root hair development (Zhang et al., unpublished data). The growth model of root hair is mostly similar to that of pollen tube, so we presume that AtVLN4 may be also participating in pollen tube.

It is well accepted that gelsolin/fragmin family members are generated by mRNA alternative splicing from plant villin (Fan et al., 2004; Huang et al., 2004; Staiger and Hussey, 2004). Gelsolin is composed of six gelsolin homology domains (G1–G6) and has Ca^{2+}-stimulated F-actin-severing activity. Gelsolin also caps the barbed ends of actin filaments and nucleates new filaments. Gelsolin-like proteins have been identified by immunoblotting in maize (*Zea mays*) and *Lilium longiflorum* pollen (Wu and Yan, 1997; Tao and Ren, 2003). Recently, this hypothesis is confirmed by the discovery of actin binding protein 29 (ABP29) in lily pollen. In their assay, Xiang et al. (2007) has cloned a 1006 bp full-length cDNA sequence from *Lilium* pollen, apart from the 16 bp sequence starting with GT before the stop codon TAA and the whole 3′UTR, which is absolutely identical to P-135-ABP. The sequence encodes a 29 kDa protein (ABP29) that merely contains G1 and G2 domains, which is the smallest member in villin/gelsolin/fragmin superfamily. ABP29 can sever, nucleate, cap F-actin *in vitro* and these activities are all Ca^{2+} and PIP_2 regulated. Further study shows that the specific sequences of ABP29 are derived from the intron of the gene encoding P-135-ABP. In addition, GT at acceptor (5′) splice site is a conserved sequence for the majority of introns, together strongly suggests that ABP29 is an mRNA alternative splicing product of a pre-ended transcription from plant villin. Lately, the expression pattern of the villin/gelsolin/fragmin superfamily proteins during lily pollen tube development was detected using the gel blot analysis. Due to the high homology among this superfamily, the purified anti-LdABP41 antibody can recognize ABP29 (Xiang et al., 2007), LdABP41 (Fan et al., 2004), ABP80 (Huang et al., 2004), ABP115 (Nakayasu et al., 1998; Yokota et al., 2003) and ABP135 (Yokota and Shimmen, 1999) from dehydrated, hydrated or germinating lily pollen grains, however, their expressed levels changed greatly in the different stages. It is showed that LdABP41 is abundant in the ungerminated pollen, however, the amount of LdABP41 decreases dramatically, and ABP80, ABP115 and ABP135 merely turn up after the pollen germinated, but ABP29 levels remain almost constant. Furthermore, the specific expression patterns of different members correlate highly with actin architecture corresponding to different pollen stages (Xiang et al., 2007; Wang et al., 2008).

Capping proteins

The turnover of actin filaments is also modulated by a class of proteins that bind and cap filament ends, called capping proteins. The best characterized of these proteins in plants is the heterodimeric capping protein from *Arabidopsis* (AtCP) (Huang et al., 2003, 2006). AtCP binds with nanomolar affinity to filament plus-ends and prevents subunit loss and addition at those ends (Huang et al., 2003). It also inhibits end-to-end annealing of filaments (Huang et al., 2003) and competes with formin for binding at filament ends (Michelot et al., 2005). So the presence of capping protein (CP) in pollen is one of contributing factors in providing a large monomer pool and small filament pool (Staiger and Blanchoin, 2006). In other systems, several CP-interacting proteins have been identified; in some cases, their interaction leads to CP being removed from filament ends (Cooper and Sept, 2008). No such proteins from plants or pollen have been identified yet; nevertheless, AtCP was shown to bind to and be regulated by PIP_2 and phosphatidic acid (PA) (Huang et al., 2003; Huang et al., 2006), which may be relevant to the motility of organelles or the plasma membrane, given that the inactivation of CP by the phospholipids is predicted to lead to the polymerization of actin filaments near the surface of organelles or the plasma membrane.

Formin proteins

The formation of actin nuclei is a rate-limiting step during spontaneous filament assembly. Within the cell, actin nucleation factors are responsible for the generation of actin nuclei, providing a mechanism for the cell to regulate when and where to assemble actin filaments. Formin is a large family of proteins sharing the evolutionarily conserved formin homology domains FH1 and FH2 that are present in nearly all eukaryotes (Cvrcková et al., 2004; Chalkia et al.,

2008; Blanchoin and Staiger, 2010). The FH2 domain is essential for actin filament nucleation, whereas the FH1 domain recruits profilin–actin complexes to the assembly machine. In addition to nucleating filament formation, many formins are processive assembly motors, remaining attached to the plus-end as they supply new monomers to the elongating filament. Apart from the two FH domains, several other domains were identified in the N-terminus and C-terminus of different yeast or animals formin proteins, such as formin homology 3 (FH3) domain, GTPase binding domain (GBD), Dia-autoregulatory domain (DAD), Bud6p binding site (BBS), trans-membrane domain (TM) (for review, see Guo and Ren, 2006). However, the domain composition of plant formin is quite different from its counterparts in other organism, which has no FH3, GBD or DAD for localization and activation.

Formin forms dimers and acts as a processive or "leaky" cap at the barbed ends in yeast and animals nucleates actin filaments (for a review see Faix and Grosse, 2006; Kovar, 2006). In addition, it also binds the side of the actin filament, leading to fragmentation of the filament (Harris and Higgs, 2004) and inducing the formation of actin bundles *in vitro* (Harris et al., 2006; Moseley and Goode, 2005). Recently, significant progress toward understanding the cellular and molecular functions of class I formins including AtFH1, AFH3, AFH4, AFH5, AtFH8, and a class II formin from *Arabidopsis* AFH14. Studies on *Arabidopsis* formin homologs (AtFHs) have shown that some of the members are conserved in nucleating, partial capping or bundling activities. Like the counterparts from yeast, animal and fungi, plant formins, the FH1FH2 domain of AtFH1, AFH4, AFH5, AtFH8 can nucleate actin filaments to form unbranched filaments, and the FH2 domain is the functional domain in nucleation (Deeks et al., 2005; Ingouff et al., 2005; Michelot et al., 2005; Yi et al., 2005). The AtFHs FH1FH2 constructs also associate with the barbed end and change the rate of polymerization and depolymerization in a partial capping mode (Ingouff et al., 2005; Michelot et al., 2005; Yi et al., 2005). But the capping activity of AtFH1 is a little special in that the FH1FH2 construct works as a "leaky cap" , but the FH2 construct is a tight cap that only allows filament elongation in the pointed end (Michelot et al., 2005). Bundling activity is also identified for AtFH1 (Michelot et al., 2005) and AtFH8 (our unpublished data), and AtFH8 FH1FH2 construct can also sever actin filaments, which is the only one reported in plant formins (Yi et al., 2005).

Miroarray analyses (Zimmermann et al., 2004) indicate that as a group actin-nucleating proteins are expressed at very low levels in pollen compared with proteins that regulate actin dynamics (e.g., actin depolymerizing factors and profilins) or with signaling molecules that mediate pathways that regulate actin dynamics (e.g., Rho GTPases) (Cheung et al., 2008; Lee et al., 2008; Kost, 2008), implying that nascent F-actin synthesis must be maintained at relatively low levels. Microarray data (Zimmermann et al., 2004) and promoter activity assays (Cheung and Wu, 2004) showed that multiple formins are expressed in pollen, with the Group I AFH3 and AFH5 being predominant, and two Group II formins among the more highly represented. FH3 is specific to pollen, whereas AFH5 is broadly expressed but at even lower levels than in pollen. The Group I formin AtFH1 stimulate actin assembly along the pollen-tube cell membrane and deregulate actin nucleation activity that disrupts the tip growth process (Cheung and Wu, 2004). Similar results are obtained when AtFH8 is overexpressed in *Arabidopsis* root hairs (Yi et al., 2005). RNAi knockdown of AFH3 in *Arabidopsis* resulted in reduced abundance of the axially-aligned actin cables and inhibited *in vitro* pollen tube growth (Ye et al., 2009). In providing nascent F-actin, actin-nucleating proteins should conceivably contribute to the assembly of higher order actin structures. AFH5 localizes to the apical dome of elongating pollen tubes, stimulates actin assembly most prevalently around the subapical membrane, and plays a crucial role in controlling pollen-tube tip growth by facilitating assembly of the subapical actin structure and apical vesicular trafficking (Cheung et al., 2010). The type II formins are targeted to the apical domain via a PTEN-like domain located N-terminal of the FH1-FH2 domains (Vidali et al. 2009). The work in our laboratory demonstrates that AFH14 is involved in meiosis through regulation of microtubule structures required for the generation of microspores (Li et al., 2010). Because AFH14 is also expressed in the pollen, we presume that AFH14 may also participate in the process of pollen tube growth.

Actin depolymerizing factors (ADFs)

ADF is a central regulator of actin dynamics in numerous eukaryotic systems (for reviews see Maciver and Hussey, 2002; Staiger and Blanchoin, 2006; Bamburg and Bernstein, 2008), which binds to both G-actin and F-actin with a marked preference for ADP–G-actin (Carlier et al., 1997; Blanchoin and Pollard, 1999), and disassemble actin filaments by a complex mechanism. Recent data from time-lapse TIRFM demonstrates unambiguously the capacity of ADF to disassemble filaments through severing activity (Andrianantoandro and Pollard, 2006). ADF can also nucleate actin filaments when present at high concentrations (Andrianantoandro and Pollard, 2006); therefore, it becomes critical to know the cellular concentration of ADF under all circumstances. ADFs were first identified in plants during search for pollen specific transcripts in *Lilium longiflorum* (Kim et al., 1993), and are present as a small multigene family in maize and *Arabidopsis*.

The properties of ADF are modulated via phosphorylation, phosphoinositides, pH, and other ABPs. Phosphorylated forms of ADF have been reported in both tobacco and lily pollen, and phospho-ADF accumulation depends on Rac/Rop activity (Chen et al., 2004). The phosphoinositide lipid, PIP_2, binds to ADF resulting in inactivation of membrane-associated ADF; and, conversely, ADF can affect polyphosphoinositide turnover by inhibiting phospholipase C activity

(Gungabissoon et al., 1998). This could be an important mode of regulation for ADF at the extreme apex of pollen tubes, where PIP_2 is abundant (Kost et al., 1999; Helling et al., 2006). ADF activity in plant cells is pH dependent (Gungabissoon et al., 2001; Allwood et al., 2002); at alkaline pH, it has high depolymerizing activity; under acidic conditions, it binds F-actin. The cellular concentration of ADF in pollen is likely to be an abundant cytoplasmic protein, similar to the situation in *Arabidopsis* leaf and suspension-cultured cells where ADF is present at equimolar ratios with total actin (Chaudhry et al., 2007). In lily and tobacco pollen, both GFP-ADF and immunocytochemistry with state-of-the art preservation methods and anti-ADF sera decorate actin filaments and show an accumulation of ADF in the cortical cytoplasm of the subapical region (Chen et al., 2002, 2003; Lovy-Wheeler et al., 2006; Wilsen et al., 2006). ADF is recruited to this region by the oscillatory alkaline band (Lovy-Wheeler et al., 2006). In model, suggested by Lovy-Wheeler et al. (2006) ADF features as a central player regulating the turnover of actin filaments in the cortical fringe by enhancing polymerization at alkaline pH and destabilizing filaments under neutral or acidic pH conditions. ADF is certain to be a key player in the oscillatory behavior of cortical actin filaments in the apical and subapical region, but additional evidence for this will require simultaneous imaging of actin dynamics and pH oscillations *in vivo* (Staiger et al., 2010). We believe that much could be learned from *adf* loss-of-function mutants using reverse-genetic experiments especially if attention is focused on the pollens specific, class IIa genes, *ADF7* and *ADF10* (Pina et al., 2005; Ruzicka et al., 2007).

Signaling pathways in the pollen tubes

The actin organization in the growing pollen tube is regulated by numerous molecules in diverse pathways. Besides different ABPs, which act as the most direct regulators, many upstream signals are also involved in the process and thus they form two main signaling pathways in pollen tubes: $[Ca^{2+}]_c$/Ca^{2+}-CaM pathway and phosphoinositide pathway. These two pathways are not paralleled and they are intersecting in many parts. They and their signaling switches, small GTPases, construct the complex networks of signaling transduction in pollen tubes.

$[Ca^{2+}]_c$/Ca^{2+}-CaM pathway

Cytosolic free calcium ($[Ca^{2+}]_c$) is a key element in the regulation of pollen tube elongation and guidance (Malhó et al., 2006). The importance of $[Ca^{2+}]_c$ presents not only that $[Ca^{2+}]_c$ operates as an independent signal but also as the central signaling molecule in the whole signal networks in pollen tubes. The distribution of $[Ca^{2+}]_c$ is not even in the pollen tube that a tip-focused $[Ca^{2+}]_c$ gradient has been detected in growing pollen tubes (Franklin-Tong, 1999). It has been widely acknowledged that the apical $[Ca^{2+}]_c$ gradient derives from localized influx through active $[Ca^{2+}]_c$ channels at the pollen tube tip (Kühtreiber and Jaffe, 1990; Malhó et al., 1994, 1995; Pierson et al., 1994; Holdaway-Clarke et al., 1997; Messerli and Robinson, 1997). Treatments that eliminate the apical $[Ca^{2+}]_c$ gradients result in the reversible inhibition of pollen tube growth (Rathore et al., 1991; Pierson et al., 1994; Malhó and Trewavas, 1996). In contrast, a basal level of $[Ca^{2+}]_c$ concentration exists in the subapical and basal part of pollen tubes. The dissipation of $[Ca^{2+}]_c$ behind the apex is thought to be regulated by Ca^{2+}-ATPases located in ER membrane and thus the ER represents a large sink which is capable of rapidly sequestering $[Ca^{2+}]_c$ (Sze et al., 1999; Franklin-Tong, 1999).

It has revealed that $[Ca^{2+}]_c$ at least participates in two main associated events during the apical growth of pollen tubes. One is the control of cytoplasmic streaming, while the other is regulation of polarized exocytosis. The mechanism of Ca^{2+} inhibition of cytoplasmic streaming has been known to be attributed to both inactivation of myosin and fragmentation of actin. The fragmenting activities of F-actin are certainly brought about by specific ABPs such as villin (Vidali et al. 2001), gelsolin (Huang et al. 2004; Xiang et al. 2007) and profilin (Vidali and Hepler, 1997), all of which probably works in combination with ADF (Cai and Cresti, 2008). These ABPs all contain the binding sites of Ca^{2+} and they present fragment characteristic to reduce the actin filaments length under high level of $[Ca^{2+}]_c$ in tips of pollen tubes and consequently slow the motion of vesicles and help them dock and fuse with the plasma membrane.

$[Ca^{2+}]_c$ participating in Ca^{2+}-CaM pathway provides another important mean by which it can regulate some other activities in pollen tubes. Calmodulin (CaM), which has four Ca^{2+} binding domains, is a Ca^{2+} sensor known to modulate the activity of many proteins. Since CaM activity is depend on binding of Ca^{2+}, different Ca^{2+} concentration may result in different activity of CaM. Rato et al. (2004) showed that CaM activity is higher in the apex of growing pollen tubes where the dense concentration of tip-focused $[Ca^{2+}]_c$ exists. Furthermore, it was found that CaM activity oscillates with a period similar to $[Ca^{2+}]_c$ (40–80 s) (Malhó et al., 2006). Malhó and Trewavas (1996) found that a decrease in CaM levels in one side of the apical dome led to growth axis reorientation to the opposite side, revealing that this result should be consistent with a decease in $[Ca^{2+}]_c$ level in the same side of the apical dome. All above suggest that $[Ca^{2+}]_c$ and CaM have close relationship with each other and they may co-regulate pollen tubes growth and guidance harmoniously. Recently, Chen et al. (2009) showed that in *Picea meyeri* pollen tube growth, Ca^{2+}-CaM dysfunction induced serial cytological responses and temporal changes in protein expression profiles.

Phosphoinositide pathway

Phosphatidylinositol-4,5-bisphosphate (PIP_2) and its

production of hydrolyzation by phospholipase C (PLC), phosphatidyl inositol 1,4,5-trisphosphate (IP_3), are the most important molecules in phosphoinositide pathway (Franklin-Tong et al., 1996; Malhó, 1998). PIP_2 has been shown to act in a common pathway with Rac/Rho GTPases (Kost et al., 1999). As it has been identified as Rac/Rho effector, PIP_2 presents regulation activities in actin organization, vesicle trafficking and ion transport (Cremona et al., 1999; Stevenson et al., 2000). Through PLC, PIP_2 generates IP_3 and diacylglycerol (DAG). The former is a potent mobilizer of Ca^{2+} from intracellular stores such as ER. The later binds with plasma membrane to active protein kinase C (PKC) which can enhance the transcription of some special genes after being activated. DAG can also be converted to phosphatidic (PA) through DAG kinase (Munnik, 2001). PIP_2 is also known to control PLD activity leading to elevated PA formation (Powner and Wakelam, 2002). Multiple PLD genes have been identified in plants and the proteins they code for seem to be regulated by Ca^{2+} and G-proteins (Zheng and Yang, 2000; Munnik, 2001).

IP_3 is possibly the most studied signaling phosphoinositide and its dominant role is as a potent mobilizer of Ca^{2+} from intracellular stores (Martin, 1998). In pollen tubes, IP_3 receptors are reckoned to have an asymmetric activity depending on their spatial localization that they undergo an intrinsic inactivation in the apex where Ca^{2+} is elevated and undergo an intrinsic activation in subapical regions to increase Ca^{2+} release to cytoplasmic matrix (Dawson, 1997). The main IP_3 receptor is IP_3-gated Ca^{2+} channel on ER. The interaction of IP_3, $[Ca^{2+}]_c$ and intracellular Ca^{2+} are complex. On the one hand, $[Ca^{2+}]_c$ may enhance the affinity of IP_3 receptors to IP_3 and thus results in increasing intracellular Ca^{2+} release; on the other hand, with the increasing concentration of $[Ca^{2+}]_c$ it reversely declines the affinity of IP_3 receptors to IP_3 and inhibits IP_3-mediated intracellular Ca^{2+} release. Consequently, Ca^{2+} and IP_3 can be regarded as coagonists for Ca^{2+} release (Dawson, 1997).

PA is a product of PLD activity that can also arise as an end product of PIP_2 hydrolysis (Monteiro et al., 2005a). As part of a feedback loop, PA can also promote PIP_2 formation by phosphatidylinositol 4-phosphate 5-kinase (Anderson et al., 1999). PA promotes membrane curvature and formation of secretory vesicles along with a crucial role in the structural integrity of the Golgi apparatus (Sweeney et al., 2002) and cytoskeleton reorganization (O'Luanaigh et al., 2002). The activity of PLD and PA can be blocked by primary alcohols, like 1-butanol. In pollen tubes, the inhibition of PLD and PA (by 1-butanol) has been shown to reversibly halted polarity (Monteiro et al., 2005a). Monteiro et al. (2005b) suggested that this is due to the actin cytoskeleton with no discernable directionality and Dhonukshe et al. (2003) found the interference of pollen tube growth can be restored by taxol treatment, indicating that microtubules may be another target. Thus, this phenomenon may result from both the abnormality of actin filaments and microtubules.

Among the possible targets for the phosphoinositide pathway, actin filaments and ABPs are particularly important ones (Monteiro et al., 2005a). In animal cells, PIP_2 is reckoned to be associated with membrane-cytoskeleton interaction. It has been suggested that plasma membrane PIP_2 controls dynamic membrane functions and cell shape by locally increasing and decreasing the adhesion between the actin-based cortical cytoskeleton and the plasma membrane (Raucher et al. 2000). The commonly accepted mechanism of this phenomenon is that PIP_2 inhibits the activity of actin-severing proteins such as cofilin, gelsolin, or profilin, and also activates vinculin, talin, α, β-catenin, α-actinin, and thus enhances the interaction between the cell membrane and cytoskeleton (Hong et al., 2009). *In vitro*, Goldschmidt-Clermont et al. (1990) found that profilin binds to PIP_2 and inhibits its hydrolysis by PLC. This phenomenon soon was demonstrated as a way of regulating the levels of PIP_2 in plants (Drøbak et al., 1994). The inhibition of PLC decreases the production of IP_3 and the consequent release of Ca^{2+}. The actin cytoskeleton cannot be remodeled by the ABPs which need Ca^{2+} as a promoter. Consistently, it has been revealed on the other side that expression of PLC in pollen tubes of *Petunia inflata* raised the apical Ca^{2+} gradient, which disorganized the actin filaments (Dowd et al., 2006).

GTP binding protein (GTPase) as signaling switches

Small GTPases are versatile signaling switches responsible for an extensive communication and cooperation between signal transduction pathways (Bar-Sagi and Hall, 2000). The role of GTPase as signaling switches is carried out by the transformation between GTP-bound forms and GDP-bound forms. This superfamily is structurally classified into at least five families: the Ras, Rho/Rac/Cdc42, Rab, Sar1/Arf, and Ran families. Plants do not contain a true Ras GTPase as those are pivotal signaling switches in animals and yeast (Vernoud et al., 2003; Gu et al., 2004), but, interestingly, they contain a special subfamily of Rho family named Rho-related GTPase from plants (ROP) (Yang, 2002), which regulates a wide range of cellular processes such as the growth of pollen tubes and the reaction to ABA.

Because of no Ras homolog in plant genomes, ROP plays a dominant role in transmitting extracellular signals in botanies. *Arabidopsis* contains 11 members of ROP which are divided into four phylogenetic groups (Zheng and Yang, 2000; Yang, 2002). Although most of them have distant function individually, some are suspected to be functionally redundant. As in pollen, while 7 ROPs (ROP1/3/5/8/9/10/11) are expressed (Li et al., 1998; Gu et al., 2003), ROP1, ROP3 and ROP5 are demonstrated to have overlapping roles because of sharing closed homology (Kost et al., 1999).

In both *Arabidopsis* and some other plants such as pea and tobacco, ROPs were found to accumulate at the plasma membrane and cytosol of pollen tube tips and similar observations were made in root hairs (Molendijk et al., 2001; Gu et al., 2004) indicating that they may be related with

the growth of polarized cells. Actually, ROPs involve in both the orientation and elongation of pollen tubes. In pollen, the constitutively active, GTP-bound form led to swollen tip growth, whereas the dominant-negative, GDP-bound form led to cessation of tube growth (Malhó et al., 2006). A simple explanation for the observed ROP functions in both growth and polarity control is the localized activation of a ROP pathway in the apical plasma membrane region (Yang, 2002). Li et al. (1999) propose a positive feedback model of ROPs recruitment in pollen tube tips. They reckon that after the activation of a basal level of tip-localized ROPs, more ROPs are recruited to the site of activation and thus form a positive feedback loop. Different upstream regulators of ROPs may affect the orientation and elongation of pollen tubes through the influence on this feedback loop.

Current evidence suggests that the mechanism through which ROPs regulate the pollen tube growth contains at least two downstream pathways: the assembly of dynamic tip actin cytoskeleton and the generation of tip-focused Ca^{2+} gradients (Li et al., 1999; Fu et al., 2002). The former may target vesicles to the site of growth, whereas the latter may regulate vesicle fusion with the apical plasma membrane region. First, ROPs promote the assembly of a fine and dynamic type of cortical actin filaments that are localized to the polar site of cell growth (Gu et al., 2004; Fu et al., 2002). Transient expression of the mutant GTP and GDP-bound proteins in tobacco pollen tubes leads to the formation of extensive and reduced actin cables, respectively, which shows that ROPs play a role in the regulation of actin dynamics (Kost et al., 1999). Secondly, ROPs seem to activate phosphatidylinositol kinase leading to the formation of PIP_2, which could function in release of Ca^{2+} (Malhó et al., 2006). The oscillatory increase in Ca^{2+} concentration may activate the villin/gelsolin/profilin group, shifting the equilibrium of actin toward the monomeric form, thus cause the actin disassembly (Cai and Cresti, 2008). Consequently, the integrated affect of ROPs is that they provide the dynamic tip actin cytoskeleton which is essential for the development of pollen tubes. It is proposed that ROPs activate the above two coordinate downstream pathways respectively (Fu et al., 2001). This hypothesis is supported by identification of two structurally distinct putative ROP1 targets in the control of polar growth in pollen tubes, RIC3 and RIC4 (Wu et al., 2001).

Conclusions

Clearly we have learned a lot about the organization and regulation of the actin cytoskeleton in pollen tubes. Predominantly, this has come from a powerful combination of biochemistry, advanced imaging methods, pharmacological studies, and reverse-genetic approaches. However, how the actin cytoskeleton functions is still poorly understood. Although advances in the characterization of actin binding proteins from pollen tubes and the signaling pathways reveal unique features of them compared to mammalian or yeast proteins, little is known about the connection of these insights with an understanding of the dynamic properties and the exact functions of actin filament structures in pollen tubes. To draw this picture more precisely, determination of the cellular concentration and intracellular localization of these players in pollen tube and how they may coordinate with each other and involved in signaling pathway to modulate actin dynamics is also necessary. We should also learn the molecular level details about how individual actin filaments are organized, where they polymerize, and how they turn over regulated by the ABPs or signaling molecules during the pollen tube elongation. Some processes, which are likely to depend on actin-based force generation by the dynamic actin cytoskeleton in pollen tubes, such as secretory/endocytic vesicle traffic and polarized cell expansion exist, while the force generation by the dynamic actin cytoskeleton in the process of pollen tube has not been well studied. These issues may be provided more insights in the near future by using of fluorescent fusion proteins and the application of techniques like VAEM or spinning disk confocal microscopy.

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