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The Actin Cytoskeleton in Pollen Tubes; Actin and Actin Binding Proteins

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Abstract Actin cytoskeleton is well known to be a key element for the germination and the elongation of pollen tubes. It has been appreciated that the cytoplasmic streaming for conveying secretory vesicles to the tube apex is a primary function of the actin cytoskeleton in pollen tubes. Recently growing evidence has revealed that highly dynamic populations of actin cytoskeleton are present in apical and subapical regions of tubes and are involved intimately in polar elongation of tubes. Tip-focused Ca^{2+}-gradient and tip-localized small GTPases (Rop/Rac) are believed to control such actin dynamics through the various kinds of actin binding proteins (ABPs). In the present chapter, we focus on the organization of actin in elongating pollen tubes and characterization of ABPs identified from pollen. We further discuss their roles, with special emphasis on recently identified proteins of the gelsolin family, regulating actin dynamics and organizing actin architecture in pollen tubes.

1 Introduction

Actin is one of the most ubiquitous and highly conserved proteins among eukaryotic cells. Under physiological ionic conditions, monomeric actin (G-actin) is polymerized into filamentous polymers (F-actin or actin filament) through the nucleation step for forming intermediates, such as dimers and trimers. F-actin is a polar filament that adds preferentially G-actin on its barbed (plus) end, and tends to release G-actin from opposite pointed (minus) end. Many kinds of ABPs are responsible for regulating and modulating actin turnover or dynamic balance between polymerization and depolymerization and for organizing actin filaments into highly ordered structures. Myosin is an actin-based molecular motor moving and sliding along actin filaments. These three components form the actin cytoskeleton, which is involved in various cellular functions and activities in eukaryotes.

2 Actin

In higher plants, actin genes constitute a multi-gene family and are subdivided into two classes, which differ in their expression pattern, reproductive and vegetative. *Arabidopsis* has at least eight expressed actin genes, *ACT1*, *ACT2*, *ACT3*, *ACT4*, *ACT7*, *ACT8*, *ACT11*, *ACT12* (Meagher et al. 2000). *ACT2*, *ACT7* and *ACT8* are strongly expressed in vegetative tissues, while *ACT1*, *ACT3*, *ACT4*, *ACT11* and *ACT12* in reproductive tissue and pollen. The cytosolic concentrations of actin in pollen were estimated to be around 100 μM and 250 μM, corresponding to about 5% and 2.3% of total extractable protein in maize (Gibbon et al. 1999) and poppy (Snowman et al. 2002), respectively, and did not change significantly during the germination. In lily pollen, however, concentration before germination was estimated to be ~ 25 μM and increased about 70% after 2 hr of germination (Vidali and Hepler 1997). In either case, actin is an abundant protein in pollen. The concentration of F-actin, however, was estimated to be in the order of tens of μM in maize (Gibbon et al. 1999) and poppy pollen (Snowman et al. 2002), which is significantly lower than that of G-actin. This indicates that large part of the actin protein is in the monomeric form.

Functionally active actin was isolated from maize pollen by two independent methods, acetone-treatment (Liu and Yen 1992, 1995), commonly used in preparing the actin from muscle tissues, and a poly-L-proline (PLP) affinity column using human recombinant profilin (Ren et al. 1997). In the later case, G-actin in a crude protein extract from pollen binds to exogenously added human profilin and then is adsorbed on the PLP column via an association between profilin and PLP (see section below). Subsequently, G-actin is selectively eluted with high ionic strength solution from the column while profilin remains bound. This method is generally used to prepare pollen actin, because of rapid, reproducible and convenient procedure. The biochemical characterization revealed that pollen actin binds to subfragments of muscle myosin, shows kinetics of polymerization similar to muscle actin, and can be incorporated into the array of actin filaments in living plant cells, such as stamen hair cells of *Tradescantia*. Hence, pollen actin has similar properties to non-plant actin. Interestingly, pollen actin was predominantly incorporated into pollen F-actin in vitro and into the array of actin filaments in stamen hair cells (Ren et al. 1997; Jing et al. 2003). In contrast, animal G-actin preferentially co-polymerized with animal G-actin and F-actin. These results indicate the functional diversity between animal and pollen actin, despite high conservation (up to 80%) in their primary structures.

3
Organization of Actin Cytoskeleton in Pollen and Pollen Tubes

During the germination of pollen, the organization and architecture of actin filaments are dramatically altered (Haslop-Harrison and Heslop-Harrison 1992; Smertenko et al. 2001). Before hydration, actin seems to be tightly packed in fusiform or spicule structures and aggregates, presumably storage forms of actin. During the hydration, actin filaments dissociate from these structures, and then are organized into fine bundles dispersed randomly in grains. A network of actin filaments concentrates on the germinal aperture(s) before the germination, and eventually actin-filament bundles are ramified in the pollen tubes.

In elongating pollen tubes, there appears to be three major regions of distinct actin-filament arrangement and architecture: apical, subapical and shank region (Vidali and Hepler 2001). In the shank of tubes, actin filaments are organized into bundles arranged in a longitudinal or a helical orientation throughout the cytoplasm. These actin filaments, together with myosin, generate the cytoplasmic streaming for transport of organelles and Golgi-driven secretory vesicles containing cell wall materials (Geitmann and Steer, this volume), which are fused at the tip and participate in the elongation of tubes. The cytoplasmic streaming in pollen tubes frequently exhibits a reverse fountain pattern with the streaming occurring acropetally along the edge or side of tubes, and basipetally in central or core region (Vidali and Hepler 2001).

The actin organization in apical and subapical regions is still unclear with contradictory reports, most likely a result of different methodologies and species under study (Vidali and Hepler 2001). The expression of the F-actin binding domain of talin tagged with GFP (Kost et al. 1998), provided the first live images of the pollen tube actin cytoskeleton and made it possible to trace and analyze the dynamics in close to real time. However, tube growth after GFP-talin expression is strongly perturbed raising questions about the validity of this methodology. In spite of the ongoing debate, it is accepted that a few short actin filaments or fine and thin bundles are dispersed with random orientation in the apical regions, while a ring-like arrangement of actin filaments is found in the subapical regions (Fig. 1). This ring-like arrangement has been coined as collar (Kost et al. 1998), subapical mesh (Chen et al. 2002; Geitmann et al. 2000) basket (Snowman et al. 2002), funnel (Vidali et al. 2001) or fringe (Lovy-Wheeler et al. 2005). Recently, this arrangement has been carefully evaluated with state-of-the-art fixation regimes (Lovy-Wheeler et al. 2005) and has been found to consist of longitudinal actin filaments concentrated densely in the tube cortex. The apical regions are devoid of large organelles and exhibit a chaotic-like cytoplasmic streaming. Therefore, this area occupied by numerous secretory vesicles is also called clear zone. The actin cytoskeleton in the apical region seems to relate with the suppression of cytoplasmic streaming in this region with thinner and transient microfil-

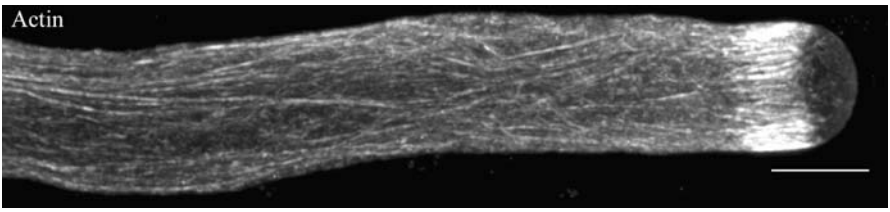

Fig. 1 Pollen tubes of lily exhibit a marked cortical fringe of actin in the apical domain. In the shank, back from the tip, actin microfilaments are evenly dispersed throughout the thickness of the tube. The pollen tube was prepared first by rapid freeze fixation, and freeze substitution. It was then rehydrated, and labeled with anti-actin antibody. (from Lovy-Wheeler et al. 2005, reprinted with permission)

aments occuring next to the plasma membrane within 5 μm from the tip (Fu et al. 2001). The absence of microfilaments at the tip of the growing tube was suggested to be due to the tip-focused Ca^{2+} gradient indispensable for growth (Hepler et al., this volume).

4
Characterization of ABPs Identified in Pollen

ABPs bind to and interact with both G-actin and F-actin in various manners (e.g., side-binding, end-capping, and cross-linking of filaments). In pollen tubes, ABPs are intimately involved in the arrangement of actin filaments into bundles and the regulation of actin dynamics or turnover, polymerization or depolymerization. Thus far, three types of ABPs, have been identified from pollen: profilin, ADF (actin depolymerizing factor)/cofilin and gelsolin family proteins – fragmin, gelsolin and villin. Their biochemical properties and localization in pollen or pollen tubes have been extensively studied (Drøbak et al. 2004; Staiger and Hussey 2004); profilin and ADF/cofilin regulate actin filament assembly and act as increasing the turnover of filaments while the gelsolin family proteins function in both regulating actin dynamics and organizing architectures of actin filaments.

4.1
Profilin

Profilin is small G-actin-binding protein (12 to 15 kDa) that forms a 1 : 1 complex with G-actin, and also binds to phosphoinositides, proline-rich proteins and PLP (Drøbak et al. 2004; Staiger and Hussey 2004). This protein was originally identified as an allergen of birch pollen (Valenta et al. 1991). Plant profilin comprises a multigene family containing 5 to 10 isoforms in many plant tissues, and the pollen is no exception. *Arabidopsis* has five isoforms of profilin,

PRF1 to PRE5 (Kandasamy et al. 2002). Among them, PRF4 and PRF5 are specifically expressed in mature pollen and pollen tubes, but not found in other cell types (including microspores), while PRF1, PRF2 and PRF3 are constitutive in all vegetative tissue at various stages of development. These isoforms are also expressed predominantly in ovules and microspores at the early stages of miscrosporogenesis. In maize, five isoforms, ZmPRO1 to ZmPRO5, are expressed in pollen (Kovar et al. 2000a). ZmPRO1 is abundant in pollen, while ZmPRO4 and ZmPRO5 are predominantly expressed in vegetative cells or tissues and insufficiently in pollen. In pollen tubes, profilin is uniformly distributed throughout the cytoplasm in lily (Vidali and Hepler 1997), *Arabidopsis* and tobacco (Kandasamy et al. 2002). In tobacco pollen tubes, profilin is also localized in the vegetative nuclei, but not in sperm cells. Pollen contains profilin abundantly, and its concentration (Gibbon et al. 1999; Vidali and Hepler 1997; Snowman et al. 2002) was nearly the same as that of total actin protein, indicating that most of profilin likely forms complexes with actin monomer.

Plant profilin shares functional and structural similarity with animal profilin (Drøbak et al. 2004; Staiger and Hussey 2004), although it does not accelerate the nucleotide exchange rate of G-actin (Perelroizen et al. 1996). This protein has two opposite actions on the dynamics of actin in vitro. Profilin depolymerizes actin filaments by binding to G-actin, and then sequesters G-actin from the spontaneous nucleation of polymerization. Conversely, the profilin-actin complex promotes and enhances the polymerization of G-actin from uncapped and free barbed ends of actin filaments when a large pool of G-actin is present. Maize profilins appeared to be divided into two functional classes possessing different affinities for G-actin and PLP (Kovar et al. 2000a). Class II profilin, ZmPRO4 and ZmPRO5, showed higher affinity for PLP and stronger sequestering activity of G-actin than those of class I profilin, ZmPRO1, ZmPRO2 and ZmPRO3. Hence, class II profilin microinjected into stamen hair cells, induced the disruption of cytoplasmic architecture and then displacement of nucleus via the depolymerization of actin filaments more efficiently and rapidly than class I profilin. Furthermore, the ability of both classes of maize profilins to sequester G-actin was enhanced by Ca^{2+}. This property is believed to contribute to the regulation of actin dynamics in apical and subapical regions of pollen tubes as described below. Interestingly, it was reported that poppy pollen profilin alters the phosphorylation level of several pollen proteins (Clarke et al. 1998). This evidence raised a possibility that pollen profilin is involved in signaling pathway by regulating protein kinase and phosphatase activities.

4.2 ADF/cofilin

ADF/cofilin is another small ABP (15 to 19 kDa) which enhances and modulates actin filament dynamics (Maciver and Hussey 2002; Drøbak et al. 2004;

Staiger and Hussey 2004). It increases actin-filament ends available for polymerization by severing filaments and dissociates actin monomers from the pointed ends of filaments for assembly at the barbed ends. In *Arabidopsis*, at least nine members of ADF/cofilin proteins (AtADF1 to AtADF9) are known to be present (Dong et al. 2001). Some isoforms are expressed predominantly in vegetative tissues, others primarily in reproductive organs. In maize, three genes encoding this protein, ZmADF1, ZmADF2 and ZmADF3, have been characterized, and are differently expressed in maize plant (Lopez et al. 1996). ZmADF1 and ZmADF2 are expressed exclusively in pollen and ZmADF3 in other tissues. The localization of ADF/cofilin in pollen was shown to be altered during maturation and germination (Smertenko et al. 2001), similarly to actin. In mature pollen grains, the protein co-localizes with actin filament arrays whereas in dehydrated pollen grains, some was co-localized with the storage form of actin (e.g., spicules or aggregates). During the germination, ADF/cofilin was accumulated at the sites of pollen tube emergence, and this pattern seemed to depend on the actin network. In pollen tubes, ADF/cofilin distributed evenly in the cytoplasm but interestingly, at points of adhesion of the pollen tube tip to an adjacent substrate, the protein appeared to bind to actin filaments (Smertenko et al. 2001). In contrast to these findings, when tobacco pollen-specific ADF/cofilin (NtADF1) tagged with GFP was expressed at moderate level in tobacco pollen, it bound and decorated predominantly subcortical mesh of actin filaments and their bundles in the shank of pollen tubes (Chen et al. 2002).

The actin-severing and -depolymerization activities of plant ADF/cofilin are sensitive to pH, and further regulated by phosphorylation and phosphoinositide 4,5 bisphosphate (PtdIns(4,5)P_2) (Maciver and Hussey 2002; Drøbak et al. 2004; Staiger and Hussey 2004). At low pH, this protein binds preferentially and cooperatively to actin filaments, while at higher pH, it promotes the rapid depolymerization of filaments by severing and accelerating mononer dissociation from the pointed ends of filaments. The depolymerizing activity of pollen-specific ADF/cofilin, lily LIADF1 (Allwood et al. 2002) and tobacco NtADF1 (Chen et al. 2002) was also enhanced at higher pH. The Ser 6 in vegetative type maize ADF/cofilin, ZmADF3 molecule, was phosphorylated by the calmodulin (CaM)-like domain protein kinase (CDPK), and as a result, its actin-binding and depolymerizing activities were reduced (Smertenko et al. 1998; Allwood et al. 2001). Similar reduction of activities of ZmADF3 (Smertenko et al. 1998) and AtADF1 (Ressad et al. 1998), a vegetative type of *Arabidopsis* ADF/cofilin protein, was induced when Ser 6 of these ADF/cofilin was replaced with Asp to mimic a phosphorylation state. In the elongating pollen tubes, a high activity of Ca^{2+}-dependent protein kinase was reported in the apex (Moutinho et al. 1998). These data suggests that the activity of ADF/cofilin in pollen tubes is also controlled by CDPK through phosphorylation and dephosphorylation. However, lily pollen LIADF1 was not found to be phoshorylated by CDPK (Allwood et al. 2002). In tobacco pollen NtADF1 re-

placement of Ser 6 by Asp led to a significant reduction in the interaction with actin filaments (in vitro and in vivo) (Chen et al. 2002). Both phosphorylated and unphosphorylated ADF/cofilins were present at similar concentrations in mature pollen of tobacco (Chen et al. 2003). These results suggested that the phosphorylation at Ser6 of NtADF1, which is not catalyzed by CDPK, is an important regulatory mechanism for its activity in pollen tubes. Furthermore, the Rop/Rac signaling pathway, (Hwang and Yang, this volume) was suggested to be involved in the phosphorylation of ADF/cofilin. Overexpression of tobacco pollen Rac1, NtRac1 induced the isotropic growth resulting in pollen tubes with ballooned tips and a disrupted actin cytoskeleton; these phenomena were diminished by the co-expression of tobacco pollen NtADF (Chen et al. 2003). When Ser 6 was replaced with Asp, the activity of NtADF1 to counteract the effect of NtRac1 decreased. Moreover, the binding activity of expressed NtADF1 to actin filament arrays in pollen tubes was suppressed by co-expression of NtRac1 or constitutively active form of NtRac1 while the phosphorylation level of NtADF1 increased. These results further support that the phosphorylation of Ser 6 in ADF/cofilin plays a critical role in controlling its activity, and suggested that ADF/cofilin is one of down regulators in signaling pathway of Rop/Rac in pollen tubes (Chen et al. 2003).

The binding activity of LIADF1 to f-actin was also inhibited by specific phosphoinositides, such as PtdIns(4,5)P_2 (Allwood et al. 2002), probably by direct binding to the actin-binding site(s). In addition to these regulatory mechanisms described above, the depolymerizing activity of LIADF1 was found to be dramatically enhanced in the presence of another pollen protein called actin-interacting protein 1 (AIP1; Allwood et al. 2002). There are two AIP1 genes, *AtAIP1-1* and *AtAIP1-2*, in *Arabidopsis*. AtAIP1-1 is expressed in floral tissues but not in vegetative organs, whereas AtAIP1-2 is in all tissues containing floral tissues. In daffodil pollen, AIP1 protein showed a distribution patter similar that of to ADF/cofilin during the maturation, hydration, and germination of pollen (Smertenko et al. 2001). These observations raise the possibility that AIP1 regulates and/or modulates the activity of ADF/cofilin in pollen tubes.

4.3 Gelsolin Family Protein

Gelsolin family proteins are generally composed of several gelsolin (or gelsolin/severin) repeat domains; fragmin and gelsolin have three and six of those domains, respectively, while villin has a headpiece domain in addition to the six gelsolin repeat domains (Drøbak et al. 2004; Staiger and Hussey 2004). Gelsolin family proteins show various functions in regulating actin dynamics and organizing actin filaments in a Ca^{2+}-dependent manner. In the presence of Ca^{2+}, these proteins bind to G-actin and form the complex which works as a nucleator for polymerization of actin, thus resulting in the

acceleration of polymerization (referred to as nucleating activity). Furthermore, they sever actin filaments and remain attached the barbed ends of severed filaments as a cap. As a result, short actin filaments that can not anneal with each other and not elongate at their barbed ends, are produced. PtdIns(4,5)P_2 dissociates gelsolin family proteins from G-actin and barbed ends of filaments. In addition, villin arranges actin filaments into bundles at low concentrations of Ca^{2+} through its headpiece domain. Plant homologues of villin (P-135-ABP and P-115-ABP; Vidali et al. 1999; Yokota et al. 2003), gelsolin (PrABP80; Huang et al. 2004) and fragmin (LdABP41; Fan et al. 2004) were identified from the pollen of *Lilium longiflorum*, *Papaver rhoeas* and *Lilium davidii*, respectively.

80-kDa PrABP80 and 41-kDa LdABP41 were originally isolated from pollen by using a DNase I affinity column chromatography (DNase I possesses strong binding activity to G-actin, in addition to DNA degradation activity) (Huang et al. 2004; Fan et al. 2004). In the presence of Ca^{2+}, both gelsolin family proteins bound to endogenous G-actin in the crude pollen protein extract, and adsorbed on the column via the interaction of G-actin with DNase I. In a final separation step, PrABP80 and LdABP41 were eluted from the column with EGTA, while G-actin remained bound to the column. Lily villin in the crude protein extract exhibited a behavior on this column similar to that of PrABP80 and LdABP41 (Yokota et al. 2005). The biochemical properties of PrABP80 have been extensively studied, though its localization in pollen has not been shown. PrABP80 could act as a nucleus for actin polymerization, resulting in the acceleration of polymerization, higher rate of barbed end capping and Ca^{2+}-dependent severing (Huang et al. 2004). Furthermore, this protein exhibited preferential binding to certain phosphoinositides, such as PtdIns(3,5)P_2, PtdIns(3,4)P_2 and PtdIns(5)P, and phosphatidic acid, but relatively less binding to PtdIns(4,5)P_2 and PtdIns(3,4,5)P3 (Drøbak et al. 2004). The influence of these phosphoinositides and phosphatidic acid on the activity of PrABP80 has however not been studied.

Fragmin-like protein, LdABP41, also exerted G-actin-nucleating and actin-filament-depolymerizing activities in the presence of Ca^{2+} (Fan et al. 2004). This protein was found to be concentrated preferentially in the apical and subapical regions of elongating pollen tubes (Fan et al. 2004) and microinjection of the antibody against LdABP41 into the germinating pollen induced the suppression of pollen tube elongation. In the *Arabidopsis* genome database, no genes for gelsolin and fragmin have been found (Huang et al. 2004; Staiger and Hussey 2004; Fan et al. 2004). Based on the crossreactivity with antibody against villin and an analysis of partial amino acid sequences, PrABP80 and LdABP41 were suggested to be expressed by alternative splicing from villin gene(s).

Plant villins, P-135-ABP and P-115-ABP, have been originally identified as actin-filament bundling factors from the germinating pollen of lily (Yokota et al. 1998; Nakayasu et al. 1998). They arrange actin filaments into bundles

with same polarity (Yokota and Shimmen 1999; Yokota et al. 2003), and this activity was suppressed in the presence of both Ca^{2+} and calmodulin (CaM), but not by Ca^{2+} alone (Yokota et al. 2000, 2003). P-135-ABP co-localized well with actin-filament bundles in the shank region of lily pollen tubes (Yokota et al. 1998; Vidali et al. 1999). P-135-ABP and P-115-ABP contained in the pollen crude protein extract from lily also associated with a DNase I column in the presence of Ca^{2+} and eluted with EGTA, indicating that both ABPs bind to G-actin in a Ca^{2+}-dependent manner (Yokota et al. 2005). Furthermore, P-135-ABP showed nucleating, end capping and depolymerizing activities, although the severing activity has not been found. However, the nucleation and depolymerization efficiencies of P-135-ABP appeared to be lower than those of PrABP80. In *Arabidopsis*, five villin isoforms are known to be present (AtVLN1∼AtVLN5), and AtVLN2 and AtVLN5 are mainly expressed in pollen (Huang et al. 2005). In VILLIN1 (AtVLN1) the amino acid sequences deduced to provide Ca^{2+}-sensitivity are poorly conserved (when compared to other *Arabidopsis* isoforms), and the recombinant protein showed only F-actin binding and bundling activities in a Ca^{2+}- and Ca^{2+}-CaM-insensitive fashion (Huang et al. 2005). The F-actin bundles formed by AtVLN 1 were stable and resistant to the depolymerizing action of *Arabidopsis* ADF/cofilin, AtADF1.

5
Actin Dynamics in Pollen Tubes

Several recent studies have revealed that pollen tube growth depends not only on the presence of stable actin bundles for cytoplasmic streaming, but also on the dynamic state of actin in the tip region. Actin depolymerizing drugs, latrunculin B and cytochalasin D (Gibbon et al. 1999; Vidali et al. 2001), and G-actin binding proteins, profilin and DNase I (Vidali et al. 2001) microinjected into pollen, inhibited pollen tube growth at concentrations lower than those needed to suppress the cytoplasmic streaming. Furthermore, an aberrant actin array was frequently observed in tip regions of tubes treated with these reagents. Despite the potential problems of the technique, images of the actin cytoskeleton in live tobacco pollen tubes expressing talin-GFP, showed that the short and fine actin-filament bundles in the apical and sub-apical regions are a highly dynamic structure (Fu et al. 2001). The emergence of short actin bundles was generally associated with reduction or disappearance of actin collar and vice versa. Moreover, the appearance of these structures appeared to oscillate alternately. In elongating pollen tubes, a tip-focused Ca^{2+} gradient (Hepler et al., this volume) and Rops (Hwang and Yang, this volume), are well known to be prominent factor for regulating actin dynamics and organization in tip regions, and so to be indispensable for the tube growth. When the Ca^{2+} gradient was diminished by caffeine, actin-filament

bundles extended and invaded the apical regions of pollen tubes (Miller et al. 1996). In contrast, actin filaments in the pollen tubes were fragmented and depolymerized throughout cytoplasm when cytosolic Ca^{2+} concentrations were elevated in the self-incompatibility response of poppy pollen (de Graaf et al., this volume) or by a Ca^{2+} ionophore, A23187 (Kohno and Shimmen 1987; Snowman et al. 2002). Active Rops localized in the apical region also have a role in regulating the organization and the dynamics of tip localized F-actin (Hwang and Yang, this volume).

Actin dynamics in apical and subapical regions of tubes was shown to oscillate in time and was suggested to be in opposite phase with tube elongation and Ca^{2+} changes (Fu et al. 2001); the peak of polymerization appeared to precede peaks of elongation and elevation of Ca^{2+} concentrations. Recently, it was proposed that Rop1 controls two counteracting pathways regulating the actin dynamics in these regions; one promotes assembly of actin filaments, while a second promotes disassembly by Ca^{2+}-dependent processes (Gu et al. 2005; Hwang and Yang, this volume). If these hypothesis hold true, it seems plausible to postulate the following mechanism for depolymerization of actin filaments: (1) the G-actin binding and sequestering-activities of profilin are enhanced; (2) gelsolin family proteins sever and depolymerize actin filaments, accompanying by capping of the barbed ends of filaments; (3) the polymerization of G-actin from the barbed ends induced by profilin-actin complex is significantly blocked. Furthermore, it is possible that ADF/cofilin also promotes the release of G-actin from the pointed ends of filaments, and profilin sequesters further G-actin, if ADF/cofilin is not phosphorylated in the Rop/Rac signaling network. The depolymerizing activity of ADF/cofilin could increase in subapical regions, due to a constitutive alkaline band (Feijó et al. 1999). However, the existence of such band is still disputed (Messerli et al. 1999). To exert a Ca^{2+}-induced depolymerization by a villin, such as P-135-ABP, micromolar CaM should be needed in addition of Ca^{2+}. CaM was distributed throughout the cytoplasm but it was also reported that the protein appears to bind significantly the sub-apical region in a sort of V-shaped collar (Moutinho et al. 1998). The concentration of CaM in the germinating pollen tubes of lily was estimated to be about 15 μM (Yokota et al. 2004), enough for the depolymerization activity of P-135-ABP. However, the depolymerizing efficiency appeared to be lower than that of gelsolin, suggesting that gelsolin or fragmin predominantly play a main role in fragmentation and depolymerization of actin filaments. Significant actin-filament fragmentation and depolymerization induced by the elevation of cytosolic Ca^{2+} concentrations upon self-incompatible response in poppy pollen is likely to be induced by a mechanism similar to the one described above.

In contrast, the mechanism for promotion of polymerization and elongation of actin at the pollen tube apex is obscure. A key point to consider is that almost all of G-actin should be complexed with profilin, and consequently spontaneous polymerization is tightly suppressed and eliminated. The nucle-

ation activity of gelsolin family proteins, e.g., P-135-ABP (Yokota et al. 2005), can not work when G-actin is saturated and bound to profilin. Two mechanisms for polymerizing and elongating actin filaments are speculated. First, G-actin bound profilin is added to uncapped barbed ends of pre-existing actin filaments (Vidali and Hepler 2001). Second, actin filaments are newly polymerized and created by potential nucleation factor, such as actin related protein Arp2/3 complex or formin. An inactivation of ADF/cofilin by Rop/Rac mediated by phosphorylation is also conceivable to promote actin polymerization.

As to the first mechanism, gelsolin family proteins (or heterodimeric capping proteins) should be released, promoting G-actin addition to free barbed ends. Decreasing Ca^{2+} concentrations is ineffective in dissociating these proteins from actin because their capping activity was almost Ca^{2+}-insensitive. Hence, an additional factor(s), such as PtdIns(4,5)P_2, may be involved in releasing these proteins from barbed ends of filaments. It has been shown that phosphatidylinositol monophosphate kinase that synthesizes PtdIns(4,5)P_2 acts as an effector of tip-localized Rac in pollen tubes (Kost et al. 1999). Hence, dissociating and releasing capping proteins from barbed ends of actin filaments by PtdIns(4,5)P_2 near or at these areas will trigger the elongation of actin filaments. Indirect confirmation of such hypothesis was obtained by localized caged photolysis of PtdIns(4,5)P_2 (Monteiro et al. 2005) which led to a reinforcement of the actin mesh in the sub-apical region of pollen tubes. However, PrABP80 showed relatively low binding ability to PtdIns(4,5)P_2 (Drøbak et al. 2004). Further studies are thus needed to elucidate the mechanism regulating the interaction of gelsolin family proteins with actin.

For the second hypothetical mechanism, two factors, Arp2/3 complex and formin protein, are considered to be good candidates to promote and regulate the polymerization of actin filaments. Arp2/3 complex contains two actin-related proteins, Arp2 and Arp3, and five additional subunits, and the *Arabidopsis* genome contains putative homologues of all seven subunits (Mathur 2005; Szymanski 2005). This complex preferentially binds to the side of pre-existing actin filaments and nucleates daughter actin filaments. This nucleation activity of Arp2/3 complex is enhanced and activated by the WASP (Wiscott-Aldrich syndrome protein)/WAVE (WASP family Verprolin homologous protein)/SCAR (Suppressor of cAR: *Dictyosrelium* homologue of WAVE) family member and recent works identified several genes in *Arabidopsis* encoding plant homologues of some components or subunits of SCAR/WAVE (reviewed in Szymanski 2005). Proline rich domain in these activators is considered to interact with profilin. Moreover, plant homologues, AtSCAR and ZmSCAR distantly related to SCAR/WAVE protein were also identified from *Arabidopsis* and maize, respectively, and found to interact with some components of plant homologue WAVE complex. The components of WAVE complex and Arp2/3 complex were shown to be expressed in floral tissues of *Arabidopsis* but, except for Arp2 (Klahre and Chua 1999), it has not been confirmed whether these proteins are expressed in pollen.

The formin molecule consists of a formin 1 domain (FH1), responsible for profilin binding, and a formin 2 domain (FH2) responsible for actin nucleation (Deeks et al. 2002). *Arabidopsis* has 21 isoforms of formin homology protein (AFH) divided into 2 groups. Group I formin (AFH1 to AFH11) is characterized by N-terminal proline rich glycosylated extracellular domain and a transmembrane domain that targets the formin to the cell membrane (Cheung and Wu 2004; Favery et al. 2004; Ingouff et al. 2005). Overexpression of AFH1 in pollen tubes of tobacco induced the formation of abundant actin cables projecting from the cell membrane, and concomitantly induced pollen tube swelling, growth depolarization and growth arrest (Cheung and Wu 2004). Moreover, the deformation of cell membrane in the apical regions occurred in overexpressing pollen tubes. AFH1 was shown to interact with profilin (Banno and Chua 2000) and recently, the analysis of actin interaction with recombinant constructs containing FH2 alone or FH1 and FH2 domains of AFH1 showed that AFH1 has abilities to promote the polymerization of G-actin bound to profilin (Michelot et al. 2005). The same work revealed that AFH1 interacts with barbed ends of actin filaments forming a "leaky capper" that allows actin polymerization from barbed ends, and binds to the side of filaments for bundling them. AFH5 (AtFH5) has also nucleation and capping activities (Ingouff et al. 2005). AFH2 was expressed in vegetative and floral organs (Banno and Chua 2000), suggesting that AFHs are present in pollen. However, predicted small GTPase binding domain from non-plant formin lacks AFHs. Further studies are needed to elucidate whether the activity of AFHs is controlled by the Rop/Rac signaling pathway and whether AFHs are indeed expressed in pollen tubes.

It remains to be determined how the actin dynamics in tip regions relates to the elongation of pollen tubes. It is possible that dynamic actin assembly provides oscillatory polar guidance to secretory vesicles and determines the position of their fusion at apical cell membrane. A recent work suggested that Ca^{2+} channels at the plasma membrane in tip regions, which are involved indispensably in influx of Ca^{2+} for generating tip-focused Ca^{2+} gradient, are controlled by actin filaments (Wang et al. 2004), and this could generate a positive feed-back for tip growth.

6
The Arrangement of Actin Filaments into Bundles

In the shank regions of pollen tubes where $[Ca^{2+}]_c$ is lower, large parts of the actin filaments are organized into bundles driving the "reverse-fountain" streaming. The direction of movement is dictated by the polarity of actin filaments so they are expected to show opposite directions of polymerization (pointed to barbed ends) at the center and edge of the tubes (Vidali and Hepler 2001). How this is achieved is presently unknown because if it was solely

based on the action of villin (e.g., P-135-ABP and P-115-ABP), the polymerization should be uniform.

In the sub-apical region, the collar structure could be maintained by a Ca^{2+}-insensitive type of villin, such as AtVIL1, which could stabilize the actin bundles and incorporate newly created actin filaments into pre-exist bundles. Fimbrin, another type of F-actin-crosslinking protein, was also identified from plants (McCurdy and Kim 1998), although the presence of this protein in pollen has not been reported. Fimbrin arranged actin filaments into aggregates, instead of bundles, in a Ca^{2+}-insensitive manner (Kover et al. 2000b). If it is expressed in pollen, it is possible that fimbrin is involved in collar formation in subapical regions of tubes.

7
Myosin, the Actin-based Molecular Motor for Cytoplasmic Streaming

Myosin generates the motive force along actin filaments (Shimmen and Yokota 2004) and is the main motor for cytoplasmic streaming and transport of organelles and vesicles in pollen tubes. Recently, microtubule-based molecular motors (kinesins) were also identified and shown to be responsible for a low-speed transport of some organelles and vesicles in pollen (Yokota and Shimmen, this volume).

Myosin is composed of heavy and light chains and is grouped into at least 18 classes on the basis of the primary structure of the heavy chain genes in eukaryotes (Seller 2000; Berg et al. 2001). From plant cells, three classes of myosins, VIII, XI and XIII, have been identified (Reichelt and Kendrick-Jones 2000; Reddy and Day 2001). To date, class XIII myosin has been found only in green alga *Acetabularia* and myosin VIII has not yet been reported in pollen. In *Arabidopsis*, there are four subclasses of myosin VIII, while myosin XI forms a large gene family with at least 13 isoforms classified into three subclasses, MYA1, MYA2 and MYA3.

A subclass of myosin XI has been biochemically isolated from pollen (Yokota et al. 1999a; Shimmen and Yokota 2004). This 170-kDa myosin was composed of a 170-kDa heavy chain and CaM as the light chain. The sliding velocity of actin filaments induced by this myosin in vitro was comparable to the velocity of cytoplasmic streaming observed in living pollen tubes (Yokota and Shimmen 1994). The 170-kDa polypeptide with similar antigenicity to lily 170-kDa myosin heavy chain was also present in tobacco pollen and vegetative cultured cells, and located around surfaces of organelle with various sizes (Yokota et al. 1995, 1999b). These results demonstrated that this myosin is a motor for generating motive force in pollen tube cytoplasmic streaming and that Myosin XI is a general molecular motor for streaming and organelle transport also in vegetative cells (Shimmen and Yokota 2004). The motile activity and actin-activated ATPase activity of 170-kDa myosin were suppressed

reversibly by micromolar concentrations of Ca^{2+} through CaM light chain (Yokota et al. 1999a). When Ca^{2+} concentrations were elevated up to 10^{-5} M, the CaM light chain was dissociated from heavy chain but this phenomenon is not physiologically relevant, because $[Ca^{2+}]_c$ concentration is not so high in pollen tubes (Hepler et al., this volume). Taken together, these properties explain why an organized streaming is not observed in the apical region, it is doubled blocked by a Ca^{2+}-inhibited myosin activity and actin fragmentation.

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