**Oryza sativa** actin-interacting protein 1 is required for rice growth by promoting actin turnover

Meng Shi\(^1\), Yurong Xie\(^1\),†, Yiyun Zheng\(^1\),†, Junmin Wang\(^3\), Yi Su\(^4\), Qiuying Yang\(^1\) and Shanjin Huang\(^1\), *†

\(^1\)Key Laboratory of Plant Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China, \(^2\)University of Chinese Academy of Sciences, Beijing 100049, China, \(^3\)Institute of Crop and Nuclear Technology Utilization, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China, and \(^4\)Hunan Agricultural University, Changsha, 410128, China

Received 31 May 2012; revised 18 October 2012; accepted 1 November 2012; published online 17 January 2013.

*For correspondence (e-mail sjhuang@ibcas.ac.cn).
†These authors contributed equally to this work.

**SUMMARY**

Rapid actin turnover is essential for numerous actin-based processes. However, how it is precisely regulated remains poorly understood. Actin-interacting protein 1 (AIP1) has been shown to be an important factor by acting coordinately with actin-depolymerizing factor (ADF)/cofilin in promoting actin depolymerization, the rate-limiting factor in actin turnover. However, the molecular mechanism by which AIP1 promotes actin turnover remains largely unknown in plants. Here, we provide a demonstration that AIP1 promotes actin turnover, which is required for optimal growth of rice plants. Specific down-regulation of OsAIP1 increased the level of filamentous actin and reduced actin turnover, whereas over-expression of OsAIP1 induced fragmentation and depolymerization of actin filaments and enhanced actin turnover. In vitro biochemical characterization showed that, although OsAIP1 alone does not affect actin dynamics, it enhances ADF-mediated actin depolymerization. It also caps the filament barbed end in the presence of ADF, but the capping activity is not required for their coordinated action. Real-time visualization of single filament dynamics showed that OsAIP1 enhanced ADF-mediated severing and dissociation of pointed end subunits. Consistent with this, the filament severing frequency and subunit off-rate were enhanced in OsAIP1 over-expressors but decreased in RNAi protoplasts. Importantly, OsAIP1 acts coordinately with ADF and profilin to induce massive net actin depolymerization, indicating that AIP1 plays a major role in the turnover of actin, which is required to optimize F-actin levels in plants.

**Keywords:** actin, actin-binding protein, ADF, actin turnover, AIP1, *Oryza sativa*.

**INTRODUCTION**

Actin dynamics has been implicated in many fundamental physiological processes, such as endocytosis, cytokinesis, intracellular transport, cell motility and polarized cell growth (Pollard and Cooper, 2009; Thomas et al., 2009). Controlling the turnover of single actin filaments is at the heart of actin dynamics. How actin turnover is precisely controlled is a central question in cell biology. The turnover rate of actin filaments *in vivo* is much higher than that of actin filaments assembled *in vitro* (Zigmond, 1993), suggesting that actin turnover is accelerated by other cellular factors. Indeed, actin dynamics is coordinated by various actin-binding proteins (Pollard et al., 2000; Pollard and Cooper, 2009; Thomas et al., 2009; Blanchoin et al., 2010), of which actin-depolymerizing factor (ADF) and actin-interacting protein 1 (AIP1) act coordinately to accelerate actin depolymerization, the rate-limiting factor for actin turnover.

Actin-interacting protein 1 was originally identified from *Saccharomyces cerevisiae* as a protein that interacts with actin (Amberg et al., 1995), and has been implicated in many essential physiological processes, including muscle contraction, cytokinesis, cell motility, etc. (Konzok et al., 1999; Ono, 2001; Gerisch et al., 2004; Fujibuchi et al., 2005; Kato et al., 2008). However, the developmental and growth defects caused by loss of AIP1 are markedly different among organisms (Konzok et al., 1999; Rodal et al., 1999; Ketelaar et al., 2004), suggesting that it is necessary to analyze the growth and developmental defects caused by loss
of AIP1 carefully among organisms. AIP1 from *S. cerevisiae* was shown to interact with cofilin and to co-localize with cofilin on actin patches (Rodal et al., 2006), and micro-injection of excess Aip1 disturbs the cortical localization of cofilin in *Xenopus* embryos (Okada et al., 1999), indicating that AIP1 may act coordinately with cofilin to control actin dynamics in vivo.

In vitro biochemical analysis showed that AIP1 alone does not have an obvious effect on actin dynamics, but it promotes ADF/cofilin-mediated actin depolymerization (Aizawa et al., 1999; Okada et al., 1999, 2006; Rodal et al., 1999; Mohri and Ono, 2003; Clark et al., 2006; Mohri et al., 2006). Biochemical fractionation of thymus extract identified AIP1 as one of the factors that promote the cofilin-mediated disassembly of *Listeria* actin comet tails (Brieher et al., 2006), which supports these observations. Direct visualization of single actin filaments showed that Caenorhabditis elegans AIP1 (UNC-78) enhances filament severing by ADF (Ono et al., 2004). However, although ADF has been shown to promote dissociation pointed end subunits (Carlier et al., 1997), it remains to be resolved whether AIP1 can also enhance ADF-mediated dissociation of pointed end subunits. Additionally, *Xenopus* AIP1 (XAIP1) enhanced cofilin-induced actin polymerization only by capping filament barred ends (Okada et al., 2002), in contrast to a study showing that barred end capping is not required for the function of UNC-78 (Ono et al., 2004). Thus, it remains to be determined for each AIP1 whether it caps the barbed end and whether the capping activity is required for its coordinated action with ADF/cofilin. The variation in biochemical activities of AIP1 among organisms emphasizes the importance of analyzing its biochemical activities on a case-by-case basis and at the single-filament level prior to attempting to understand its function in vivo.

Down-regulation of AIP1 in Arabidopsis induced developmental defects and caused the formation of more thick bundles (Ketelaar et al., 2004), implicating AIP1 as an important factor in promoting actin turnover in Arabidopsis. Indeed, a recent study showed that AIP1 promotes actin dynamic remodeling in Physcomitrella patens (Augustine et al., 2011). However, the mechanism by which AIP1 and ADF coordinate actin dynamics remains largely unknown in plants, apart from one study, which showed that AtAIP1-1 promotes Lilium longiflorum ADF1 (LUAADF1)-mediated actin depolymerization (Allwood et al., 2002). The detailed mechanism by which AIP1 and ADF coordinate actin dynamics awaits further characterization.

*Oryza sativa* has become an increasingly important monocot model system for studying the mechanisms underlying plant growth and development due to the rapid development of resources in recent years, including a gene expression atlas and co-expression network analysis, full-length cDNA and mutant collections, and a high-efficiency transcription system (Xie et al., 2005; Toki et al., 2006; Zhang et al., 2006; Miyao et al., 2007; Nobuta et al., 2007; Piffanelli et al., 2007; Lu et al., 2008; Childs et al., 2011). Additionally, recent studies have verified rice as a good system to study the function of the actin cytoskeleton (Yang et al., 2011; Zhang et al., 2011b). Here, we found that *OsAIP1* is required for cell elongation and rice growth by promoting actin turnover.

**RESULTS**

**Alteration of *OsAIP1* expression affects rice growth**

To uncover potential cellular and developmental functions for *OsAIP1*, *OsAIP1* over-expression (OE) and RNAi transgenic lines were generated. The expression level was significantly increased in OE lines and reduced in RNAi lines (Figure S1A,B). Both *OsAIP1* over-expression and RNAi decreased the height of adult rice plants (Figure S1C–H). Further characterization showed that both *OsAIP1* OE and RNAi lines had reduced epidermal cell length (Figure S1I), suggesting that *OsAIP1* mis-expression inhibits anisotropic cell expansion. The inhibitory effect of *OsAIP1* mis-expression on cell length is positively and inversely related to the amount of *OsAIP1* transcript in the RNAi and OE lines, respectively. We also determined the effect of *OsAIP1* mis-expression on polarized root hair growth. No major defect in root hair morphology was detected in *OsAIP1* RNAi plants other than a significantly increased frequency of root hairs with a swollen tip (Figure 1b and Figure S2), compared to control plants harboring the empty vector TCK303 (Figure 1a). However, most *OsAIP1* OE root hairs do not grow straight and appear swollen (Figure 1c). Compared to controls (Figure 1d), the frequency of root hairs greater than 200 μm long decreased in RNAi plants (Figure 1e), and the frequency of short root hairs increased substantially in OE plants (Figure 1f). The mean length of *OsAIP1* RNAi and OE root hairs reduced significantly (P < 0.05 by Student’s t test). Additionally, the width of both RNAi and OE root hairs increased significantly (Figure 1g), suggesting that *OsAIP1* is also crucial for polarized root hair growth. Taken together, the data suggest that *OsAIP1* is required for maximal cell elongation and rice growth.

**Up-regulation of *AIP1* induces actin depolymerization whereas down-regulation of *AIP1* promotes actin polymerization**

We next examined the effect of *OsAIP1* mis-expression on the actin cytoskeleton in rice cells. Actin filaments behave as longitudinal cables in the shank of control root hairs (Figure 1h and Figure S3A,B), similar to the pattern of actin distribution in Arabidopsis root hairs (Ketelaar et al., 2004). However, the actin cytoskeleton became very prominent and highly bundled in the shank of *OsAIP1* RNAi root hairs (Figure 1h and Figure S3C–F). This was confirmed by
measurements showing that the mean fluorescence pixel intensity of actin staining in this region increased significantly from 19.8 ± 2.5 (n = 11) for the control to 41.5 ± 11.6 (n = 10) for OsAIP1 RNAi (P < 0.01 by Student’s t test), and the widths of fluorescence peaks increased significantly from 0.25 ± 0.03 (n = 137) for the control to 0.37 ± 0.01 (n = 156) for OsAIP1 RNAi (P < 0.01 by Student’s t test), suggesting that down-regulation of OsAIP1 promotes actin assembly. Additionally, large bundles invade the apical region of OsAIP1 RNAi root hairs (Figure 1h), which, to some extent, explains why the growth of OsAIP1 RNAi root hairs was inhibited. By contrast, actin filaments became few and scattered, and the fluorescence of actin filaments was very dim in OsAIP1 OE root hairs under identical image-acquisition conditions (Figure 1h and Figure S3G–I), suggesting that OsAIP1 over-expression induced actin depolymerization. By contrast, actin filaments became denser and more highly bundled in OsAIP1 RNAi pollen grains (Figure 2c). The F-actin level was decreased in OsAIP1 OE pollen grains but increased in OsAIP1 RNAi pollen grains (Figure 2d), and the F-actin level inversely correlated with the amount of OsAIP1 transcript (Figure S1A,B), suggesting that OsAIP1 inhibits actin polymerization. We also applied latrunculin B treatment and found that the rate of loss of F-actin was decreased in OsAIP1 RNAi pollen grains but increased in OE pollen grains (Figure S4), suggesting that OsAIP1 promotes actin turnover. Taken together, the data suggest that OsAIP1 reduces actin polymerization in pollen and root hair by promoting actin turnover.

Figure 1. Down-regulation of OsAIP1 promotes actin assembly, whereas up-regulation of OsAIP1 inhibits actin assembly in root hairs. (a–c) Micrographs of root hairs. (a) Control; (b) OsAIP1 RNAi line 8-3; (c) OsAIP1 OE line 8-2. The white arrow in (b) indicates a root hair with a swollen tip. Scale bar = 50 μm. (d–f) Length distribution of root hairs (n ≥ 200). (d) Control; (e) OsAIP1 RNAi line 8-3; (f) OsAIP1 OE line 8-2. (g) Both OsAIP1 RNAi and OE increase the width of root hairs at the tip. Asterisks indicate a significant difference compared with control (**P < 0.01 by Student’s t test, n ≥ 128). (h) The actin cytoskeleton in root hairs. Scale bar = 10 μm. Additional representative images of the actin filament distribution in control, OsAIP1 RNAi and OE root hairs are shown in Figure S3.
OsAIP1 enhances AtADF1-mediated filament severing and dissociation of pointed end subunits

To examine how OsAIP1 and AtADF1 coordinately modify single-filament dynamics, we initially determined the effect on filament length distribution. The result showed that OsAIP1 reduced the filament length in a dose-dependent manner in the presence of AtADF1, but that OsAIP1 alone had no effect (Figure S7). To distinguish whether OsAIP1 promotes AtADF1-mediated filament severing and/or pointed end subunit dissociation, direct visualization of single-filament dynamics by total internal reflection fluorescence microscopy (TIRFM) was performed. Compared to the buffer control (Figure 5a and Movie S1), addition of 50 nM AtADF1 generated more breaks along actin filaments (Figure 5b and Movie S2), suggesting that AtADF1 severs filaments. However, compared to 50 nM AtADF1, addition of 250 nM OsAIP1 and 50 nM AtADF1 generated more breaks along filaments (Figure 5c and Movie S3) and OsAIP1 increased the number of breaks in a dose-dependent manner (Figure 5e), suggesting that OsAIP1 promotes AtADF1-mediated filament severing. Addition of 500 nM OsAIP1 alone did not generate more breaks along filaments compared to the buffer control (Figure 5d and Movie S4), suggesting that OsAIP1 cannot sever filaments on its own.

Actin filaments that remain fixed at the constriction point on an NEM (N-ethylmaleimide)-myosin-coated cover slip were selected for the measurement of subunit off-rate, thus excluding sliding actin filaments from the analysis. Based on previous studies showing that the subunit dissociation rate of the barbed end is faster than that of the pointed end for control actin filaments in the absence of ADF/cofilin (Kuhn and Pollard, 2005), the faster shortening end of the measured filament was designated as the pointed end, respectively. The pointed end subunit dissociation, direct visualization of single-filament dynamics by total internal reflection fluorescence microscopy (TIRFM) was performed. Compared to the buffer control (Figure 5a and Movie S1), addition of 50 nM AtADF1 generated more breaks along actin filaments (Figure 5b and Movie S2), suggesting that AtADF1 severs filaments. However, compared to 50 nM AtADF1, addition of 250 nM OsAIP1 and 50 nM AtADF1 generated more breaks along filaments (Figure 5c and Movie S3) and OsAIP1 increased the number of breaks in a dose-dependent manner (Figure 5e), suggesting that OsAIP1 promotes AtADF1-mediated filament severing. Addition of 500 nM OsAIP1 alone did not generate more breaks along filaments compared to the buffer control (Figure 5d and Movie S4), suggesting that OsAIP1 cannot sever filaments on its own.

We next determined the biochemical basis for the function of OsAIP1. As OsAIP1 shares reasonable homology with characterized AIP1 proteins and contains some conserved residues (Figure S5), it is reasonable to speculate that OsAIP1 may retain conserved functions. Recombinant OsAIP1 bound to actin filaments (Figure 3c), but did not affect the amount of sedimented actin (Figure 3d), implying that OsAIP1 does not affect actin polymerization. Indeed, OsAIP1 did not affect the kinetics of spontaneous actin assembly (Figure 3e). As AIP1 proteins from various organisms have been shown to act coordinately with ADF/cofilin to modify actin dynamics, we examined the synergistic effect of OsAIP1 with ADF. The well-characterized protein AtADF1 (Carlier et al., 1997) was included in these assays. Consistent with its role in promoting actin depolymerization, AtADF1 increased the amount of actin in the supernatant (Figure 4a). Addition of OsAIP1 enhanced the depolymerizing activity of AtADF1 substantially (Figure 4a), and promoted AtADF1-mediated depolymerization in a dose-dependent manner at both pH 6.8 and pH 8.0 (Figure 4b). Given that UNC-78 has been shown to have preference for a specific ADF isoform (Mohri and Ono, 2003; Ono et al., 2011), we tested whether OsAIP1 prefers rice ADF. We generated recombinant proteins of two rice ADFs, OsADF2 and OsADF9, that are expressed in vegetative and reproductive tissues, respectively (Figure S6A). However, the results showed that OsAIP1 did not have a preference for either OsADF2 or OsADF9 (Figure S6B–D), implying that OsAIP1 may not have preference for specific ADF isovariants. Taken together, the data suggest that OsAIP1 alone does not have an obvious effect on actin dynamics, but promotes ADF-mediated actin depolymerization.

OsAIP1 assists ADF-mediated depolymerization in vitro

We next determined the biochemical basis for the function of OsAIP1. As OsAIP1 shares reasonable homology with characterized AIP1 proteins and contains some conserved residues (Figure S5), it is reasonable to speculate that OsAIP1 may retain conserved functions. Recombinant OsAIP1 bound to actin filaments (Figure 3c), but did not affect the amount of sedimented actin (Figure 3d), implying that OsAIP1 does not affect actin polymerization. Indeed, OsAIP1 did not affect the kinetics of spontaneous actin assembly (Figure 3e). As AIP1 proteins from various organisms have been shown to act coordinately with ADF/cofilin to modify actin dynamics, we examined the synergistic effect of OsAIP1 with ADF. The well-characterized protein AtADF1 (Carlier et al., 1997) was included in these assays. Consistent with its role in promoting actin depolymerization, AtADF1 increased the amount of actin in the supernatant (Figure 4a). Addition of OsAIP1 enhanced the depolymerizing activity of AtADF1 substantially (Figure 4a), and promoted AtADF1-mediated depolymerization in a dose-dependent manner at both pH 6.8 and pH 8.0 (Figure 4b). Given that UNC-78 has been shown to have preference for a specific ADF isoform (Mohri and Ono, 2003; Ono et al., 2011), we tested whether OsAIP1 prefers rice ADF. We generated recombinant proteins of two rice ADFs, OsADF2 and OsADF9, that are expressed in vegetative and reproductive tissues, respectively (Figure S6A). However, the results showed that OsAIP1 did not have a preference for either OsADF2 or OsADF9 (Figure S6B–D), implying that OsAIP1 may not have preference for specific ADF isovariants. Taken together, the data suggest that OsAIP1 alone does not have an obvious effect on actin dynamics, but promotes ADF-mediated actin depolymerization.
the subunit dissociation rate for the pointed end increased in a dose-dependent manner, but OsAIP1 alone did not enhance the pointed end subunit dissociation rate (Table S1). Taken together, the data suggest that OsAIP1 enhances AtADF1-mediated actin filament severing and subunit dissociation from the pointed end.

OsAIP1 caps filaments in the presence of AtADF1, but capping activity is not a prerequisite for their coordinated action

To determine whether the synergistic effect of AIP1 and ADF on reducing filament length is due to the capping activity, an actin elongation assay was performed. AtADF1 promoted actin elongation (Figure 6a), consistent with its role in generating more barbed ends by severing filaments. However, on addition of OsAIP1, the initial elongation rate was inhibited in a dose-dependent manner (Figure 6a). As OsAIP1 enhances AtADF1-mediated filament severing, addition of OsAIP1 together with AtADF1 should generate more filaments than AtADF1 alone. Theoretically, the actin polymerization rate resulting from barbed end elongation should be faster in the presence of OsAIP1 and AtADF1 than in the presence of AtADF1 alone if the barbed ends are free. However, the result is the opposite, suggesting that the concentration of free barbed ends decreased on addition of OsAIP1 together with ADF. The interpretation may be that OsAIP1 has barbed end capping activity. However, OsAIP1 alone does not affect actin elongation (Figure 6a), suggesting that the capping activity of OsAIP1 requires the presence of ADF. As barbed end capping allows filament stabilization by preventing subunit loss upon dilution, a dilution-mediated actin depolymerization assay was performed to test this. Compared to actin alone, AtADF1 enhanced dilution-mediated depolymerization (Figure 6b), consistent with its role in promoting depolymerization as shown previously (Carlier et al., 1997). However, on addition of OsAIP1 together with AtADF1, dilution-mediated depolymerization was inhibited in a dose-dependent manner.
manner (Figure 6b), probably due to the capping activity shown in Figure 6(a). However, OsAIP1 inhibits depolymerization, although it does not have capping activity on its own (Figure 6b), probably due to its filament side binding activity, as shown in Figure 3.

To determine whether capping activity is required for the coordinating effect of OsAIP1 and ADF as proposed by Okada et al. (2002), the actin barbed end was pre-capped using CytoD (Cooper, 1987) and Arabidopsis capping proteins (AtCP) (Huang et al., 2003). We initially demonstrated that both CytoD and AtCP were able to cap the barbed end of actin filaments (Figure S8). Pre-capping the barbed end using either CytoD or AtCP did not inhibit the coordinating effect of OsAIP1 and AtADF1 on shortening filaments (Figure S9). The results were further extended by visualizing single-filament dynamics directly, showing that pre-capping by AtCP did not inhibit the effect of OsAIP1 and AtADF1 on enhancing severing and dissociation of pointed end subunits (Figure S10 and Table S2). Taken together, the data suggest that occupation of the actin barbed end by another capping agent does not enhance or prevent the synergistic effect of OsAIP1 and AtADF1, implying that barbed end capping is not a prerequisite for the co-ordinating effect of OsAIP1 and AtADF1.

**Actin filaments are turned over rapidly by AIP1 and ADF in the presence of a high concentration of polymerizable actin**

The concerted action of OsAIP1 and AtADF1 led us to examine whether they cooperate with the monomer-sequestering effect of profilin to maintain the actin monomer pool. To test this, a high-speed co-sedimentation assay was performed. Combination of all three components led to the greatest extent of depolymerization compared to either AtADF1 plus OsAIP1 or profilin plus OsAIP1 (Figure S11), suggesting that OsAIP1 and AtADF1 act synergistically with profilin to enhance depolymerization. We next determined how AIP1/ADF drive rapid actin dynamics in the presence of a high concentration of actin/profilin at the single filament level. Perfusion of 5 μM actin/profilin or 5 μM actin/profilin together with 1 μM OsAIP1 had negligible effect on filament severing (Figure S12 and Movies S5 and S6). However, more severing events were detected after perfusion of 50 nM AtADF1 together with actin/profilin (Figure S12 and Movie S7), suggesting that AtADF1 is able to sever filaments in the presence of high concentrations of polymerizable actin. However, more severing events occurred when OsAIP1 was added together with AtADF1 in the presence of a high concentration of actin/profilin (Figure S12 and Movie S8). The severing frequencies were significantly higher than that for 50 nM AtADF1 alone (Figure S12). Additionally, OsAIP1 increased the pointed end subunit dissociation rate in a dose-dependent manner in the presence of 50 nM AtADF1 (Table S3). Taken together, the data suggest that AIP1 and ADF drive rapid actin turnover in the presence of a high concentration of actin/profilin, implicating AIP1 as an important factor in maintaining the surprisingly low amount of F-actin in plant cells.

**OsAIP1 promotes both filament severing and depolymerization in vivo**

The compelling in vitro evidence for the coordinated effect of OsAIP1 and ADF on regulating single-filament dynamics led us to examine the function of OsAIP1 in regulating the
dynamics of single actin filaments in vivo. Protoplasts derived from 14-day-old dark-grown rice sheaths and stems of control, OsAIP1 OE and RNAi plants were transformed with a GFP–ABD2–GFP plasmid in which expression of the fusion was driven by the ubiquitin promoter (Yang et al., 2011). Real-time visualization of control protoplasts showed that single filaments were severed frequently and depolymerized rapidly after being severed (Figure 7a, Movie S9 and Table S4). The phenomenon is very similar to that in Arabidopsis hypocotyl epidermal cells and BY-2 suspension cells (Staiger et al., 2009; Smerlenko et al., 2010). However, actin filaments are very stable in OsAIP1 RNAi protoplasts: most filaments became heavily bundled, and filament-severing events were hardly detected within the time window of image acquisition (Figure 7b and Movie S10). By contrast, filaments are extremely dynamic in OsAIP1 OE protoplasts (Figure 7c and Movie S11). Both the severing frequency and the subunit dissociation rate increased in OsAIP1 OE protoplasts, but decreased significantly in RNAi protoplasts (Figure 7d,e and Table S4). Consequently, the maximum filament lifetime increased in OsAIP1 RNAi protoplasts and decreased significantly in OsAIP1 OE protoplasts (Table S4). Taken together, these in vivo findings suggest that OsAIP1 is

![Figure 5. Direct visualization of AtADF1-mediated filament severing by TIRFM.](image)

(a) Time-lapse images of actin filaments after injection of 1× TIRFM buffer.
(b) Time-lapse images of actin filaments after injection of 50 nM AtADF1 in 1× TIRFM buffer.
(c) Time-lapse images of actin filaments after injection of 50 nM AtADF1 and 250 nM OsAIP1 in 1× TIRFM buffer. Movie S3 shows the entire series.
(d) Time-lapse images of actin filaments after injection of 500 nM OsAIP1 in 1× TIRFM buffer.
(e) Mean severing frequencies were determined as described in Experimental procedures and plotted. Values are means ± SE (n = 3). Asterisks indicate significant differences (**P < 0.01 by Student’s t test).
important for modulating the stochastic dynamic behavior of actin filaments by enhancing filament severing and subunit dissociation.

**DISCUSSION**

**OsAIP1 enhances AtADF1-mediated severing and dissociation of pointed end subunits**

Our results demonstrate unambiguously that OsAIP1 enhances both ADF-mediated severing and dissociation of pointed end subunits (Figure 5 and Table S1). However, how OsAIP1 achieves this remains elusive. Previous studies showed that ADF/cofilin sever filaments by weakening the lateral contacts between sub-domain 1 (SD1) and sub-domain 2 (SD2) of adjacent actin subunits (Galkin et al., 2003). Further electron cryomicroscopy analysis showed that binding of cofilin substantially disorders and displaces SD2 of actin (Galkin et al., 2011) and increases the flexibility of actin filaments (Orlova and Egelman, 1993). Therefore, the promoting effect of AIP1 on ADF-mediated severing may be due to the promoting effect of AIP1 on ADF-induced weakening of lateral contacts with adjacent actin subunits. It has been hypothesized that binding of cofilin creates a conformational change in F-actin conducive to AIP1 interaction (Clark et al., 2006). However, a very recent study showed that cofilin severs filaments at the junction between bare and cofilin-decorated regions, and found that ADF/cofilin binding is not necessary to induce filament severing, but the frequency of filament severing correlates with boundary density (Suarez et al., 2011). A previous study showing that ADF/cofilin only severs filaments at relative low concentrations (Andrianantoandro and Pollard, 2006) supports this. Therefore, the promoting effect of AIP1 on filament severing may be due to either the increase in the density of boundaries and/or the shear stress that accumulates at the boundaries between bare and ADF/cofilin-decorated regions. Additionally, the additive effect of a structural change of F-actin in the presence of AIP1 may promote dissociation of actin subunits from the pointed end. Indeed, it was suggested that the conformational change induced by ADF/cofilin binding may propagate to the bare regions (Galkin et al., 2003). Future structural analysis of F-actin decorated with ADF and AIP1 will provide insights into this, and direct visualization of actin filaments and ADF/cofilin simultaneously in the presence of AIP1 will also provide clues as to the coordinated mode of AIP1 and ADF/cofilin, as performed by Suarez et al. (2011).

**Barbed end capping is not a prerequisite for the coordinated action of OsAIP1 and AtADF1**

Our results showed that OsAIP1 caps the barbed end in the presence of ADF (Figure 6a,b). It remains unknown why AIP1 only caps the barbed end in the presence of ADF/cofilin. It may be that the binding of ADF/cofilin induces a conformational change on actin filaments as observed previously (McGough et al., 1997; McGough and Chiu, 1999; Galkin et al., 2011), to allow binding of AIP1 to the barbed end. Indeed, it has been hypothesized that binding of cofilin creates a conformational change in actin filaments that is conducive to AIP1 interaction, and AIP1 may bind to newly formed barbed end after cleaving of filaments by ADF/cofilin (Clark et al., 2006). However, our studies showed that barbed end capping is not required for the coordinated action of OsAIP1 and AtADF1 (Figures S9 and 10), similar to the finding for UNC-78 (Ono et al., 2011).

Figure 6. OsAIP1 has barbed end capping activity.

(a) OsAIP1 inhibits actin elongation in the presence of AtADF1. Ten micromoles of F-actin were incubated with 10 μM AtADF1 and various concentrations of OsAIP1 at room temperature for 5 min, and the mixture was then diluted tenfold with 2 μM actin monomers (10% pyrene-labeled) in 1× F-buffer (10 mM KCl, 10 mM MgCl2, 10 mM EGTA, and 100 mM Tris-HCl, pH 8.0) to initiate elongation at pH 8.0.

(b) OsAIP1 inhibits dilution-mediated actin depolymerization in the presence of AtADF1. NBD (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole)-labeled actin was used in this assay because quenching of NBD-labeled actin fluorescence by AtADF1 is not very obvious compared to the quenching of pyrene-labeled actinfluorescence by AtADF1. Five micromoles of pre-assembled actin filaments (50% NBD-labeled) together with 2.5 μM AtADF1 were incubated with various concentrations of OsAIP1 at pH 8.0, and the mixtures were then diluted 12.5-fold in buffer G (5 mM Tris-Cl, pH 8.0, 0.2 mM ATP, 0.2 mM CaCl2, 0.1 mM DTT).
but different from that for XAIP1 (Okada et al., 2002). This is also in contrast to results showing that barbed end capping with either CytoD or CapZ inhibited the endwise bursting disassembly induced by AIP1 together with coronin and cofilin (Kueh et al., 2008). Although barbed end capping is not a prerequisite for the coordinated action of OsAIP1 and AtADF1 in vitro, it may be important in plant cells (discussed below).

**The potential role of AIP1 and ADF in maintaining the actin monomer pool**

The biochemical activities of OsAIP1 and AtADF1 led us to assume that they may coordinate with the monomer sequestering activity of profilin to cause net actin depolymerization. The fact that addition of an actin-severing and barbed end-capping protein PrABP80 together with profilin to pre-assembled filaments induced massive depolymerization in vitro supports this hypothesis (Huang et al., 2004). Our in vitro reconstitution experiments also support this (Figure S11). Additionally, our study showed that OsAIP1 drives rapid actin dynamics in the presence of a high concentration of actin/profilin (Figure S12), which may explain why single filaments turnover rapidly in the cortical region of plant cells (Staiger et al., 2009; Smertenko et al., 2010). As actin/profilin can add to the barbed end of actin filaments just as well as actin alone (Pantaloni and Carlier, 1993) and the concentration of profilin/actin may be as high as 100-200 μM in plant cells (Chen et al., 2009), extremely rapid actin elongation will therefore deplete the actin monomer pool very rapidly if barbed

![Figure 7. Both filament severing frequency and subunit off-rate are enhanced in OsAIP1 OE protoplasts but decreased in OsAIP1 RNAi protoplasts.](attachment:image.png)

(a) Time-lapse images of actin filaments in control protoplasts.
(b) Time-lapse images of actin filaments in OsAIP1 RNAi line 8-3 protoplasts.
(c) Time-lapse images of actin filaments in OsAIP1 OE line 8-2 protoplasts. Colored arrows indicate breaking events along actin filaments, which are indicated by colored dots. Scale bar = 2 μm.
(d) Plot of filament severing frequency, expressed as the number of breaks per unit filament length per second. Values are means ± SD. Asterisks indicate significant differences (**P < 0.01 by Student’s t test).
(e) Plot of subunit off-rate. Values are means ± SD. Asterisks indicate significant differences (**P < 0.01 by Student’s t test).
ends are free. The observation that the actin elongation rate reaches approximately 2 μm sec⁻¹ in the cortical region of etiolated hypocotyl cells supports this speculation (Staiger et al., 2009). In this regard, AIP1/ADF may play a role in maintaining the large actin monomer pool by capping filaments and preventing elongation.

Actin-depolymerizing factor/cofilins also play roles in promoting actin assembly, very likely through severing and nucleating actin assembly (Andriani Rantanoandro and Pollarid, 2006). This has been demonstrated in vivo, where activation of cofilin promoted actin assembly in cells (Ghosh et al., 2004). Therefore, AIP1 may play a role in promoting rapid actin assembly in vivo through its severing activity (Figure 5 and Movies S1–S4). However, cells must have a strategy to uncap AIP1-capped actin filaments.

**OsAIP1 promotes actin turnover in vivo**

Direct visualization of single filaments in protoplasts derived from control, OsAIP1 OE and OsAIP1 RNAi transgenic plants demonstrated unambiguously that OsAIP1 promotes filament severing and subunit dissociation (Figure 7, Movies S9–S11 and Table S4). This study, together with the recent report on Arabidopsis ADF4 (Hentty et al., 2011), indicates that AIP1/ADF play important roles in modulating the stochastic dynamic behavior of cortical actin filaments. Consistent with this, we found that OsAIP1 over-expression decreased the F-actin level whereas OsAIP1 RNAi increased the F-actin level (Figure 2). Additionally, more heavy actin bundles appeared in OsAIP1 RNAi root hairs and pollen grains (Figures 1h and 2, and Figure S3C–F), similar to the study in Arabidopsis (Ketelaar et al., 2004). This is very likely due to an increase in the amount of F-actin, which consequently increases the frequency of filament bundling spatially. Certainly, as OsAIP1 has filament side binding activity (Figure 3b,c), the possibility of an increase in filament binding in OsAIP1 RNAi cells by bundling factors such as villin, fimbrin and LIMs (LIM domain-containing proteins) (Thomas et al., 2009) cannot be completely ruled out.

Both OsAIP1 RNAi and OE lines inhibit cell expansion and rice growth (Figure S1), supporting previous observations that cell expansion requires a tightly controlled actin cytoskeleton (Ramachandran et al., 2000; Baluska et al., 2001; Dong et al., 2001; Collings et al., 2006; Kandasamy et al., 2009; Hentty et al., 2011; Yang et al., 2011; Zhang et al., 2011a,b). Additionally, OsAIP1 mis-expression inhibited root hair elongation and caused depolarized growth (Figure 1a–g), suggesting that AIP1-mediated actin dynamics is crucial for polarized cell growth. In contrast to the dramatic plant developmental defect caused by expression of an inducible AIP1 RNAi construct in Arabidopsis (Ketelaar et al., 2004), the phenotype of OsAIP1 RNAi plants is quite moderate. The reason may be that only transgenic plants with moderate developmental defects were selected during the transformation step. Analysis of OsAIP1 knock-out mutants will help to address this issue in the future.

**EXPERIMENTAL PROCEDURES**

**Plasmid construction**

To construct the protein expression vector, full-length OsAIP1 cDNA was amplified using the cDNA J033106G21 (Salk Institute Genomic Analysis Laboratory) as the template, with primers OsAIP1F and OsAIP1R (Table S5). After sequencing, the fragment was inserted into pET28b (Novagen, http://www.merkmillipore.com) digested with HindIII/Xhol to create pET28b-OsAIP1. To construct OsAIP1 RNAi and OE plasmids, an 800 bp gene-specific fragment and the full-length OsAIP1 coding sequence were amplified using the following primer pairs: OsAIP1,800F/OsAIP1,800R and OsAIP1OE1/OsAIP1OE2, respectively (Table S5). After sequencing, the sequences subsequently cloned into the TCK303 vector, in which expression is driven by a constitutive maize Ubi-1 promoter as previously described (Wang et al., 2004).

**Rice transformation and growth**

Transformation of rice (Zhonghua 10) embryonic calli was performed as described previously (Toki et al., 2006). T3 homozygous OsAIP1 OE and RNAi transgenic plants were used for phenotypic analysis. The rice plants were grown under natural conditions in the growing season (from May to early October) in Beijing, China. Plants with termination of elongation were photographed, and the lengths of the panicle and internodes were measured. Student’s t test implemented in the R programming language (version 2.11.0) was used for statistical analysis in this study.

**Observation of rice internode epidermal cells**

Epidermal cells from the middle portion of the upper 4th internode were incubated in chloral hydrate solution (8 g trichloroacetic-aldehyde monohydrate, 1 ml glycerol and 2 ml water) at room temperature for 5 days. They were then observed directly under an BX51 TRF microscope (Olympus, http://www.olympus-global.com) equipped with a 60× objective, and images were acquired using a Retiga EXi Fast 1394 CCD camera (QImaging, http://www.qimaging.com) using IMAGE-PRO EXPRESS 6.3 software (Media Cybernetics, http://www.mediacy.com). The length of the epidermal cell was measured as the distance between two longitudinal cork cells.

**Root hair determination and actin staining**

After germination for 4 days at 28°C in darkness, roots approximately 1 cm in length were cut from seedlings. Root hairs were observed under a microscope (Olympus BX51 TRF) equipped with 10× objectives, and images were acquired. More than 500 root hairs were selected for length and width measurement for each genotype. The actin staining procedure in root hairs has been described previously (He et al., 2016). Actin filaments were observed under an LSM META 510 laser scanning confocal microscope (Zeiss, http://www.zeiss.com), fluorescence was excited using a 488 nm blue argon laser, and the optical Z-series sections were collected in 0.5 μm steps. The images presented are projections of the optical sections through an individual root hair. The image collection setting was kept identical in order to compare the relative amount of F-actin between control and transgenic root hairs. F-actin staining and quantification in pollen grains were per-
formed as described previously (Ye et al., 2009; Zhang et al., 2010).

Direct visualization of actin dynamics in rice protoplasts

To visualize the dynamics of single actin filaments in vivo, the plasmid pCambia1300-ubi-EGFP-ABD2-EGFP (Yang et al., 2011) was transformed into rice protoplasts derived from control, OsAIP1 RNAi and OE lines. Transformation of rice protoplasts was performed as described previously (Bart et al., 2006), with minor modifications. Briefly, rice seeds were grown on half-strength MS medium for 14 days in the dark. The sheath and stem were cut into 0.5 cm pieces using sharp razors. The dissected tissues were immediately incubated with enzyme solution (10 mM MES, pH 5.7, 0.6 M mannitol, 1.5% cellulase RS (Yakult, http://www.yakult.co.jp), 0.75% macerozyme R10 (Yakult, http://www.yakult.co.jp), 0.1% bovine serum albumin, 1 mM CaCl2 and 5 mM b-mercaptoethanol) and shaken gently (40 rpm) for 4 h in the dark. An equal volume of HCl, pH 8.0, 50 mM KCl), aliquoted, frozen in liquid nitrogen and stored at -80°C. OsAIP1 was transformed into rice protoplasts derived from different conditions.

Protein production

To generate recombinant OsAIP1, the pET28b-OsAIP1 construct was transformed into the BL21 DE3 strain of Escherichia coli. Expression of OsAIP1 was induced for 16 h at 16°C with addition of 0.3 mM isopropyl thi-o-p-galactoside. 6His-tagged OsAIP1 was purified by Ni2+-NTA agarose column (Novagen, http://www.merckmillipore.com) according to the manufacturer’s instructions. The eluted OsAIP1 was dialyzed against dialysis buffer (5 mM Tris-HCl, pH 8.0, 50 mM KCl), aliquoted, frozen in liquid nitrogen and stored at -80°C. OsAIP1 was clarified at 200 000 g for 1 h before use, and its concentration was determined using the Bradford assay (Bio-Rad, http://www.bio-rad.com) with bovine serum albumin as the standard. Arabidopsis ADF1 (AtADF1), Arabidopsis capping proteins (AtCP), Arabidopsis profilin 2 (AtPRF2), OsFH5 FH2 and human profilin 1 (HRP01) were purified as described previously (Fedorov et al., 1994; Carlier et al., 1997; Gibbon et al., 1997; Huang et al., 2003; Yang et al., 2011).

Biochemical assays to determine the effect of OsAIP1 on actin

High-speed co-sedimentation assays were performed to examine the binding of OsAIP1 to actin filaments and determine the coordinated effect of OsAIP1 with AtADF1 on actin depolymerization, as previously described (Kovar et al., 2000) with minor modifications: the incubation and centrifugation were performed for 30 and 45 min, respectively. The actin elongation assay, dilution-mediated depolymerization assay and direct visualization of actin filaments by fluorescence microscopy were performed as described previously (Okada et al., 2002).

Quantification of actin filament severing and depolymerization by TIRFM

Direct visualization of single actin filaments by TIRFM was performed as described previously (Amann and Pollard, 2001). The flow chamber was pre-incubated with 25 nm NEM-myosin and subsequently incubated with 1% bovine serum albumin and washed with 1× TIRFM buffer (10 mM imidazole, pH 7.0, 50 mM KCl, 1 mM MgCl2, 1 mM EGTA, 50 mM dithiothreitol, 0.2 mM ATP, 50 mM CaCl2, 15 mM glucose, 20 µg ml-1 catalase, 100 µg ml-1 glucose oxidase and 0.5% methylcellulose) (Kovar and Pollard, 2004). Pre-assembled F-actin (100% rhodamine-labeled, 40 nM) was injected into the flow chamber and subsequently washed with 1× TIRFM buffer. To determine the effect of OsAIP1 and/or AtADF1 on actin dynamics, AtADF1 and/or OsAIP1 at various concentrations were injected into the perfusion chamber. Time-lapse images of actin filaments were acquired at every 3 sec. To determine the effect of AtADF1 and/or OsAIP1 on actin dynamics in the presence of high concentrations of actin/profilin, various concentrations of OsAIP1 and/or 50 nM AtADF1 together with 5 µM AtPRF2 and 5 µM G-actin in 1× TIRFM buffer were injected into the perfusion chamber. Quantification of severing frequency was determined as described by Andrianantoandro and Pollard (2006). To determine whether barbed end capping by ACP affects the promoting effect of OsAIP1 on AtADF1-mediated filament severing and dissociation of pointed end subunits, 100 nM ACP was included in the injection solutions.

ACKNOWLEDGEMENTS

We thank Kang Chong (Institute of Botany, Chinese Academy of Sciences, Beijing, China) for providing the TCK303 vector, Zuhua He (Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Beijing, China) for providing the plasmid pCambia1300-ubi-EGFP-ABD2-EGFP and RIKEN (Tokyo, Japan) for providing OsAIP1 full-length cDNA. We are grateful to Christopher J. Staiger (Department of Biological Sciences, Purdue University, Indiana, USA) for constructive comments on improving this manuscript, and Youjun Wu for critical reading. M.S. thanks Shaojie Cui for invaluable suggestions on rice protoplast transformation and Xiaolu Qu for operating the spinning disc confocal microscope. This work was supported by grants from the Ministry of Science and Technology of China (2013CB945100 and 2011CB444600) and the National Natural Science Foundation of China (31125004, 31121065 and 30770988).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.
Figure S1 Alteration of OsAIP1 expression inhibits rice growth.

Figure S2 OsAIP1 RNAi increases the frequency of root hairs with a swollen tip.

Figure S3 Actin cytoskeleton distribution in control, OsAIP1 RNAi and OsAIP1 OE root hairs.

Figure S4 OsAIP1 enhances actin turnover in pollen grains.

Figure S5 Comparison of the protein sequence of OsAIP1 with that of other AIP1 proteins.

Figure S6 OsAIP1 enhances both OsADF2- and OsADF9-mediated actin depolymerization.

Figure S7 OsAIP1 shortens actin filaments in a dose-dependent manner in the presence of 500 nM AtADF1.

Figure S8 AtCP and CytoD cap the barbed end of actin filaments.

Figure S9 Pre-capping by CytoD or AtCP does not inhibit the effect of OsAIP1 on enhancing the shortening of AtADF1-mediated filaments.

Figure S10 Pre-capping by AtCP does not inhibit AIP1/ADF-mediated filament severing.

Figure S11 OsAIP1 cooperates with AtADF1 and AtPRF2 to induce massive net actin depolymerization.

Figure S12 Direct visualization of ADF1-mediated filament severing in the presence of a high concentration of actin/AtPRF2.

Table S1 Mean subunit off-rate from the barbed and pointed ends of F-actin in the presence of 50 nM AtADF1 and various concentrations of OsAIP1.

Table S2 Mean subunit off-rate from the barbed and pointed ends of F-actin in the presence of 50 nM AtADF1 and various concentrations of OsAIP1 together with 100 nM AtCP.

Table S3 Mean subunit off-rate from the barbed and pointed ends of F-actin in the presence of 50 nM AtADF1 and various concentrations of OsAIP1 together with 5 μM G-actin and 5 μM AtPRF2.

Table S4 Actin dynamics parameters in protoplasts.

Table S5 Primer sequences used in this study.

Movie S1 Time-lapse series of actin filaments dynamics after injection of 1× TIRFM buffer.

Movie S2 Time-lapse series of actin filaments dynamics after injection of 50 nM AAADF1.

Movie S3 Time-lapse series of actin filaments dynamics after injection of 50 nM AAADF1 and 250 nM OsAIP1.

Movie S4 Time-lapse series of actin filaments dynamics after injection of 500 nM OsAIP1.

Movie S5 Time-lapse series of actin filaments dynamics after injection of 5 μM actin and 5 μM AtPRF2.

Movie S6 Time-lapse series of actin filaments dynamics after injection of 5 μM actin and 5 μM AtPRF2 together with 200 nM OsAIP1.

Movie S7 Time-lapse series of actin filaments dynamics after injection of 5 μM actin and 5 μM AtPRF2 together with 50 nM AtADF1.

Movie S8 Time-lapse series of actin filaments dynamics after injection of 5 μM actin and 5 μM AtPRF2 together with 50 nM AtADF1 and 200 nM OsAIP1.

Movie S9 Dynamic remodeling of actin filaments in control protoplasts.

Movie S10 Dynamic remodeling of actin filaments in OsAIP1 RNAi protoplasts.

Movie S11 Dynamic remodeling of actin filaments in OsAIP1 OE protoplasts.

REFERENCES


Oryza sativa AIP1 promotes actin turnover 759


Yang, W., Ren, S., Zhang, X., Gao, M., Ye, S., Qi, Y., Zheng, Y., Wang, J.,
Zeng, L., Li, Q., Huang, S. and He, Z. (2011) BENT UPPERMOST INTER-
NODE1 encodes the class II formin FH5 crucial for actin organization and
Arabidopsis formin3 directs the formation of actin cables and polarized
RMD: a rice mutant database for functional analysis of the rice genome.
*Nucleic Acids Res.* 34, D745–D748.
Zhang, H., Qu, X., Bao, C., Khurana, P., Wang, Q., Xie, Y., Zheng, Y., Chen,
an actin filament bundling and severing protein, is necessary for normal
VILLIN4 is involved in root hair growth through regulating actin organi-
zation in a Ca2+-dependent manner. *New Phytol.* 190, 687–692.
Zhang, Z., Zhang, Y., Tan, H., Wang, Y., Li, G., Lieng, W., Yuan, Z., Hu, J.,
Ren, H. and Zhang, D. (2011b) RICE MORPHOLOGY DETERMINANT
encodes the type II formin FH5 and regulates rice morphogenesis. *Plant
Zigmond, S.H. (1993) Recent quantitative studies of actin filament turnover