ABP41 is Involved in the Pollen Tube Development via Fragmenting Actin Filaments

Ting Wang², Yun Xiang², Jian Hou and Hai-Yun Ren¹

Key Laboratory of Cell Proliferation and Regulation Biology of Ministry of Education, Beijing Normal University, Beijing 100875, People's Republic of China

ABSTRACT ABP41 is identified as a novel member of plant villin/gelsolin/fragmin superfamily proteins from lily pollen, which binds stoichiometrically to actin filaments and severs them in vitro. To further understand its in-vivo function and the potential molecular mechanisms, biochemical analysis, fluorescence microscopic observation and microinjection assays were performed. Different biochemical measurements showed that ABP41 maintained actin filaments in forms of short F-actin in vitro. Microinjection of ABP41 into pollen tubes could fragment the pre-existing actin filaments, inhibit the velocity of cytoplasmic streaming, and shorten the length of the clear zone of pollen tube. In addition, it was found that the endogenous ABP41 expressing level was dynamically corresponding to the short actin filament structure in pollen at different stages of pollen germination. Our results suggest that ABP41 is involved in the regulation of actin dynamics during the pollen germination process via maintenance of short dynamic actin filaments.

Key words: ABP41; actin filament fragmentation; pollen germination; actin-binding protein.

INTRODUCTION

Pollen tubes, which deliver male gametes to the ovule during sexual reproduction in higher plants, are a fascinating model system for the study of highly polarized cell growth (Franklin-Tong, 1999; Taylor and Hepler, 1997). It is well known that the actin cytoskeleton suffers dynamic changes in actin array during pollen hydration and the germination process. In the dehydrated dormant state of pollen grains, actin filaments exist mostly as crystalline fusiform bodies or speculate structure, in readiness for assembling the extensive actin array necessary for pollen tube extension (Xu et al., 2004). In germinated pollen tubes, actin structure was described characteristically as three forms: the regular actin bundles arrayed in the shank region that provide tracks of the rapid cytoplasmic streaming driven by myosin, ensuring the delivery of cytoplasmic particles (Parton et al., 2001), less organized or collar-like filament bundles at the sub-apex responsible for reversing cytoplasmic streaming direction and the highly dynamic array of actin filament, especially the short F-actin fragments in the apical region that play a central role in polarized pollen growth (Fu et al., 2001; Gibbon et al., 1999; Hepler et al., 2001; Vidali and Hepler, 2001). It is suggested that the dynamic actin organization was regulated by many regulating factors, such as Ca²⁺, which was reported to inhibit cytoplasmic streaming at the 10⁻⁵-M level (Kohno and Shimmen, 1988) and cause fragmentation of actin filaments in the lily pollen tubes (Yokota et al., 2005).

It is widely accepted that there are many actin binding proteins participating in the regulation of actin dynamics in pollen grains and the tubes (Ren and Xiang, 2007). Among them, profilin, ADF, and villin/gelsolin/fragmin superfamily proteins are relatively extensively characterized. Profilin, a simple sequestering protein by binding to actin monomers and preventing spontaneous actin nucleation and polymerization in the presence of capped barbed end, is essential for tip growth and proper organization of the F-actin network (Vidali et al., 2007). Another well characterized ABP is ADF that interacts with both monomeric and filamentous actin and enhances depolymerization by increasing the off rate of actin monomers at the pointed end and by inducing filament severing (Bamburg, 1999; Carlier et al., 1997). A characterized pollen-specific ADF–LIADF showed decorating F-actin in pollen grains to maintain the storage form of actin arrays (Iwano et al., 2004; Smertenko et al., 2001). Villin/gelsolin/fragmin superfamily proteins are typified by the possession of three or six
gelsolin-like domains existing abundantly in pollen grain or the tube. P-115-ABP and P-135-ABP, identified from pollen tube as members of the villin/gesolin superfamily, show co-localization with the filamentous actin in the pollen tube (Yokota and Shimmen Ki, 1998) and are responsible for the stable actin bundles in the shank region (Yokota et al., 2005). P-135-ABP and P-115-ABP, identified from pollen tube as members of the villin/gesolin superfamily, show co-localization with the filamentous actin in the pollen tube (Yokota and Shimmen Ki, 1998) and are responsible for the stable actin bundles in the shank region (Yokota et al., 2005). A gelsolin-like 80-kD protein from poppy pollen (Huang et al., 2004) and a fragmin-like protein from *Mimosa pudica* (Yamashiro et al., 2001) have been identified and characterized to sever the actin filament in vitro. Recently, two low-molecular-weight members of the family, LdABP41 (Fan et al., 2004) and ABP29 (Xiang et al., 2007), have been identified in *Lilium* pollen. LdABP41 (ABP41) is a fragmin-like protein containing three gelsolin-like domains, nucleates actin and severs prepolymerized lily pollen F-actin into short actin filament in a Ca\(^{2+}\)-sensitive manner. ABP29 contains only two gelsolin-like domains, but has similar biochemical characteristics with ABP41 in actin monomer binding and actin filament severing. However, their functional differences in pollen with different states are still unclear.

In the present study, we found that ABP41 could maintain the actin filaments in short forms in vitro. Microinjection assays revealed that ABP41 could fragment actin filaments in vivo. In addition, the dynamic expressing level of ABP41 corresponded to the short actin filament structure during the pollen hydration and germination process. All together, the above results suggest that ABP41 is involved in the regulation of actin dynamics during the pollen germination process via fragmenting actin filament.

### RESULTS

**Actin Filaments Maintained in Short Forms in the Presence of ABP41 In Vitro**

Purified ABP41 with purity above 90% shown in Figure 1A, Lane 2, was applied to the following series of functional assays. In our experimental conditions, actin alone was polymerized to a length of 16.77 ± 4.37 μm (n ≥ 200) (Figure 1B). When G-actin incubated with 300 nM ABP41 in the presence of 200 μM Ca\(^{2+}\), actin filament arrayed in the form of very short F-actin with length of 1.82 ± 0.53 μm (Figure 1C) and the number of actin filaments increased per field. When G-actin incubated with 300 nM ABP41 in the presence of 2 mM EGTA (50 nM Ca\(^{2+}\)), the length of F-actin recovered to 11.09 ± 3.61 μm (Figure 1D), which indicated that the effect of ABP41 was Ca\(^{2+}\)-dependent. The increase of actin filament numbers by ABP41 was correlated with the nucleating activity of ABP41 identified before (Fan et al., 2004). However, the shortage of the actin filaments by ABP41 could also come from actin filament severing, capping, and depolymerization activity of ABP41. To verify if the effect of ABP41 involved these activities, the following assays were performed, respectively.

Initially, we tested whether ABP41 could sever populations of prepolymerized F-actin labeled with Alexa488-phalloidin. As shown in Figure 1F, ABP41 significantly reduced the length of actin filament to 3.45 ± 1.17 μm after incubation in the presence of 200 μM Ca\(^{2+}\) for 30 min when compared with control (average mean = 12.58 ± 4.22 μm) (Figure 1E). When the prepolymerized F-actin incubated with 300 nM ABP41 in

---

**Figure 1.** Microscope Observation of Alexa488-phalloidin-Labeled Actin Filaments in the Presence of 300 nM ABP41.

(A) Lane 1—Actin (about 43 kDa) eluted with 3 M urea from the DNase I affinity column. Lane 2—Targeting protein ABP41 purified from the DNase I affinity column.

(B) 5 μM monomeric actin was polymerized alone.

(C) 5 μM monomeric actin incubated with 300 nM ABP41 in the presence of 200 μM Ca\(^{2+}\).

(D) 5 μM monomeric actin incubated with 300 nM ABP41 in the presence of 2 mM EGTA.

(E) 5 μM prepolymerized actin filaments alone.

(F) 5 μM prepolymerized actin filaments incubated with 300 nM ABP41 in the presence of 200 μM Ca\(^{2+}\).

(G) 5 μM prepolymerized actin filaments incubated with 300 nM ABP41 in the presence of 2 mM EGTA. Bar = 10 μm.
the presence of 2 mM EGTA (50 nM Ca$^{2+}$), the mean actin filament length recovered to 9.16 ± 3.46 μm (Figure 1G). The results indicated that ABP41 could sever actin filament in a Ca$^{2+}$-dependent manner and maintain actin array in the short F-actin form.

The subsequent seed elongation assays were performed to examine whether ABP41 could cap the barbed end of actin seeds and inhibit the F-actin elongation from the barbed end. In our experiment, 1 μM G-actin saturated with 4 μM human profilin I was added to initiate actin elongation at the barbed end in the presence of 200 μM free Ca$^{2+}$. The results showed that the pyrene fluorescence was decreased corresponding to the substoichiometric amounts of ABP41, which suggested that ABP41 blocks the elongation of actin filament in a dose-dependent manner and, in the presence of 50 nM ABP41, the F-actin elongation process was almost suppressed completely (Figure 2A). The above result is consistent with that of ABP29 (Xiang et al., 2007). However, the complete inhibition of the F-actin elongation for ABP29 is at 400 nM concentration, indicating that the inhibition effect of ABP41 is stronger than that of ABP29. Similar experiments were performed in the presence of 50 nM ABP41 and various amounts of EGTA to test the Ca$^{2+}$ dependence of capping activity. As shown in Figure 2B, the pyrene fluorescence increased with the increased EGTA concentration compared to that of control, especially when, in the presence of 2 mM EGTA, the capping activity of ABP41 was arrested significantly.

The influence of ABP41 on actin filament depolymerization was investigated by diluting solutions of pyrene-labeled F-actin into buffer and monitoring the decrease in fluorescence. The results in Figure 2C showed that the depolymerization rate was increased by adding various amounts of ABP41 in the presence of 200 μM free Ca$^{2+}$, compared with that of actin alone. It is revealed that ABP41 could accelerate F-actin depolymerization.

**Introduction of ABP41 Leads to the Fragmentation of Microfilaments in Pollen Tubes**

Microinjection assay was performed to understand the role of ABP41 in pollen tube growth. Firstly, we investigate the effect of ABP41 on the cytoplasmic streaming rate and length of clear zone. The statistic data in Figure 3A and Figure 3B showed that 10 min after microinjection of 15 μM ABP41, the visible clear zone was gradually shortened and the rate of active cytoplasmic streaming became slower obviously compared with that of the control in which active cytoplasmic streaming and obvious clear zone were maintained. The above effects caused by ABP41 were significantly enhanced when 25 μM ABP41 was applied; some samples even showed the disappearance of the clear zone. It is suggested that the introduction of ABP41 shortened the clear zone and caused the slowdown of the cytoplasmic streaming rate effectively in a concentration-dependent manner.

The subsequent experiment was performed to test the influence of ABP41 on the dynamic actin structure in the growing pollen tube and Alexa488-phalloidin was applied by double-microinjection to label the actin filament. The results showed that in the control pollen tube, actin organization was arranged in parallel regular actin bundles in the shank and characteristic clear zone in the tip region without observable F-actin (Figure 3C). After microinjection of 25 μM ABP41, the visible short F-actin fragment appeared in the extreme tip of the pollen tube (Figure 3D) and the F-actin bundles in the shank became unstable and disordered, mostly in the form of short and thin actin filament, although the long F-actin bundles were still present. Increasing the protein

---

**Figure 2.** ABP41 Inhibits Actin Filament Elongation from the Barbed End and Accelerates Actin Filament Depolymerization.

(A) Preformed F-actin (0.4 μM) seeds were incubated with ABP41 at different concentrations, and 1 μM G-actin saturated with 4 μM human profilin I was added to initiate actin elongation at the barbed ends in the presence of 200 μM Ca$^{2+}$. The change in pyrene-actin fluorescence accompanying polymerization is plotted versus time after the addition of G-actin.

(B) Preformed F-actin (0.4 μM) seeds were incubated with 50 nM ABP41 in the presence of various EGTA concentrations.

(C) 5 μM polymerized F-actin (50% pyrene-labeld) was incubated with ABP41 of various concentrations in the presence of 200 μM Ca$^{2+}$ and then the solution was diluted 25-fold into buffer G. The change in pyrene-actin fluorescence accompanying actin depolymerization is plotted versus time after dilution.
concentration (45 \mu M ABP41), actin filament was fragmented into short segments dispersed uniformly in the whole tubes and, even in the shank region, no obvious long F-actin bundles were observed (Figure 3E), which indicated that the effect of exogenous ABP41 on the actin organization in vivo was concentration-dependent. It is revealed from the above results that excess ABP41 in the pollen tube potentially disrupts the dynamic actin structure and fragments the pre-existing actin filament into short F-actin.

Expressing Level of ABP41 in Corresponding to the Short Actin Filament Structure during Pollen Germination

To study the relationship between the dynamic expression level of ABP41 and the actin organization in the pollen development, the expressing level of ABP41 and the actin filament structure during different pollen germination process were simultaneously detected and analyzed. The actin in Figure 4A, Lane 1, could not be recognized by anti-ABP41 antibody, which confirmed that anti-ABP41 antibody was specific for the villin/geslolin/fragmin superfamily. It was shown that short actin fragments were the main form in the hydrated pollen (Figure 4B) in correspondence to the large amount of ABP41 (Figure 4A, Lane 3). When in conditions of shake culture for 5 min, the dynamic actin network appeared and the short actin fragment changed into thin and long filamentous actin (Figure 4C); it is shown in Figure 4A, Lane 4, that the corresponding amount of ABP41 was decreased obviously. When this dynamic actin network gradually transformed to the regular parallel F-actin bundles (Figure 4D), the corresponding amount of ABP41 (Figure 4A, Lane 5) was much less than

Figure 3. Effect of ABP41 on the Pollen Tube Growth and the Actin Cytoskeleton.
(A) Effect of ABP41 on the length of clear zone in the growing pollen tube. \( L \) = the length of clear zone before injection; \( L' \) = the length of clear zone after injection.
(B) Effect of LdABP41 on the rate of cytoplasmic streaming. \( V \) = rate of cytoplasmic streaming before injection; \( V' \) = rate of cytoplasmic streaming after injection. According to the two-tailed \( t \)-test, the difference between the control and ABP41 at both concentrations is significant \((P < 0.05)\). Error bars represent standard error (SE).
(C) The actin organization in pollen tubes after microinjection of buffer alone.
(D) The actin organization after microinjection of 25 \mu M LdABP41.
(E) The actin organization after microinjection of 45 \mu M LdABP41.
(C’–E’) The corresponding DIC images, respectively. Bar = 20 \mu m.
the former one. Upon germination, the ordered actin bundles encircled in a parallel array in the shank of the newly growing tube and highly dynamic F-actin formed in the tip (Figure 4E); the corresponding ABP41 contents (Figure 4A, Lane 6) were cut down to a steady level and still maintained at this level for the further tube growth (data not shown). In our experiment, anti-actin antibody was also applied and the result showed that the amount of actin during the pollen germination process is maintained at a constant (Figure 4A, Lanes 7–12).

DISCUSSION

Our previous studies have identified a novel plant villin/gelsolin/fragmin superfamily member-ABP41 from lily pollen and showed that it had actin nucleating and actin filament severing activities (Fan et al., 2004). Our present data suggest that ABP41 maintained actin array in the form of short F-actin fragment, probably by varieties of activities, such as nucleating, severing, capping, and depolymerization activity, which might be involved in regulating the dynamic balance between G-actin and F-actin level. Our results confirm that ABP41 possess the conserved function of the gelsolin/fragmin superfamily in vitro (dos Remedios et al., 2003; McGough et al., 2003), which is also consistent with the study of PrABP80 (Huang et al., 2004). However, in the same profilin- or diluted-mediated depolymerization assay, our newly identified gelsolin/fragmin superfamily member ABP29 inhibit actin depolymerization (Xiang et al., 2007), which is different from that of ABP41 that promote actin depolymerization, suggesting that the capping activity of ABP29 is stronger than that of ABP41, but monomer binding or severing activities of ABP41 are stronger. As ABP41 contains the presumably intact G1–G3 domain, but ABP29 just contains G1–G2, G3 domain is assumed to be the main consideration for the above difference.

Several members of the villin/gelsolin/fragmin superfamily protein have been identified and well characterized in vitro in higher plants (Huang et al., 2004; Xiang et al., 2007; Yokota et al., 2003). However, how they functioned in vivo is still not clear. It is found in our previous work that microinjection of ABP41 and anti-ABP41 antibody into germinated lily pollen could lower the elongation rate of growing pollen tube, which demonstrated that ABP41 play a important role in the pollen tube growth (Fan et al., 2004), but the potential mechanism of effect of ABP41 on the pollen tube growth is not explained. Our present data revealed that both the clear zone and the cytoplasmic streaming rate are influenced by the introduction of ABP41. Furthermore, it is demonstrated that exogenous ABP41 not only disrupt the tip dynamic actin polymerization correlated to the fusion of the small vesicles, but also influence the actin bundles in the shank region, which offers the track...
for the cytoplasmic streaming, which could be responsible for the significant effect of ABP41 on the clear zone and the cytoplasmic streaming rate. The above results indicated that exogenous ABP41 could functionally bind and fragment the actin filament in vivo and further inhibit the pollen tube growth via the disruption of actin organization. However, the substantial role of endogenous ABP41 in pollen tube growth needs to be further explored.

The analysis of the dynamic expression pattern of ABP41 corresponding to the unique actin structure during the pollen germination process indicates that there is a large amount of ABP41 in the hydrated pollen. Once cultured in germination medium, the ABP41 level descends gradually and then reaches a steady level when pollen grows to the mature tube. Synchronously, the actin filament structures change from short fragments in the pollen grain to long actin filament bundles in the shank of the tube and maintain dynamic fragments in the tip region, suggesting that the ABP41 level during the pollen germination process is possibly involved in the transformation of actin dynamics and required for the normal pollen tube germination. After growing to the mature pollen tube, the amount of ABP41 is still maintained at a low level invariably, which indicates that the rational quantity of ABP41 in the growing pollen tube is important for the dynamic regulation of actin organization in the tip region, especially the extreme tip region in which dynamic actin polymerization and depolymerization are under control (Cardenas et al., 2005; Gibbon et al., 1999). In addition, it is found that during the pollen germination process, the content of ABP29 maintains at the constant level and the expression patterns of ABP80, P-115-ABP, and P-135-ABP are quite different from that of ABP41, which indicated their distinctive role within the process. However, the potential significance of these diverse protein expression patterns of the villin/gelsolin/fragmin superfamilly is still unknown needs to be further studied.

In conclusion, ABP41 can maintain actin filaments in the form of short F-actin in vitro and fragment the pre-existing actin filament in vivo. Together with its dynamic expressing level corresponding to the short actin filament structure during pollen germination processes, it can be assumed that ABP41 is involved in the regulation of actin dynamics during the pollen germination process via the fragmentation of actin filament.

METHODS

Purification of ABP41 from Dehydrated Lily Pollen

The method for purification of ABP41 from lily pollen was performed as described in Fan et al. (2004) with some modifications. The targeting protein was eluted with EGTA buffer (0.1 M Tris, 0.5 mM EGTA, 0.5 mM ATP, 5 mM DTT, 10 μg mL⁻¹ aprotinin, 10 μg mL⁻¹ leupeptin, and 10 μg mL⁻¹ pepstatin, pH 7.5), then ultrafiltrated and dialyzed in buffer G (2 mM Tris–HCl, 0.2 mM CaCl₂, 0.2 mM ATP, 0.2 mM DTT, pH 8.0). Extraction of the lily total pollen protein was performed as Xiang et al. (2007) described. The final protein concentration was measured using the Bradford reagent (Bio-Rad Laboratories), and BSA as the standard. The final loading volume of all the total pollen proteins was 200 μg, and the pollen actin was 50 μg.

Immunoblotting

After SDS–PAGE, proteins on the 12% polyacrylamide gel were electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore) according to the method of Towbin et al. (1992), and the sheet was blocked with Tris-buffered saline (10 mM Tris–HCl and 150 mM NaCl, pH 7.4) containing 5% BSA and 0.5% Tween 20 for 60 min. Purified anti-ABP41 polyclonal antibody and rabbit anti-actin polyclonal antibody were diluted 100-fold with Tris-buffered saline supplemented with 5% BSA and 0.1% Tween 20. Anti-rabbit IgG conjugated with alkaline phosphatase was diluted 5000-fold as secondary antibody. All the experiments in this manuscript were repeated at least three times, independently.

Fluorescence Microscope Visualization of Actin Filament

In the experiment to visualize the effect of ABP41 on the generation of actin filament during G-actin polymerization, 5 μM G-actin incubated with 300 nM ABP41 in the presence of EGTA or not was polymerized in 1 F buffer at room temperature for 30 min. In the experiment to test ABP41’s severing activity, 5 μM prepolymerized actin filament were incubated with 300 nM ABP41 in the presence of EGTA or not at room temperature for 30 min. All of the above F-actin were labeled with an equimolar amount of Alexa488-phalloidin (Molecular Probes) and then were diluted to 50 nM; the diluted sample of 2 μL was added to a 22 × 22-mm cover slip for direct observation by fluorescence microscopy. Actin filaments for static observation were viewed using a confocal laser scanning microscope (Olympus FV-300) mounted on an inverted microscope (Olympus IX-70) using a 60× oil-immersion objective, and the images were collected by Olympus Fluoview 4.0 software.

Elongation Assay to Determine the Capping Activity of ABP41 for the F-Actin Barbed End

After 0.4 μM preformed F-actin seeds were incubated with ABP41 at different concentrations at room temperature for 5 min, 1 μM G-actin (5% pyrene-labeled) saturated with 4 μM human profilin I and one-tenth volume of 10 × F buffer were added to initiate actin elongation at the barbed end of actin filament in the presence of 200 μM free Ca²⁺. Also, preformed F-actin (0.4 μM) seeds incubated with 60 nM ABP41 in the presence of EGTA at various concentrations was performed to test the Ca²⁺-dependence of capping activity. Free Ca²⁺ in the presence of EGTA was calculated with winmaxc32 software by Petesmif (P. M. Smith, University of Liverpool), which is available at http://www.stanford.edu/~cpatton/maxc.html. According to our calculation, 2 mM EGTA was equivalent to the volume of 40.5 nM free [Ca²⁺], 1 mM EGTA equal to 91.1 nM [Ca²⁺], and 0.5 mM EGTA equal to 243 nM [Ca²⁺] in all of our experiment systems. The affinity of ABP41 for the
barbed ends of actin filament was determined by the variation of the initial rate of elongation.

**Dynamics of Actin Filament Depolymerization**

Five micromolar preformed F-actin (50% pyrene-labeled) was incubated with ABP41 of various concentrations at room temperature for 5 min in the presence of 200 μM free Ca\(^{2+}\), and the solution was diluted 25-fold into buffer G (2 mM Tris–HCl, 0.2 mM CaCl\(_2\), 0.2 mM ATP, and 0.2 mM DTT, pH 8.0) at room temperature. The decrease in pyrene fluorescence accompanying actin depolymerization was monitored for 400 s after dilution.

**Observation of the Effect on Actin Organization in Lily Pollen Tube via Microinjection of ABP41**

The whole experiment was completed via microinjection performance and the specific method was described in Fan et al. (2004). Approximately 0.3 nL of agents were injected into each pollen tube. The rate of cytoplasmic streaming and the length of clear zone were measured at the time of 10 min before injection and 10 min after injection for the calculation. Three kinds of agents: ABP41 (15 μM), ABP41 (25 μM), and ABP41 (45 μM) in the presence of 20 μM Ca\(^{2+}\) and the buffer contained 5 mM Tris–HCl and 20 μM Ca\(^{2+}\) as the control. The whole microinjection process was recorded on video tape (JVC-CK-C1381EG, color video camera, Japan). At least 10 successful injections of each sample were used for the statistical analysis and then F-actin in live pollen tube were labelled by microinjection of Alexa488-phalloidin (Molecular Probe), and images were acquired with a confocal laser scanning microscope (Olympus FV300-IX70). Cells were imaged through a 60× Olympus Pla-napo objective; the fluorescent probes were excited using the 488-nm line of the Argon laser. Optical sections in 1-μm steps were collected and projected with Fluoview 4.0 software.

**Labeling of Microfilaments in Pollen Grains and Tubes**

The method for labeling F-actin in pollen and pollen tube with Alexa488-phalloindin was referred to in Xiang et al. (2007).

**FUNDING**

This work was supported by the National Basic Research Program of China (grant no. 2007CB108700, 2006CB100100) and the National Natural Science Foundation of China (30630005, 30470176) to H.R. No conflict of interest declared.

**REFERENCES**


