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Oryza sativa Actin-Interacting Protein 1 is required for Rice Growth via Promoting Actin Turnover

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SUMMARY

Rapid actin turnover is essential for numerous actin-based processes. However, how it is precisely modified remains poorly understood. AIP1 has been shown to be an important factor by acting coordinately with 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 the demonstration that AIP1 promotes actin turnover, which is required for optimal growth of rice plants. Specific downregulation of *OsAIP1* increased the level of filamentous actin and reduced actin turnover, whereas overexpression of *OsAIP1* induced fragmentation and depolymerization of actin filaments and enhanced actin turnover. *In vitro* biochemical characterization showed that, though OsAIP1 alone does not affect actin dynamics, it enhances ADF-mediated actin depolymerization. It also caps filament barbed end in the presence of ADF, but the capping activity is not required for their coordinating action. Real-time visualization of single filament dynamics showed that OsAIP1 enhanced ADF-mediated severing and pointed end subunit dissociation. Consistent with this, the filament severing frequency and subunit off-rate were enhanced in *OsAIP1* OE but decreased in RNAi protoplasts. Importantly, OsAIP1 acts coordinately with ADF and profilin to induce massive net actin depolymerization, indicating that AIP1 is a major player in the turnover of actin, which is required for optimizing F-actin levels in plants.

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 a plethora of actin-binding proteins (Blanchoin *et al.,* 2010; Pollard *et al.,* 2000; Pollard and Cooper 2009; Thomas *et al.,* 2009), in which actin depolymerizing factor (ADF) and actin-interacting protein (AIP) 1 act coordinately to accelerate the rapid actin depolymerization, the rate-limiting factor for actin turnover.

AIP1 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. (Fujibuchi *et al.,* 2005; Gerisch *et al.,* 2004; Kato *et al.,* 2008; Konzok *et al.,* 1999; Ono 2001). However, the developmental and growth defect caused by loss of AIP1 are markedly different among different organisms (Ketelaar *et al.,* 2004; Konzok *et al.,* 1999; Rodal *et al.,* 1999), suggesting that it is necessary to analyze the growth and developmental defects caused by loss of AIP1 carefully among different organisms. AIP1 from *S. cerevisiae* was lately shown to interact with cofilin and colocalize with cofilin on actin patches (Rodal *et al.,* 1999), and the microinjection of excess Aip1 disturbs the cortical localization of cofilin in *Xenopus* embryos (Okada *et al.,* 1999), it appears 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; Clark *et al.,* 2006; Mohri *et al.,* 2006; Mohri and Ono 2003; Okada *et al.,* 1999; Okada *et al.,* 2006; Rodal *et al.,* 1999). Biochemical fractionation of thymus extract identified AIP1 as one of the factors that promote the cofilin-mediated disassembly of *Listeria* actin comet tails, which supports these observations (Brieher *et al.,* 2006). Direct visualization of single actin filaments showed that *Caenorhabditis elegans* AIP1 (UNC-78) enhances filament severing by ADF (Ono *et al.,* 2004). However, considering that ADF was demonstrated to promote pointed end subunit dissociation (Carlier *et al.,* 1997), it remains to be resolved whether a given AIP1 can enhance ADF-mediated pointed end subunit dissociation as well. Additionally, *Xenopus* AIP1 (XAIP1) enhanced cofilin-induced actin fragmentation only by capping filament barbed ends (Okada *et al.,* 2002), in contrast to the study showing that barbed end capping is not required for the function of UNC-78 (Ono *et al.,* 2004). Anyhow, it remains to be determined for a given AIP1 whether it caps the barbed end and if the capping activity is required for its coordinating action with ADF/cofilin. The variation in biochemical activities of AIP1 among different organisms emphasizes the importance of analyzing its biochemical activities on a case-by-case basis and on a single filament level prior to understanding its function *in vivo*.

Downregulation 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, 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, besides one study which showed that AtAIP1-1 promotes LiADF1-mediated actin depolymerization (Allwood *et al.,* 2002). The detailed mechanism by which AIP1 and ADF coordinate actin dynamics awaits further characterization.

Oryza sativa has been becoming an increasingly important moncot model system in studying the mechanisms underlying plant growth and development due to the rapid development of resources in recent years, including a gene expression atlas and coexpression network analysis, full-length cDNA and mutant collections and a high efficiency transformation system (Childs *et al.,* 2011; Lu *et al.,* 2008; Miyao *et al.,* 2007; Nobuta *et al.,* 2007; Piffanelli *et al.,* 2007; Toki *et al.,* 2006; Xie *et al.,* 2005; Zhang *et al.,* 2006). 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 via promoting actin turnover.

RESULTS

Alteration of *OsAIP1* Expression Affects Rice Growth

To uncover the potential cellular and developmental functions for *OsAIP1*, *OsAIP1* overexpression (OE) and RNAi transgenic lines were generated. The expression level was significantly increased in OE lines and reduced in RNAi lines (Figure S1A and B). Both *OsAIP1* OE and RNAi decreased adult rice plant height (Figure S1C-H). Further characterization showed that both *OsAIP1* OE and RNAi reduced epidermal cell length

(Figure S1I), suggesting that *OsAIP1* misexpression inhibits anisotropic cell expansion. The inhibitory effect of *OsAIP1* misexpression on cell length is positively and inversely related to the amount of *OsAIP1* transcript in RNAi and OE lines, respectively. We also determined the effect of *OsAIP1* misexpression on polarized root hair growth. No major defect on root hair morphology was detected in *OsAIP1* RNAi plants besides the frequency of root hairs having swollen tip increased significantly (Figure 1B; Figure S2) compared to control plants (Figure 1A). However, most of *OsAIP1* OE root hairs can not grow straightly and appear swollen (Figure 1C). Compared to controls (Figure 1D), the frequency of root hairs greater than 200 μm 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.

Upregulation of *AIP1* Induced Actin Depolymerization whereas Downregulation of *AIP1* Promoted Actin Polymerization

We next examined the effect of *OsAIP1* misexpression on the actin cytoskeleton in rice cells. Actin filaments behave as longitudinal cables in the shank of control root hairs (Figure 1H; Figure S3A and B), similar to the pattern of actin distribution in *Arabidopis* 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; Figure S3C-F). This was

confirmed by the measurements showing that the average fluorescence pixel intensity of actin staining in this region increased significantly from 19.8 ± 2.5 (n = 11) for 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 control to 0.37 ± 0.01 (n = 156) for *OsAIP1* RNAi ($P < 0.01$ by Student's t-test), suggesting that downregulation of *OsAIP1* promotes actin assembly. Additionally, heavy bundles invade into the apical region of *OsAIP1* RNAi roots 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 is very dim in *OsAIP1* OE root hairs under the identical image acquisition conditions (Figure 1H; Figure S3G-I), suggesting that *OsAIP1* OE induced depolymerization of actin or inhibited actin assembly. To quantify the difference of F-actin level between control and *OsAIP1* transgenic rice cells, we examined the actin cytoskeleton in pollen grains, which have been shown to be a very good system to visualize the actin cytoskeleton and perform quantification (Huang *et al.,* 2006). The actin cytoskeleton appeared to have a uniformly distributed network in control pollen grain (Figure 2A), but appeared fragmented in *OsAIP1* OE pollen grain (Figure 2B), suggesting that *OsAIP1* OE induced actin depolymerization. By contrast, actin filaments became denser and highly bundled in *OsAIP1* RNAi pollen grains (Figure 2C). F-actin level was decreased in *OsAIP1* OE but increased in *OsAIP1* RNAi pollen grains (Figure 2D), and F-actin level inversely correlated with the amount of *OsAIP1* transcript (Figure S1A and B), suggesting that OsAIP1 inhibits actin polymerization. We also applied latrunculin B (LatB) treatment and found that the rate of the loss of F-actin was decreased in *OsAIP1* RNAi 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 via promoting actin turnover.

OsAIP1 Assists ADF-mediated Depolymerization *in vitro*

We next determined the biochemical basis for the function of OsAIP1. Given that OsAIP1 shares reasonable homology with the 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 it 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). Given that AIP1 proteins from various organisms were shown to act coordinately with ADF/cofilin to modify actin dynamics, we examined the synergistic effect of OsAIP1 with ADF. The well characterized AtADF1 (Carlier *et al.,* 1997) was included in the following assays. Consistent with its role in promoting actin depolymerization, AtADF1 increased the amount of actin in the supernatant (Figure 4A). The 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 8.0 (Figure 4B). Considering that UNC-78 was 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 therefore generated recombinant proteins of two rice *ADFs*, *OsADF2* and *OsADF9*, that express in vegetative and reproductive tissues, respectively (Figure S6A). However, the

results showed that OsAIP1 did not have preference for either OsADF2 or OsADF9 (Figure S6B-D), implying that OsAIP1 may not have preference for specific ADF isovariant. Taken together, the data suggest that OsAIP1 alone does not have obvious effect on actin dynamics but promotes ADF-mediated actin depolymerization.

OsAIP1 Enhances AtADF1-mediated Filament Severing and Pointed End Subunit Dissociation

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 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 that of the buffer control (Figure 5A, Movie S1), the addition of 50 nM AtADF1 generated more breaks along actin filaments (Figure 5B, Movie S2), suggesting that AtADF1 severs filaments. However, compared to that of 50 nM AtADF1, the addition of 250 nM OsAIP1 and 50 nM AtADF1 generated more breaks along filaments (Figure 5C, Movie S3) and OsAIP1 increased the number of breaks in a dose-dependent manner (Figure 5E), suggesting that OsAIP1 promotes AtADF1-mediated filament severing. The addition of 500 nM OsAIP1 alone did not generate more breaks along filaments compared to the buffer control (Figure 5D; Movie S4), suggesting that OsAIP1 cannot sever filaments on its own.

Actin filaments that remain fixed at the constriction point on a NEM-myosin coated coverslip were selected for the measurement of subunit off-rate, where the sliding actin filaments were excluded from the analysis. Based on previous studies showing that subunit dissociation rate of barbed end is faster than that of pointed end for control actin filaments in the absence of ADF/cofilin (Kuhn and Pollard 2005), the fast and slow shortening ends of each measured filament were designated as the barbed and pointed end, respectively, for actin alone. However, in the presence of ADF/cofilin, the dissociation rate of pointed end is faster than that of barbed end (Andrianantoandro and Pollard 2006). Therefore, in the presence of AtADF1, we designated the fast and slow shortening end of each measured filament as the pointed and barbed end, respectively. The pointed end subunit dissociation rate was enhanced in the presence of 50 nM AtADF1 compared to that of actin alone (Table S1). With the addition of various concentrations of OsAIP1 along with 50 nM AtADF1, the subunit dissociation rate from the pointed end increased in a dose-dependent manner, but OsAIP1 alone did not enhance 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 Coordinating Action

To determine whether the synergistic effect of AIP1 and ADF on reducing filament length could be somewhat 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, with the addition of OsAIP1, the initial elongation rate was inhibited in a dose-dependent manner (Figure 6A). Given that OsAIP1 enhances AtADF1-mediated filament severing, the addition of OsAIP1 along with AtADF1 should generate more filaments than that of AtADF1 alone. Theoretically, actin polymerization rate resulting from the barbed end elongation should be faster in the presence of OsAIP1 and AtADF1 than that of AtADF1 alone if barbed ends are free. However, the result is opposite, suggesting that the concentration of free barbed ends decreased with the addition of OsAIP1 along with ADF. The interpretation here could 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. Given that barbed end capping allows filaments stabilization by preventing subunit loss upon dilution, a dilution-mediated actin depolymerization assay was therefore 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, with the addition of OsAIP1 along with AtADF1, dilution-mediated depolymerization was inhibited in a dose-dependent manner (Figure 6B), which is very likely due to the capping activity as shown in Figure 6A. However, OsAIP1 inhibits depolymerization though it does not have capping activity on its own (Figure 6B), which is very likely due to its filament side binding activity as shown in Figure 3.

To determine whether the capping activity is required for the coordinating effect of OsAIP1 and ADF as proposed by Okada *et al.,* (2002), the actin barbed end was precapped by 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). Precapping the barbed end by 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 precapping by AtCP did not inhibit the effect of OsAIP1 and AtADF1 on enhancing severing and pointed end subunit dissociation (Figure S10; 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 coordinating effect of OsAIP1 and AtADF1.

Actin Filaments Can Be Turned Over Rapidly by AIP1 and ADF in the Presence of High Concentration of Polymerizable Actin

The concerted action of OsAIP1 and AtADF1 inspired us to examine whether they might cooperate with the monomer sequestering effect of profilin to maintain actin monomer pool. To test this, a high-speed cosedimentation assay was performed. The combination of all three components led to the greatest extent of depolymerization, compared to that of 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 high concentration of actin/profilin on a single filament level. Perfusion of 5 μM actin/profilin or 5 μM actin/profilin along with 1 μM OsAIP1 had negligible effect on filament severing (Figure S12; Movies S5 and 6). However, more severing events were detected after the perfusion of 50 nM

AtADF1 along with actin/profilin (Figure S12; Movie S7), suggesting that AtADF1 is able to sever filaments in the presence of high concentration of polymerizable actin. However, more severing events occurred while OsAIP1 was added along with AtADF1 in the presence of a high concentration of actin/profilin (Figure S12; Movie S8). The severing frequencies were significantly higher than that of 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 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 of the coordination of OsAIP1 and ADF on regulating single filament dynamics urged us to examine the function of OsAIP1 in regulating single actin filaments dynamics *in vivo*. The protoplasts derived from 14 day-old dark-grown rice sheath and stem of Ctrl, *OsAIP1* OE and RNAi were transformed with GFP-ABD2-GFP plasmid driven by ubiquitin promoter (Yang *et al.,* 2011). Real-time visualization of single filaments showed that they were severed frequently and depolymerized rapidly after being severed in Ctrl protoplasts (Figure 7A; Movie S9 and Table S4). The phenomenon is very similar to that in *Arabidopsis* hypocotyl epidermal cells and BY-2 suspension cells (Smertenko *et al.,* 2010; Staiger *et al.,* 2009). 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, Movie S10). By contrast, filaments are extremely dynamic in *OsAIP1* OE protoplasts (Figure 7C, Movie S11). Both severing frequency and subunit dissociation rate increased in *OsAIP1* OE protoplasts whereas they decreased significantly in RNAi protoplasts (Figure 7D and E, 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 important for modulating the stochastic dynamic behavior of actin filaments via enhancing filament severing and subunit dissociation.

DISCUSSION

OsAIP1 Enhances AtADF1-mediated Severing and Pointed End Subunit Dissociation

Our results demonstrate unambiguously that OsAIP1 enhances both ADF-mediated severing and pointed end subunit dissociation (Figure 5; Table S1). However, how OsAIP1 achieves this remains elusive. Previous studies showed that ADF/cofilin severs filaments via weakening the lateral contacts between subdomain 1 (SD1) and SD2 of adjacent actin subunits (Galkin *et al.,* 2003). Further electron cryomicroscopy analysis showed that the 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 could be somewhat due to the promoting effect of AIP1 on ADF-induced weakening of lateral contacts of adjacent actin subunits. It was actually hypothesized that the binding of cofilin creates a conformational change of 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 scales with boundary density (Suarez *et al.,* 2011). Previous study showed that ADF/cofilin only severs filaments at relative low concentrations (Andrianantoandro and Pollard 2006) indeed supports this. Therefore, the promoting effect of AIP1 on filament severing could be due to either the increase in the density of boundaries and/or the shear stress accumulated at the boundaries between bare and ADF/cofilin-decorated regions. Additionally, the additive effect of structural change of F-actin in the presence of AIP1 may promote the dissociation of actin subunits from the pointed end. Indeed, it was suggested that the conformational change induced by ADF/cofilin-binding can 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. Certainly, direct visualization of actin filaments and ADF/cofilin simultaneously in the presence of AIP1 will also provide clue to the coordinating mode of AIP1 and ADF/cofilin, as did by Suarez *et al.* (2011).

Barbed End Capping is not a Prerequisite for the Coordinating Action of OsAIP1 and AtADF1

Our results showed that OsAIP1 caps the barbed end in the presence of ADF (Figure 6A and B). It remains a mystery why AIP1 only caps the barbed end in the presence of ADF/cofilin. It could be that the binding of ADF/cofilin induced conformational change on actin filaments noticed previously (Galkin *et al.,* 2011; McGough and Chiu 1999; McGough *et al.,* 1997), to

consequently allow the binding of AIP1 to the barbed end. Indeed, it was hypothesized that the binding of cofilin creates a conformational change of actin filaments conducive to AIP1 interaction, and AIP1 may bind to newly formed barbed end after filaments were cleaved by ADF/cofilin (Clark *et al.,* 2006). However, our studies showed that barbed end capping is not required for the coordinating action of OsAIP1 and AtADF1 (Figure S9 and 10), which is similar to the finding for UNC-78 (Ono *et al.,* 2004), but is different from that for XAIP1 (Okada *et al.,* 2002). This is also in contrast to the results showing that barbed end capping with either CytoD or CapZ inhibited the endwise bursting disassembly induced by AIP1 along with coronin and cofilin (Kueh *et al.,* 2008). Though the barbed end capping is not a prerequisite for the coordinating action of OsAIP1 and AtADF1 *in vitro*, it might be important in plant cells (discussed forthcoming).

The Potential Role of AIP1 and ADF in Maintaining 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 the addition of an actin severing and barbed end capping protein PrABP80 along with profilin to preassembled filaments induced massive depolymerization *in vitro* supports this hypothesis (Huang *et al.,* 2004). Our *in vitro* reconstitution experiments indeed support this (Figure S11). Additionally, our study showed that OsAIP1 can drive 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 (Smertenko *et al.,* 2010; Staiger *et al.,* 2009). Considering that actin/profilin can add onto the barbed end of actin

filaments just as well as actin alone (Pantaloni and Carlier 1993) and the concentration of profilin/actin can 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 ends are free. The observation that actin elongation rate can reach ~2 μm/s 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.

ADF/cofilins also play roles in promoting actin assembly, very likely through severing and nucleating actin assembly (Andrianantoandro and Pollard 2006). This was actually demonstrated *in vivo*, where the activation of cofilin promotes 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; Movies S1-4). However, cells have to come up with 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 S9-11 and Table S4). This study, along with the recent report on *Arabidopsis* ADF4 (Henty *et al.,* 2011), placed AIP1/ADF as important players in modulating the stochastic dynamic behavior of cortical actin filaments. Consistent with this, we found that *OsAIP1* OE decreased F-actin level whereas *OsAIP1*

RNAi increased F-actin level (Figure 2). Additionally, more heavy actin bundles appeared in *OsAIP1* RNAi root hairs and pollen grains (Figure 1H, Figure S3C-F and Figure 2C), similar to the study in *Arabidopsis* (Ketelaar *et al.,* 2004). This is very likely due to the increase in the amount of F-actin, which consequently increases the frequency of filament bundling spatially. Certainly, given that OsAIP1 has filament side binding activity (Figure 3B and C), the possibility of the increase in the filament binding in *OsAIP1* RNAi cells by bundling factors, such as villin, fimbrin and LIMs (Thomas *et al.,* 2009), cannot be completely ruled out here.

Both *OsAIP1* RNAi and OE inhibit cell expansion and rice growth (Figure S1), supporting previous observations that cell expansion requires a tightly controlled actin cytoskeleton (Baluska *et al.,* 2001; Collings *et al.,* 2006; Dong *et al.,* 2001; Henty *et al.,* 2011; Kandasamy *et al.,* 2009; Ramachandran *et al.,* 2000; Yang *et al.,* 2011; Zhang *et al.,* 2011a; Zhang *et al.,* 2011b). Additionally, *OsAIP1* misexpression inhibited root hair elongation and caused depolarized growth (Figure 1A-G), suggesting AIP1-mediated actin dynamics is crucial for polarized cell growth. In contrast to the dramatic plant developmental defect caused by the expression of an inducible *AIP1* RNAi construct in *Arabidopsis* (Ketelaar *et al.,* 2004), the phenotype of *OsAIP1* RNAi plants is quite moderate. The reason could be that only the transgenic plants with moderate developmental defect were selected during the transformation step. Analysis of *OsAIP1* knockout 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 cDNA J033106G21 (Salk Institute Genomic Analysis Laboratory) as the template with primers OsAIP1F and OsAIP1R (Table S5). After being examined by sequencing, the fragment was moved into pET28b digested with *Hind*III/*Xho*I to make pET28b-OsAIP1. To construct *OsAIP1* RNAi and OE plasmids, an 800-bp gene specific fragment and the full-length *OsAIP1* coding sequence were amplified with the following primer pairs: OsAIP1$_i$800F and OsAIP1$_i$800R, OsAIP1$_{OE}$F and OsAIP1$_{OE}$R, respectively (Table S5). After verified the sequences by sequencing, they were subsequently cloned into the TCK303 vector which is driven by a constitutive maize *Ubi*-1 promoter as previously described (Wang *et al.,* 2004).

Rice Transformation and Growth

The transformation of rice (Zhonghua 10) embryonic calli was performed according to a previously published method (Toki *et al.,* 2006). T3 homozygous *OsAIP1* OE and RNAi transgenic plants were used for phenotypic analysis. The rice plants grew under natural conditions in the growing season (from May to early October) in Beijing, China. The plants with the termination of elongation were photographed and the length of panicle and internodes were measured. Student's *t*-test in the R programming language (version 2.11.0) was adopted for statistical analysis in this study.

Rice Internode Epidermal Cell Observation

Epidermal cells torn from the middle portion of the upper fourth internode were marinated in chloral hydrate solution (trichloroacetaldehyde monohydrate: glycerol: water = 8 g: 1 mL: 2 mL) at room temperature for 5 d. They were then observed directly under an Olympus microscope (BX51TRF) equipped with a 60× objective, and the images were acquired with a Retiga EXi Fast 1394 CCD camera using Image-pro Express 6.3 software. The length of the epidermal cell was measured as the distance between two longitudinal cork cells.

Root Hair Determination and Actin Staining

After germinated for 4 d at 28 □ in darkness, roots with about 1 cm in length were cut from seedlings. Root hairs were observed under a microscope (Olympus BX51TRF) equipped with 10× objectives and the images were acquired. More than 500 root hairs were selected for length and width measurement for each genotype. The actin staining procedure in root hair was according to He et al. (2006). Actin filaments were observed under a Zeiss LSM META 510 laser scanning confocal microscope, the fluorescence was excited using a 488 nm blue argon laser, the optical Z-series sections were collected at 0.5 μm steps. The presented images were projections of the optical sections through an individual root hair. The image collection setting was 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 was according to the published method (Ye *et al.,* 2009; Zhang *et al.,* 2010).

Direct Visualization of Actin Dynamics in Rice Protoplasts

To visualize single actin filament dynamics *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. The transformation of rice protoplasts was roughly according to Bart *et al.,* (2006). Briefly, rice seeds were grown on 1/2 MS medium for 14 d 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% cellulose RS, 0.75% macerozyme R10, 0.1% bovine serum albumin (BSA), 1 mM $CaCl_2$ and 5 mM β-mercaptoethanol) and shaked gently (40 rpm) for 4 h in the dark. An equal volume of W5 solution (2 mM MES, pH 5.7, 154 mM NaCl, 125 mM $CaCl_2$ and 5 mM KCl) was subsequently added into the digestion solution. The protoplasts were collected by passing through a 35 μm nylon mesh filter followed by centrifugation at 300 *g* for 5 min. The protoplasts were washed once with W5 solution and resuspended at 2×10^6 cells/mL in Mmg solution (4 mM MES, pH 5.7, 15 mM $MgCl_2$ and 0.6 M mannitol). For transformation, the pCambia1300-ubi-EGFP-ABD2-EGFP plasmid (20 μg) was added into 100 μl protoplasts followed by the addition of equal volume of PEG solution (0.6 M mannitol, 100 mM $CaCl_2$ and 40% (v/v) PEG 4000), and incubated for 20 min at room temperature. The protoplasts were then washed with 10 volumes of W5 solution, and resuspended in incubation buffer (4 mM MES, pH 5.7, 4 mM KCl and 0.6 mM mannitol) and the incubation was extended for 20 h at room temperature in the dark. The dynamics of actin filaments were observed under an Olympus BX61 inverted microscope equipped with a 100× 1.4 NA UPLSAPO objective. The images were collected by spinning disk confocal

(equipped with a Yokogawa CSU-X1 spinning disk head) with a 512 ×512 Andor iXON electron multiplying CCD camera. GFP was excited with a 488-nm laser, and fluorescence emission was collected through 525/50-nm band-pass filter. The time-lapse Z-series images (with a step at 0.5 μm) were collected with the time interval at 100 millisecond with Andor IQ2, and the time interval for the whole Z-series collection was set at 5 s. Filament severing frequency and maximum filament lifetime were determined according to Henty *et al.,* (2011).

Protein Production

To generate recombinant OsAIP1, the pET28b-OsAIP1 construct was transformed into the BL21 DE3 strain of *E. coli*. The expression of OsAIP1 was induced for 16 h at 16℃ with the addition of 0.3 mM isopropyl b-D-thiogalactopyranoside (IPTG). 6His-OsAIP1 was purified by a Ni^{2+}-NTA-agarose column according to the manufacturer's instruction. 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℃. OsAIP1 was clarified at 200 000 *g* for 1 h before use and its concentration was determined with the Bradford assay (Bio-Rad) with bovine serum albumin as the standard. *Arabidopsis* ADF1 (AtADF1), *Arabidopsis* capping proteins (AtCP), *Arabidopsis* profilin 2 (AtPRF2), Os FH5 FH2 and Human profilin 1 (HPRO1) were purified according to previously published methods (Carlier *et al.,* 1997; Fedorov *et al.,* 1994; Gibbon *et al.,* 1997; Huang *et al.,* 2003; Yang *et al.,* 2011).

Biochemical Assays to Determine the Effect of OsAIP1 on Actin

High-speed cosedimentation assays were performed to examine the binding of OsAIP1 to actin filaments and determine the coordinating effect of OsAIP1 with AtADF1 on actin

depolymerization, which is roughly according to previous published method (Kovar et al., 2001). The actin elongation assay, dilution mediated depolymerization assay and direct visualization of actin filaments by fluorescence microscopy were performed according to Okada *et al.,* (2002).

Quantification of Actin Filament Severing and Depolymerization by TIRFM

Direct visualization of single actin filaments by TIRFM was performed according to previously published method (Amann and Pollard 2001). The flow chamber was preincubated with 25 nM NEM-myosin and subsequently incubated with 1% BSA and washed with 1×TIRFM buffer (10 mM imidazole, pH 7.0, 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 50 mM DTT, 0.2 mM ATP, 50 µM $CaCl_2$, 15 mM glucose, 20 µg/mL catalase, 100 µg/mL glucose oxidase and 0.5% methylcellulose) (Kovar and Pollard 2004). Preassembled F-actin (100% rhodamine-labeled, 40 nM) was injected into 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 s. To determine the effect of AtADF1 and/or OsAIP1 on actin dynamics in the presence of high concentration of actin/profilin, various concentrations of OsAIP1 and/or 50 nM AtADF1 along with 5 µM AtPRF2 and 5 µM G-actin in 1×TIRFM buffer were injected into the perfusion chamber. Quantification of severing frequency was according to (Andrianantoandro and Pollard, 2006). To determine whether the barbed end capping by AtCP will affect the promoting effect of OsAIP1 on AtADF1-mediated filament severing and pointed end subunit dissociation, 100

nM AtCP was included in the injection solutions.

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SUPPORTING INFORMATION:

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

Table S2. Average subunit off-rate from the barbed end and pointed end of F-actin in the presence of 50 nM AtADF1 and various concentration of OsAIP1 along with 100 nM AtCP.

Table S3. Average subunit off-rate from barbed end and pointed end of F-actin in the presence of 50 nM AtADF1 and various concentration of OsAIP1 along with 5 μM G-actin and 5 μM AtPRF2.

Table S4. Actin dynamics parameters in protoplasts.

Table S5. Primer sequences used in this study.

Figure S1. The Alteration of *OsAIP1* Expression Inhibits Rice Growth

Figure S2. *OsAIP1* RNAi Increases the Frequency of Root Hairs with Swollen Tip

Movie S5. A Time-lapse Series of Actin Filaments Dynamics after Injection of 5 μM Actin and 5 μM AtPRF2

Movie S6. A Time-lapse Series of Actin Filaments Dynamics after Injection of 5 μM Actin and 5 μM AtPRF2 along with 200 nM OsAIP1

Movie S7. A Time-lapse Series of Actin Filaments Dynamics after Injection of 5 μM Actin and 5 μM AtPRF2 along with 50 nM AtADF1

Movie S8. A Time-lapse Series of Actin Filaments Dynamics after Injection of 5 μM Actin and 5 μM AtPRF2 along with 50 nM AtADF1 and 200 nM OsAIP1

Movie S9: Actin Filaments Dynamic Remodeling in Control Protoplasts

Movie S10: Actin Filaments Dynamic Remodeling in *OsAIP1* RNAi Protoplasts

Movie S11: Actin Filaments Dynamic Remodeling in *OsAIP1* OE Protoplasts

Movie Legends S1. Movie legends for Movies S1-11.

FIGURE LEGENDS

Figure 1. Downregulation of *OsAIP1* Promotes Actin assembly, whereas Upregulation of *OsAIP1* Inhibits Actin Assembly in Root Hairs

(**A**) to (**C**) Micrographs of root hairs. (**A**) Ctrl; (**B**) *OsAIP1* RNAi 8-3; (**C**) *OsAIP1* OE 8-2. White arrow in (**B**) indicates the root hair with swollen tip. Bar = 50 μm.

(**D**) to (**F**) Length distribution of root hairs. (**D**) Ctrl; (**E**) *OsAIP1* RNAi 8-3 and (**F**) *OsAIP1* OE 8-2. $n \geq 200$.

(**G**) Both *OsAIP1* RNAi and OE increased the width of root hairs at the tip. **P < 0.01 by

Student's *t*-test, $n \geq 128$.

(**H**) The actin cytoskeleton in root hairs. Bar = 10 μm. More representative images of actin filaments distribution in Ctrl, *OsAIP1* RNAi and OE roots hairs are shown in Figure S3.

Figure 2. F-actin Level is Increased in *OsAIP1* RNAi but Reduced in *OsAIP1* OE Pollen Grains

Pollen grains were subjected to Alexa-488 phalloidin staining according to Zhang *et al*., (2010). (**A**) Ctrl; (**B**) OE 8-2 and (**C**) RNAi 8-3. Bar = 10 μm. (**D**) Quantification of F-actin level. Pollen grain actin staining images were collected under the same conditions and the relative amount of F-actin was analyzed by measuring the pixel intensity of individual pollen grains. The values represent mean ± SE (n = 10), **P < 0.01 by Student's *t*-test.

Figure 3. OsAIP1 Binds to Actin Filaments but It Does Not Have an Obvious Effect on Actin Polymerization

(**A**) SDS-PAGE analysis of purified recombinant OsAIP1. The purity of OsAIP1 was greater than 95% determined by ImageJ (http://rsbweb.nih.gov/ij/, Version 1.38).

(**B**) Three micromolar preassembled actin filaments were incubated with or without 1 μM OsAIP1 for 30 min at room temperature, the mixtures were centrifuged at 100,000 × g for 35 min at 4℃. Lanes 1, 3 and 5 represent the supernatant of 3 μM actin alone, 3 μM actin + 1 μM OsAIP1 and 1 μM OsAIP1 alone, respectively. Lanes 2, 4 and 6 represent the pellet of 3 μM actin alone, 3 μM actin + 1 μM OsAIP1 and 1 μM OsAIP1 alone, respectively.

(**C**) The amount of OsAIP1 in the pellet was determined in the presence (lane 4) or absence

(lane 6) of actin filaments by densitometry. Values represent mean ± SE (n = 3), **P < 0.01 by Student's t-test.

(**D**) The amount of actin in the pellet in the absence (lane 2) or presence (lane 4) of OsAIP1 was determined by densitometry.

(**E**) OsAIP1 does not have an obvious effect on spontaneous actin polymerization. The left panel showing the SDS-PAGE analysis of purified recombinant HPRO1 (Lane 1) and OsFH5 FH2 (Lane 2), and the right panel showing the polymerization curves of 3 μM Mg^{2+}-actin (10% pyrene-labeled) in the absence or presence of OsAIP1. OsFH5 FH2 was used as the control that promotes actin assembly (Yang et al., 2011), and HPRO1 was used as the control that inhibits spontaneous actin assembly. The purity of OsFH5 FH2 and HPRO1 was greater than 95% determined by ImageJ (http://rsbweb.nih.gov/ij/, Version 1.38).

Figure 4. OsAIP1 Enhances Actin Depolymerization in the Presence of AtADF1 in a Dose-dependent Manner

(**A**) High-speed cosedimentation assay was performed to determine the effect AtADF1 and/or OsAIP1 on actin depolymerization. Lanes 1, 3, 5 and 7 represent supernatant for 3 μM actin alone, 3 μM actin + 5 μM AtADF1, actin + 1 μM OsAIP1 and actin + 5 μM AtADF1 + 1 μM OsAIP1, respectively. Lanes 2, 4, 6 and 8 represent pellet for 3 μM actin alone, 3 μM actin + 5 μM AtADF1, actin + 1 μM OsAIP1 and actin + 5 μM AtADF1 + 1 μM OsAIP1, respectively. The pH is 8.0 in this experiment.

(**B**) OsAIP1 enhances actin depolymerization in the presence of AtADF1 in a dose-dependent manner. Varying concentrations of OsAIP1 were incubated with 3 μM preassembled actin

filaments in the presence of 5 μM AtADF1 for 30 min at room temperature at both pH 6.8 and pH 8.0. The mixtures were then sedimented at 100,000 × g for 35 min at 4℃. The amount of actin in the pellet was determined by densitometry and plotted against the concentrations of OsAIP1 in the pellet. Closed circles, pH = 8.0; open circles, pH = 6.8. Values represent mean ± SE (n = 3).

Figure 5. Direct Visualization of AtADF1-mediated Filaments Severing by TIRFM

The time-series images were collected every 3 s, where different colored arrows indicate an increasing number of breaks overtime on a particular actin filament.

(**A**) Time-lapse images of actin filaments after injection of 1×TIRFM buffer. Bar = 5 μm.

(**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. See Movie S3 for the entire series.

(**D**) Time-lapse images of actin filaments after injection of 500 nM OsAIP1 in 1×TIRFM buffer.

(**E**) The average severing frequencies were determined as described in the Methods section and plotted. Values represent mean ± SE (n = 3), **P < 0.01 by Student's t-test.

Figure 6. OsAIP1 Has Barbed End Capping Activity

(**A**) OsAIP1 inhibits actin elongation in the presence of AtADF1. Ten micromolar F-actin was incubated with 10 μM AtADF1 and various concentrations of OsAIP1 at room temperature

for 5 min, the mixture was then diluted 10-fold with 2 μM actin monomers (10% pyrene-labeled) in 1×F-buffer to initiate elongation at pH 8.0.

(**B**) OsAIP1 inhibits dilution-mediated actin depolymerization in the presence of AtADF1. NBD-actin was used in this assay since the quench of NBD-actin fluorescence by AtADF1 is not very obvious compared to that of pyrene-actin. Five micromolar preassembled actin filaments (50% NBD-labeled) along with 2.5 μM AtADF1 were incubated with various concentrations of OsAIP1 at pH 8.0 (**B**), the mixtures were then diluted 12.5-fold in Buffer G.

Figure 7. Both Filament Severing Frequency and Subunit off-rate are Enhanced in *OsAIP1* OE but Decreased in *OsAIP1* RNAi Protoplasts

(A) Time-lapse images of actin filaments in Ctrl protoplasts. (**B**) Time-lapse images of actin filaments in *OsAIP1* RNAi 8-3 protoplasts. (**C**) Time-lapse images of actin filaments in *OsAIP1* OE 8-2 protoplasts. Colored arrows indicated the breaking events along actin filaments which were indicated by the corresponding colored dots. Bar = 2 μm. (**D**) Plot of filament severing frequency, which was expressed as number of breaks per unit filament length per second. Values represent mean ± SD, ** $P < 0.01$ by Student's *t*-test. (**E**) Plot of subunit off-rate. Values represent mean ± SD, ** $P < 0.01$ by Student's *t*-test.