JIPB Journal of Integrative Plant Biology

Journal of Integrative Plant Biology 2011, **53** (9): 698–709

Research Article

Adenosine Diphosphate Ribosylation Factor-GTPase-Activating Protein Stimulates the Transport of AUX1 Endosome, Which Relies on Actin Cytoskeletal Organization in Rice Root Development

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Available online on 2 June 2011 at www.jipb.net and www.wileyonlinelibrary.com/journal/jipb

doi: 10.1111/j.1744-7909.2011.01059.x

Abstract

Polar auxin transport, which depends on polarized subcellular distribution of AUXIN RESISTANT 1/LIKE AUX1 (AUX1/LAX) influx carriers and PIN-FORMED (PIN) efflux carriers, mediates various processes of plant growth and development. Endosomal recycling of PIN1 is mediated by an adenosine diphosphate (ADP)ribosylation factor (ARF)-GTPase exchange factor protein, GNOM. However, the mediation of auxin influx carrier recycling is poorly understood. Here, we report that overexpression of OsAGAP, an ARF-GTPase-activating protein in rice, stimulates vesicle transport from the plasma membrane to the Golgi apparatus in protoplasts and transgenic plants and induces the accumulation of early endosomes and AUX1. AUX1 endosomes could partially colocalize with FM4–64 labeled early endosome after actin disruption. Furthermore, OsAGAP is involved in actin cytoskeletal organization, and its overexpression tends to reduce the thickness and bundling of actin filaments. Fluorescence recovery after photobleaching analysis revealed exocytosis of the AUX1 recycling endosome was not affected in the OsAGAP overexpression cells, and was only slightly promoted when the actin filaments were completely disrupted by Lat B. Thus, we propose that AUX1 accumulation in the OsAGAP overexpression and actin disrupted cells may be due to the fact that endocytosis of the auxin influx carrier AUX1 early endosome was greatly promoted by actin cytoskeleton disruption.

Keywords: actin; adenosine diphosphate ribosylation factor-GTPase-activating protein; AUX1; endosome; polar auxin transport.

Du C, **Xu Y**, **Wang Y**, **Chong K** (2011) Adenosine diphosphate ribosylation factor-GTPase-activating protein stimulates the transport of AUX1 endosome, which relies on actin cytoskeletal organization in rice root development. *J. Integr. Plant Biol*. **53**(9), 698–709.

Introduction

The phytohormone auxin plays a pivotal role in many processes of plant growth and development (Davis 1995), including phototropism, gravitropism, aptical dominance and embryogenesis, as well as root development (Zhuang et al. 2005). Auxin is considered unique among plant hormones because it is transported through tissues in a polar manner (Tanaka et al. 2006). Polar auxin transport (PAT) involves a specialized transport system composed of influx (AUXIN-RESISTANT 1/LIKE AUX1 [AUX1/LAX] family) and efflux carriers (PIN-FORMED [PIN] family), which mediate the mobilization of auxin into and out of

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cells (Bennett et al. 1996; Gälweiler et al. 1998; Luschnig et al. 1998; Parry et al. 2001). The polarized plasma membrane (PM) protein auxin carriers are delivered to and recycled from the PM via endosomes. PIN1, the auxin efflux carrier, is constitutively recycled in a brefeldin A (BFA)-sensitive GNOM-dependent pathway. GNOM is a GTPase exchange factor (GEF) for the ADP ribosylation factor (ARF) GTPases, which localize to recycling endosomes (Geldner et al. 2003). BFA treatment induces the intracellular accumulation of PIN1 in wild-type plants but not in plants expressing engineered BFA-resistant $GNOM^{M696L}$ (Geldner et al. 2003). However, the auxin influx carrier AUX1, which depends on the endoplasmic reticulum (ER) protein AXR4, is recycled from endosomes to the PM in a BFA-insensitive GNOM-independent manner (Dharmasiri et al. 2006; Kleine-Vehn et al. 2006). PAT carrier dynamics rely on the actin filaments (Geldner et al. 2001; Kleine-Vehn et al. 2006), and auxin could affect actin filaments in *Arabidopsis* and rice (Rahman et al. 2007; Nick et al. 2009). The subcellular dynamics of AUX1 were found blocked by the auxin transport inhibitor TIBA, which acted as the actin stabilizer (Kleine-Vehn et al. 2006; Rahman et al. 2007; Dhonukshe et al. 2008). Compared with the localization and trafficking of auxin efflux carriers such as PIN1, those of AUX1 more strictly depend on intact actin filaments (Kleine-Vehn et al. 2006).

Adenosine diphosphate ribosylation factors (ARFs) are members of the ARF family of GTP-binding proteins of the Ras superfamily. They function as regulators of vesicular traffic and actin remodeling (Randazzo and Hirsch 2004). TaARF in wheat could increase growth rate (Yao et al. 2009). The ARF-GTPase-activating proteins (GAPs) are a family of proteins that induce hydrolysis of GTP-bound ARF to GDP-bound ARF, which is opposite to the ARF-GEF function at the biochemical level (Donaldson and Jackson 2000). ARF-GAPs can mediate membrane trafficking and actin remodeling through ARF. Also, they can function independently of ARF (Randazzo and Hirsch 2004). ARF-GAPs have multiple domains that can interact with signaling proteins and adopter domains. *Arabidopsis* has 15 ARF-GAPs (Vernoud et al. 2003). Characterized ARF-GAPs include SFC/VAN3 (Koizumi et al. 2005; Sieburth et al. 2006), AGD7 (Min et al. 2007), RPA (Song et al. 2006), and ZAC (Jensen et al. 2000). All localize to the Golgi complex. SFC/VAN3, which functions in leaf vein patterning, and is required for normal auxin efflux via a *trans*-Golgi network (TGN)-mediated vesicle transport system (Koizumi et al. 2005; Sieburth et al. 2006). AGD7 can promote the relocation of Golgi proteins into the ER (Min et al. 2007).

OsAGAP, a homolog of ZAC in rice, mediates root development in *Arabidopsis* and rice (Zhuang et al. 2005, 2006). Ectopic expression of OsAGAP could alter the localization of *Arabidopsis* AUX1 and has a specific role in regulating vesicle transport (Zhuang et al. 2006). Thus, similar to the ARF-GAPs in animals, ARF-GAPs in plants could also affect vesicle transport, but the mechanism is poorly understood (Jensen et al. 2000; Koizumi et al. 2005; Sieburth et al. 2006; Song et al. 2006; Zhuang et al. 2006; Min et al. 2007). Especially in rice, a monocotyledon model plant, little is known about how such proteins mediate the transport pathway. In this paper, we report that overexpressed OsAGAP disrupts the polar location of AUX1 by affecting actin cytoskeletal organization and endosome transport.

Results

OsAGAP overexpression affects actin cytoskeletal organization

ARF-GAPs are involved in the regulation of cytoskeletal structures, including focal adhesions and actin-rich membrane ruffles in animals (Randazzo and Hirsch 2004). AUX1 localization and trafficking relies on intact actin filaments (Kleine-Vehn et al. 2006). As well, auxin could stimulate its own transport by shaping actin filaments in rice (Nick et al. 2009). To test this hypothesis in plants, F-actin was stained in living rice root epidermal cells by phalloidin-fluorescein isothiocyanate (FITC). The fluorescence in OsAGAP-overexpressed root cells was reduced and the actin filaments appeared to be thinner and less bundled than in the control (**Figure 1A, B**). We also overexpressed OsAGAP in a green fluorescent protein (GFP)-tagged actin-binding domain from fimbrin (ABD2-GFP) in transgenic *Arabidopsis* root cells. With overexpressed OsAGAP, the actin filaments in transgenic *Arabidopsis* root cells were the same as in transgenic rice (**Figure 1C, D**). Thus, OsAGAP overexpression can lead to the disruption of actin cytoskeletal organization.

Despite the reduction in thickness and bundling of actin filaments with OsAGAP overexpression, the microtubule organization in rice roots detected by anti-tubulin antibody was not affected (**Figure S1**). This finding is in accordance with AUX1 localization and trafficking relying on intact actin filaments instead of microtubules.

AUX1 could localize to the early endosome after actin disruption

AUX1 could colocalize with early endosome and the microfilament (MF) disruption by Lat B leads to intracellular agglomeration of AUX1 in plant cells (Kleine-Vehn et al. 2006). To confirm whether the AUX1 endosome agglomeration in the MF-disrupted root cells is caused by faster movement as FM4–64-stained vesicles, we treated AUX1-YFP transgenic *Arabidopsis* with Lat B (20 μM, 3 h) and the protein synthesis inhibitor cycloheximide (50 μM, 0.5 h), then FM4–64 staining for 30 min.

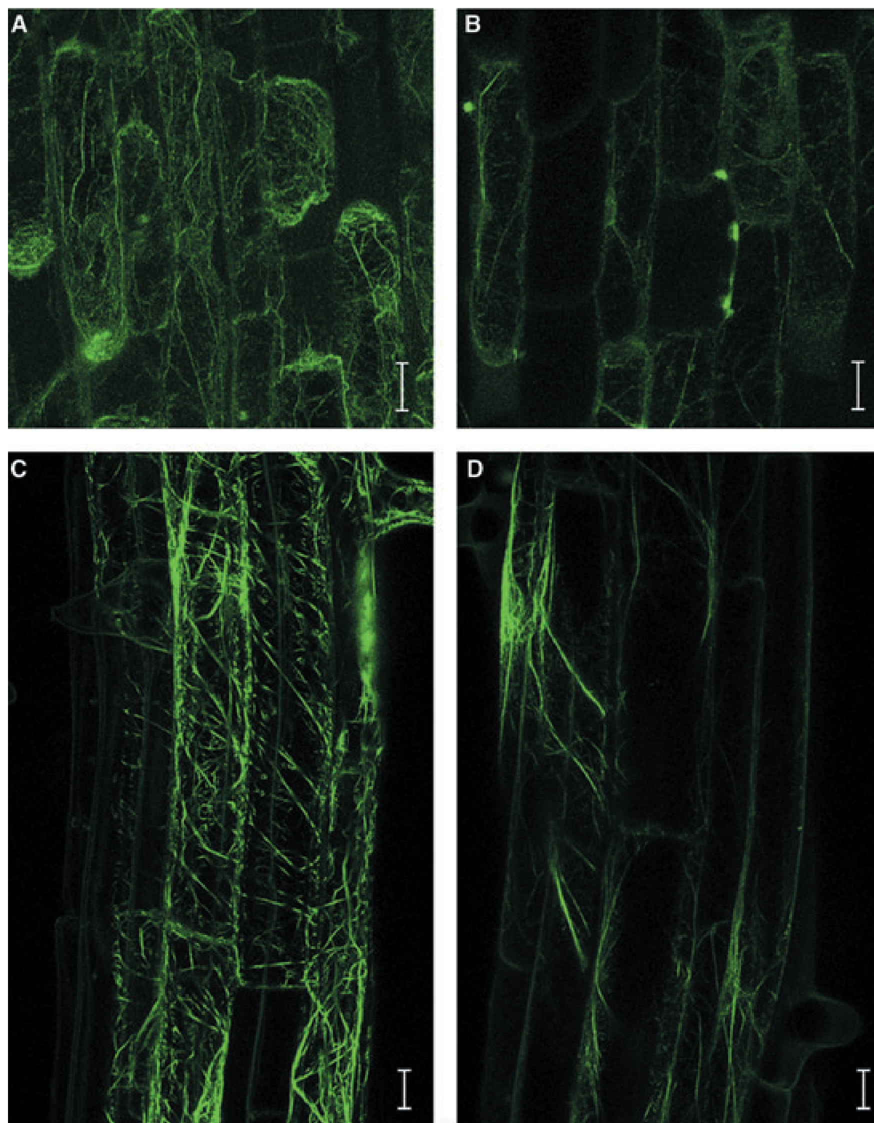

Figure 1. OsAGAP overexpression causes the disruption of actin cytoskeleton in rice and *Arabidopsis* root cells.

(A) and **(B)** F-actin stained by phalloidin-fluorescein isothiocyanate (FITC) in wild-type **(A)** and transgenic rice root cells **(B)**. **(C)** and **(D)** ABD2-GFP in 5-day-old *Arabidopsis* root cells. C, control; D, OsAGAP-overexpressed.

Bars = 10 μm.

AUX1-YFP partially colocalized with FM4–64-labeled vesicles in large patches (**Figure 2**). This finding suggested that the endocytosis of AUX1 early endosomes could also be promoted by MF disruption.

Early endosome is accumulated due to the overexpression of OsAGAP

Trans-Golgi network (TGN) is reported as early endosome in plant (Viotti et al. 2010). Thus, we investigated the TGN markers in plant protoplasts. GONST1 was widely used as TGN marker in onion, *Arabidopsis* and BY-2 cells (Baldwin et al. 2001; Tse et al. 2004) When the GFP-fused form of GONST1 was transformed into rice protoplasts, GONST1-GFP exhibited a typical punctate staining pattern (**Figure 3Aa**). However, some OsAGAP-overexpressed protoplasts (approximately 30%) showed an accumulated pattern (**Figure 3Ab**). And this result was confirmed in *Arabidopsis* protoplasts. (**Figure 3Ac, d**). The fluorescent images of the markers in rice and *Arabidopsis* revealed similar labeling patterns. Considering the transformation efficiency of protoplasts is usually 30% to 50% (data not shown), these results strongly suggest that early endosome TGN is accumulated in the OsAGAP overexpression cells.

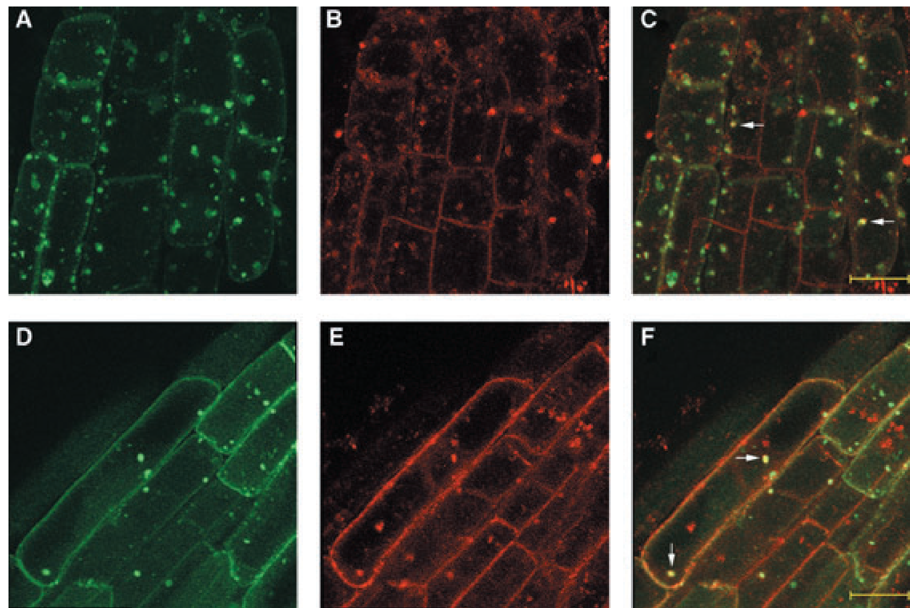

Figure 2. Subcellular localization of AUX1.

Partial colocalization of AUX1-yellow fluorescent protein (AUX1-YFP) (green) and FM4–64-stained endosomes (red) in epidermal cells after Lat B (20 μM, 3 h) treatment without (**A**–**C**) or with (**D**–**F**) cyclohexamide (CHX) (50 μM, 0.5 h). Bars = 10 μm.

Actin cytoskeleton disruption greatly stimulates the endocytosis of early endosomes

Because overexpression of OsAGAP affected vesicle transport and led to the disruption of actin cytoskeletal organization, we wondered whether microfilament disruption could affect vesicle transport. FM4–64 is a widely used endocytosis marker in living eukaryotic cells and mainly stained early endosomes within 30 min (Bolte et al. 2004). We used FM4–64 to stain microfilament-disrupted *Arabidopsis* roots treated with a *bona fide* actin inhibitor, latrunculin B (Lat B). Although 20 μM Lat B is a high concentration to depolymerize actin organization, auxin influx carrier AUX1 but not efflux carrier PIN1 could accumulate in the root cells after short time treatment of 20 μM Lat B (Kleine-Vehn et al. 2006). After Lat B (20 μM) treatment for 3 h, all actin filaments in root cells were completely disrupted (**Figure 4A, B**). Fluorescent images showed some small dots in the microfilament (MF)-disrupted root cells 10 min after FM4–64 staining, but controls showed almost no staining in root cells (**Figure 4C, D**). At 30 min after FM4–64 staining, both of the transport vesicles in root cells were stained, but in the MF-disrupted root cells, the labeled vesicles were larger (**Figure 4E–G**). Then we investigated the dynamics of FM4–64-labeled early endosomes in OsAGAP-overexpressed and actin-disrupted root cells. **Figure 5** and supplemental movies (**Figure 5**; Videos 1, 2 and 3) show that the FM4–64-labeled early endosomes moved more rapidly in the OsAGAP-overexpressed (average velocity = 0.25 μm/s) and actin-disrupted (0.47 μm/s) cells than in the control cells (0.18 μm/s). Thus, the endocytosis of FM4–64-labeled early endosomes from the PM to the Golgi apparatus was promoted in the MF-disrupted root cells, which corresponds to vesicle accumulation in the root cells.

Actin stabilizer could rescue the phenotype of OsAGAP overexpression plants

Actin stabilization could interfere with vesicle transport of AUX1 and PIN protein (Kleine-Vehn et al. 2006; Dhonukshe et al. 2008). To confirm that actin disruption by OsAGAP induced the accumulation of TGN and AUX1 endosome, we tested the effects of actin stabilization in OsAGAP overexpression plants. In OsAGAP overexpression protoplasts and *Arabidopsis* transgenic root cells, endosome and AUX1 accumulation could be rescued by actin stabilizer jasplakinolide (**Figures 3B**, **6**).

Exocytosis of AUX1 recycling endosomes in the OsAGAP-overexpressed and actin-disrupted cells

The auxin transport inhibitor TIBA can inhibit AUX1 recovery at the PM by stabilizing actin filaments (Kleine-Vehn et al. 2006, Dhonukshe et al. 2008). To examine any change in exocytosis of AUX1 in the MF-disrupted cells, we performed a fluorescence recovery after photobleaching (FRAP) experiment. We first crossed *35S::OsAGAP* transgenic *Arabidopsis* plants with

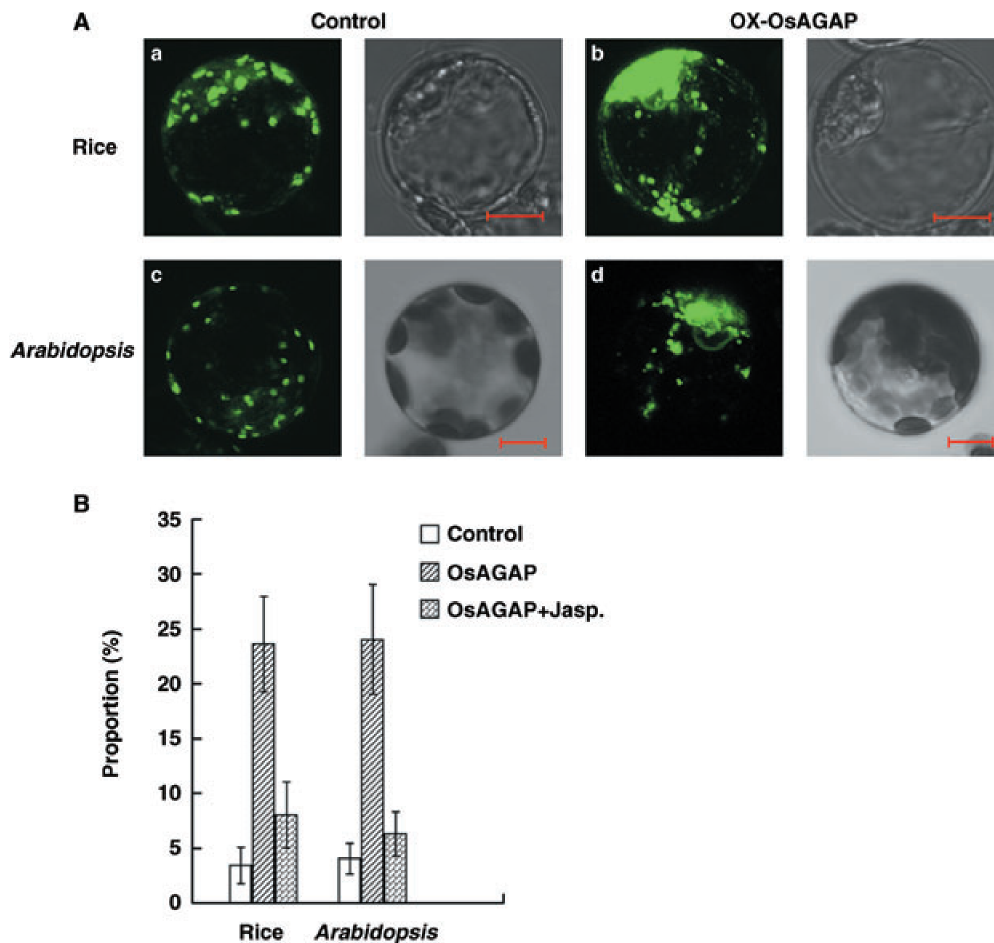

Figure 3. *Trans*-Goigi network accumulated in OsAGAP-overexpressed transgenic rice and *Arabidopsis* protoplasts.

(A) Protoplasts transformation of *trans*-Golgi network (TGN) marker GONST1-GFP. a and b are rice protoplasts. c and d are *Arabidopsis*. a and c are control; the others are OsAGAP-overexpressed transgenic plants. Right images are the differential interference contrast (DIC) of the left fluorescent images. Images were taken with a 63× water lens (rice) and 40× lens (*Arabidopsis*). Bars = 10 μm.

(B) Proportion of protoplasts which showed TGN accumulated pattern. It could be rescued by actin stabilizer jasplakinolide (Jasp.) treatment (0.1 μM, 3 h). Error bars represent *SE*. ($n > 50$, data were from three independent experiments) OX-OsAGAP: OsAGAP-overexpressed.

the *AUX1-YFP* transgenic line. However, the accumulation of AUX1-YFP is not as striking as HA-AUX1 (**Figure 6C**, Zhuang et al. 2006). In the root epidermis cells, the PM pool of AUX1-YFP recovered visibly within 30 min after bleaching (Figure S2) and was a little slower (40 min) when protein synthesis was inhibited by cycloheximide (50 μM, 0.5 h) (**Figure 7A**). The recovery of the PM pool of AUX1-YFP, in the MF-disrupted root cells by Lat B or in the OsAGAP-overexpressed root cells, was the same as in the control; the time for recovery was within 40 min (**Figure 7A–D** and supplemental movies **Figure 7**; Videos 1–4). However, the fluorescence recovery rate in actin completely disrupted cells was a little faster than in controls (**Figure 7E**). There were still some intact microfilaments after 2 μM Lat B treatment for 3 h (data not shown). Therefore, exocytosis of the AUX1 endosome was not sensitive to the actin cytoskeleton organization, and it would be slightly promoted by actin disruption. PIN2-GFP was a control in the FRAP analysis. The recovery of PIN2-GFP in wild-type root epidermal cells was slow (**Figure S3A**). PIN2-GFP recovery on FRAP did not differ among the wild-type, OsAGAP-overexpressed and Lat B-treated root cells (**Figure S3**). These results indicate that actin stabilization greatly impeded AUX1 recovery at the PM, but actin disruption only slightly and specifically promoted AUX1 exocytosis.

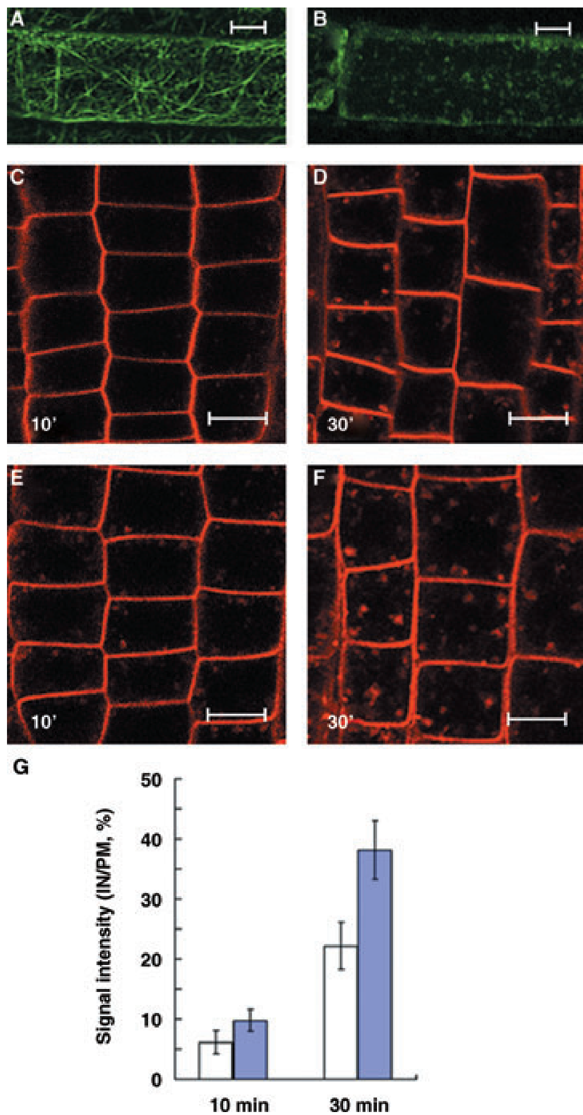

Figure 4. Actin cytoskeleton disruption stimulates the endocytosis of endosomes.

(A) and **(B)** Actin binding domain from fimbrin-green fluorescent protein (ABD2-GFP) in *Arabidopsis* roots.
(C) to **(F)** FM4–64 staining of *Arabidopsis* roots.
(A), **(C)** and **(D)** treated with dimethylsulfoxide (DMSO) for 3 h before staining (control).
(B), **(E)** and **(F)** treated with 20 μM latrunculin **(B)** (Lat B) for 3 h.
(G) Ratio of fluorescent intensity of intracellular compartments (IN) to the plasma membrane (PM) in root cells.
Error bars represent *SE*. ($n = 20$, data were from three independent experiments) Images are projections of 10 optical sections.
Bars = 10 μm.
Statistical analysis of data indicated the significant difference between the actin-disrupted and the control plant as determined by repeated-measures analysis of variance (two-sample t-test; $P < 0.05$)

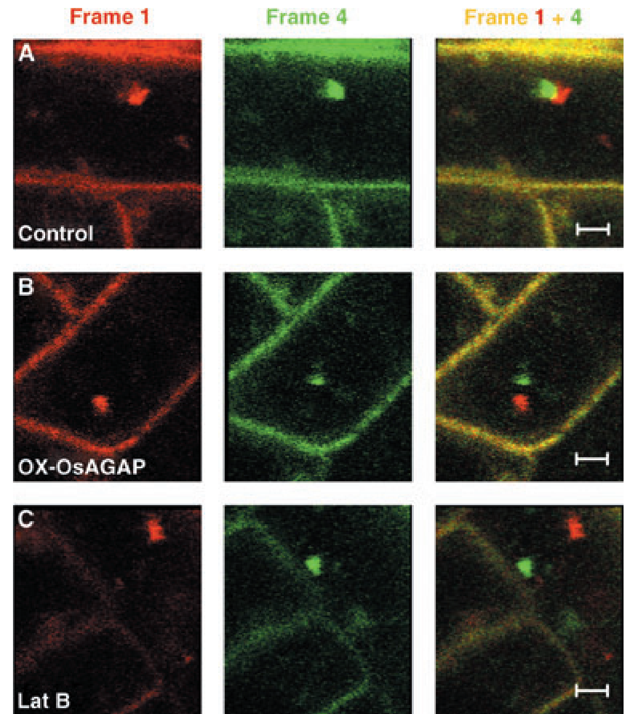

Figure 5. Effects on early endosome dynamics of OsAGAP and actin depolymerizer Lat B.

Early endosomes were stained with FM4–64 for 20 min, and images were taken with a 2.18 s interval between frames. Subcellular dynamics of FM4–64-labeled endosomes (yellow) in a control cell (dimethylsulfoxide [DMSO], **A**), an OsAGAP-overexpressed cell **(B)** and a Lat B-treated cell **(C)**. Overlay of frame 1 (red) on frame 4 (+6.54 sec; green).
Bars = 2 μm.

Discussion

Polar auxin transport, which depends on polarized subcellular distribution of AUX1/LAX influx carriers and PIN efflux carriers, mediates various processes of plant growth and development (Bennett et al. 1996; Estelle 1998; Gälweiler et al. 1998; Luschnig et al. 1998; Parry et al. 2001). AUX1 is a polarized PM protein that is delivered to and recycled from the PM via endosomes (Marchant et al. 1999; Grebe et al. 2002; Kleine-Vehn et al. 2006). Like PIN proteins, AUX1 subcellular distribution is sensitive to BFA treatment (Grebe et al. 2002). However, in contrast to PIN1, only the intracellular pool of AUX1 exhibits BFA sensitivity. AUX1 seems to be recycled from endosomes to the PM in a BFA-insensitive GNOM (an ARF-GEF)-independent manner (Geldner et al. 2003; Kleine-Vehn et al. 2006). The localization of AUX1 but not PIN protein depends on the novel ER protein AXR4 in *Arabidopsis*

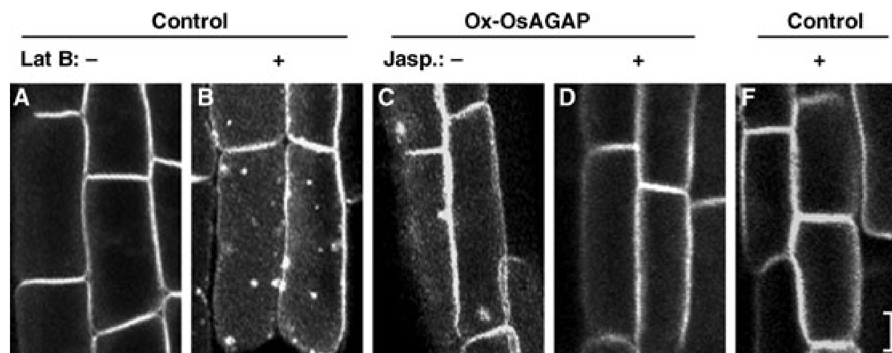

Figure 6. Effects of Lat B and jasplakinolide on AUX1 accumulation in *Arabidopsis*.

AUX1-YFP could accumulate in the actin-dirupted **(B)** and was OsAGAP overexpressed **(C)** root cells and could be rescued by actin stabilizer jasplakinolide (Jasp.) treatment (**D**; 1 μM, 3 h). Bars = 5 μm.

(Dharmasiri et al. 2006). Overexpression of OsAGAP in rice impaired polar auxin transport and interfered with both primary and lateral root development by altering AUX1 localization (Zhuang et al. 2006).

OsAGAP overexpression promotes vesicle transport between the PM and Golgi apparatus

ARF-GAPs are multifunctional proteins that could regulate membrane traffic (Randazzo and Hirsch 2004). Previous observations showed that OsAGAP could rescue the defect of vesicular transport in the yeast ARF-GAP double mutant *gcs1*Δ*glo3*Δ; therefore, OsAGAP might be involved in the regulation of vesicle transport between the ER and Golgi compartments (Zhuang et al. 2005). As well, FM4–64 staining revealed OsAGAP is involved in the regulation of vesicle trafficking (Zhuang et al. 2006). Overexpression of AGD7, an ARF-GAP in *Arabidopsis*, led to the relocation of Golgi proteins into the ER in plant cells (Min et al. 2007). Our data showed the early endosome TGN aggregated in OsAGAP-overexpressed rice and *Arabidopsis* (**Figure 3**). FM4–64 is widely used as an endocytosis marker in living eukaryotic cells and mainly stained early endosomes within 30 min (Bolte et al. 2004). AUX1 could partially accumulate with FM4–64 early endosome after actin disruption (**Figure 2**). It is hard to investigate the movement of AUX1. We had to use FM4–64 to mimic the movement of AUX1. The staining patterns at 10 min and 30 min were observed (**Figure 4**). These suggested that endocytosis of early endosomes was significantly promoted after actin disruption. Moreover, the velocity of early endosome movement, which was labeled by FM4–64, was promoted in OsAGAP-overexpressed *Arabidopsis* cells (**Figure 5**). OsAGAP induced the aggregation of FM 4–64 labeled vesicles in transgenic rice (Zhuang et al. 2006). These results support the hypothesis that OsAGAP overexpression stimulates AUX1 early endosome trafficking between the PM and Golgi apparatus. OsAGAP was localized to the plasma membrane, cytoplasm and nucleus (Zhuang et al. 2006). The OsAGAP localization at membrane and cytoplasm is consistent with its role in regulation of AUX1 localization and vesicle transport. Thus, OsAGAP may be involved in the regulation of AUX1 in endosome transport. But because there were no significant differences between the OsAGAP RNAi knockdown transgenic lines and wild-type in the number of lateral roots and response of root elongation to the various auxins, we only examined the effect in its overexpression line (Zhuang et al. 2006).

Actin cytoskeleton disruption greatly stimulates the endocytosis but not exocytosis of AUX1 endosomes

ARF-GAPs have been found to regulate cytoskeletal structures, including focal adhesions and actin-rich membrane ruffles in animals (Moss and Vaughan 1998; Randazzo and Hirsch 2004). Git2-short can reduce the amount of actin stress fibers when overexpressed in NIH3T3 cells (Mazaki et al. 2001). As well, vesicle transport relies on actin dynamics (Samaj et al. 2006). Moreover, AUX1 and PIN protein localization and dynamics require microfilaments but not microtubules. AUX1 localization and trafficking relies on intact actin filaments (Kleine-Vehn et al. 2006). We found that similar to Git2-short overexpression, OsAGAP overexpression produced thinner and less bundled actin filaments (**Figure 1**). The accumulation of early endosome and AUX1 in OsAGAP overexpression cells could be rescued by actin stabilizer jasplakinolide (**Figures 3B**, **6**). Polar localization and dynamics of AUX1 or PIN proteins require MFs. However, a high concentration of the actin depolymerizer Lat B (20 μM, 3 h), which did not affect PIN1 localization, was sufficient to strongly influence AUX1 localization (Kleine-Vehn et al. 2006). In our study, after 20 μM Lat B treatment for 3 h, all the actin filaments in the root cells

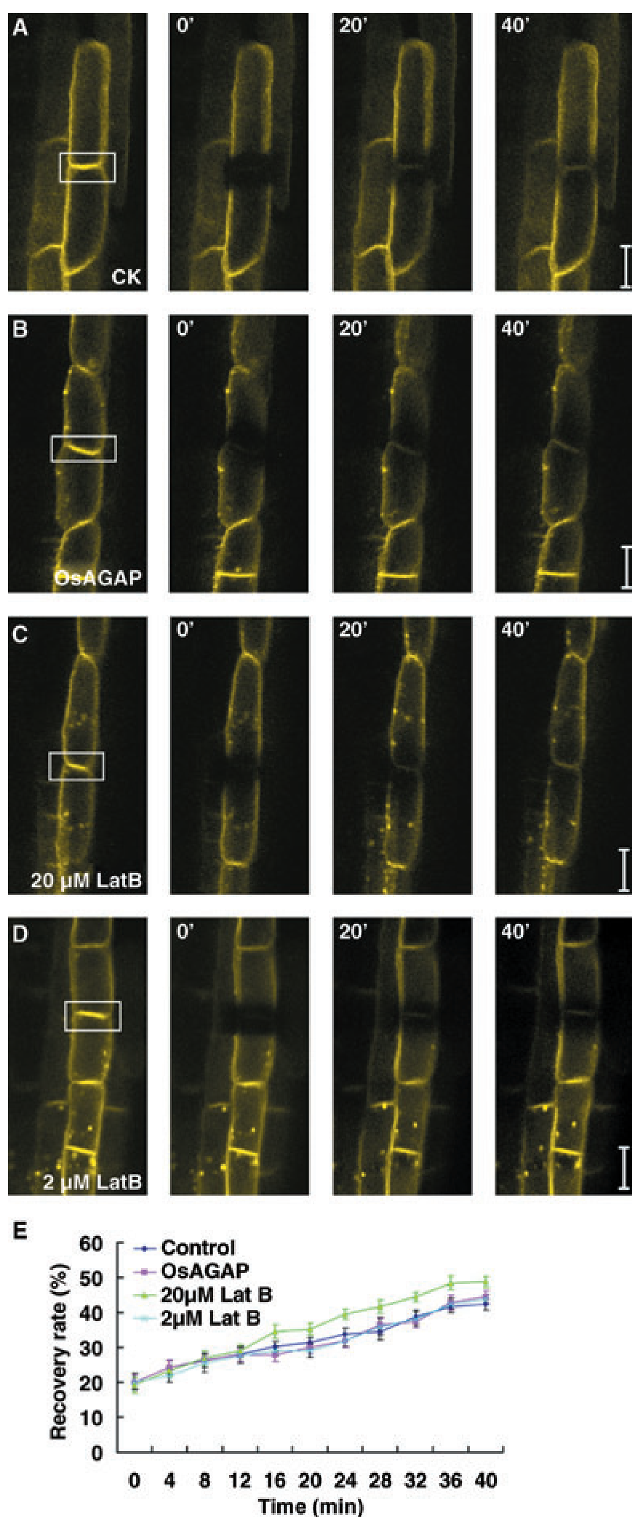

Figure 7. Fluorescence recovery after photobleaching (FRAP) analysis of AUX1-YFP dynamics.

(A) and **(B)** AUX1-YFP recovery in the root epidermal cells of wild-type **(A)** and OsAGAP-overexpressed **(B)** *Arabidopsis* treated with dimethylsulfoxide (DMSO) for 3 h with protein synthesis inhibited by cyclohexamide (50 μM, 0.5 h).

(**C**, **D**) AUX1-YFP recovery in the root epidermal cells of wild-type *Arabidopsis* treated with 20 μM (**C**) and 2 μM (**D**) Lat B for 3 h with protein synthesis inhibited by CHX (50 μM, 0.5 h).

Bars = 10 μm.

Boxes indicate the bleached area.

(E) Fluorescence recovery rate of AUX1-YFP.

Similar results were obtained in four different FRAP experiments.

Control is fluorescence quantification of **Figure 7A**(♦), OsAGAP is **Figure 7B**(■), 20 μM Lat B is **Figure 7C**(▲) and 2 μM Lat B is **Figure 7D**(×).

were disrupted (**Figure 4A,B**). However, the actin filament disruption in the OsAGAP-overexpressed *Arabidopsis* root cells was not as severe as with Lat B treatment (20 μM, 3 h) (**Figures 1D**, **4B**). Therefore, OsAGAP overexpression disrupts the localization of AUX1 but not PIN1, and the average velocity of the FM4–64-labeled early endosomes in the OsAGAP-overexpressed cells is slower than in the actin-disrupted cells (**Figure 5**).

Our studies revealed that overexpression of OsAGAP affected the vesicle transport between the PM and Golgi apparatus and led to the disruption of actin cytoskeletal organization. The FM4–64-labeled transport vesicles could move faster from the PM to the Golgi apparatus in the MF-disrupted root cells, and vesicles accumulated in the root cells (**Figures 4**, **5**). As well, AUX1-YFP partially co-localized with FM4–64-labeled vesicles in large patches in the MF-disrupted root cells (**Figure 2**). These data suggest that the endocytosis of early endosomes with AUX1 could also be stimulated by MF disruption.

Recent studies have shown that actin stabilization by the auxin transport inhibitor TIBA impairs vesicle motility in and out of cells (Kleine-Vehn et al. 2006; Dhonukshe et al. 2008). Cortical F-actin is thought to be associated with vesicle targeting but also acts as a barrier to vesicles docking to the target PM (Muallem et al. 1995; Manneville et al. 2003), and the exocytosis in the pollen tip is promoted by RIC3-mediated actin depolymerization (Lee et al. 2008). Our investigation showed that MF disruption by Lat B and OsAGAP could greatly increase endocytosis of early endosomes. But FRAP analysis revealed exocytosis of AUX1 recycling endosome only slightly promoted (**Figure 7**). These results suggest that sensitivities to the actin filaments of different organelles are different, and the endocytosis of early endosomes with AUX1 is more sensitive to the disruption of actin filaments. Thus, when actin filaments are thicker and more bundled with TIBA treatment, the created barrier may be high enough to impair vesicle motility. However, under natural conditions, the actin cytoskeleton does not quite affect the exocytosis of the AUX1 recycling endosome.

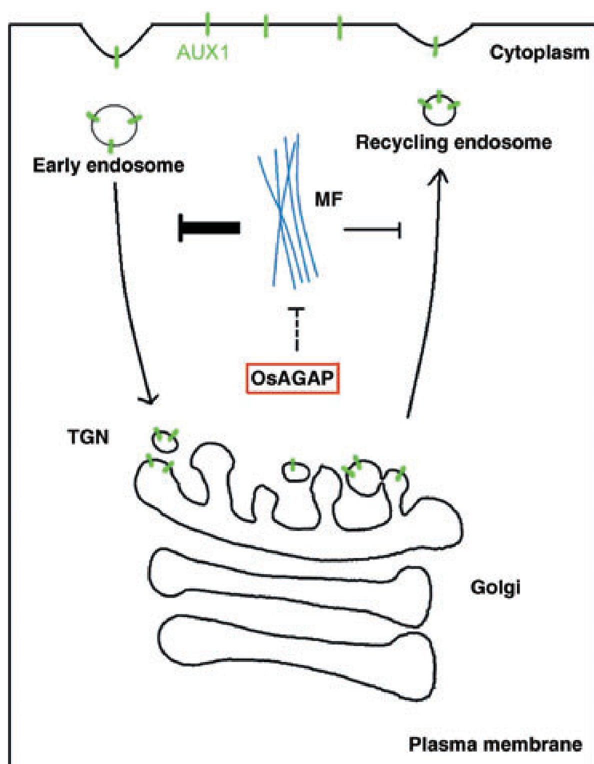

Figure 8. Model of OsAGAP affecting the AUX1-YFP dynamics.

OsAGAP regulates actin filament dynamics indirectly. With overexpression of OsAGAP, endocytosis of the auxin influx carrier AUX1 early endosomes is significantly stimulated by actin disruption, but exocytosis of AUX1 recycling endosomes is only slightly affected.

We propose a model of how OsAGAP affects AUX1-YFP dynamics in the plant cell, which depends on actin cytoskeletal organization (Figure 8). OsAGAP, an ARF-GAP in rice, is involved in the regulation of actin organization by reducing the thickness and bundling. As well, actin filaments may greatly interfere with the endocytic pathway of the auxin influx carrier AUX1 early endosome, but exocytosis of the AUX1 recycling endosome is only slightly inhibited. When the MF blockage of the AUX1 early endosome from the PM to cytoplasm is released through the OsAGAP-dependent actin disruption, it induces a burst of endocytosis. Additionally, our data suggest that AUX1 early endosome uses a novel trafficking pathway distinct from the AUX1 recycling endosome. Further studies characterizing different endosomes of AUX1 trafficking are needed.

Materials and Methods

Plant material, growth conditions and chemicals

Arabidopsis thaliana seedlings were grown on vertical agar plates for 5 d under long-day conditions at 23 °C (half-strength Murashige and Skoog [MS] agar containing 1% sucrose, pH 5.8). The following transgenic *Arabidopsis* lines were described previously: AUX1-yellow fluorescent protein (AUX1-YFP) (Swarup et al. 2004), actin binding domain from fimbrin-green fluorescent protein (ABD2-GFP) (Wang et al. 2004), PIN2-GFP (Xu and Scheres 2005) and the OsAGAP overexpressed line (35S::OsAGAP) (Zhuang et al. 2005). *otr-2* was used as OsAGAP overexpressed transgenic rice line. (Zhuang et al. 2006) For actin staining, rice seeds were grown on 1/2 MS agar plates (without sucrose, pH 5.8) in the dark at 28 °C. Latrunculin B (Lat B; Sigma), jasplakinolide (Molecular Probes), cycloheximide (Calbiochim) and FM4–64 (Molecular Probes) were used in dimethylsulfoxide (DMSO) stock solutions of 20 mM, 1 mM, 50 mM and 1 mg/mL, respectively. Control experiments contained equal amounts of solvents (DMSO).

Plasmid construction

For construction of the GONST1-GFP transient expression vector, polymerase chain reaction (PCR) was used to amplify the full-length GONST1 cDNA with the primers forward, 5′-AGCTCTAGAATGAAATTGTACGAACACGA-3′ and reverse, 5′-AGAGGTACCGGACTTCTCCCTCATTTTG-3′) with use of pGONST1-YFP (Baldwin et al. 2001). The amplified PCR fragments were digested with *Xba*I and *Kpn*I and subcloned into pGFP221.

Protoplast transformation

Plasmids were purified by use of Tiangen or Qiagen kits according to the manufacturer's protocols. *Arabidopsis* protoplasts transformation was performed as described by Jin et al. (2001). The rice protoplast transformation by polyethylene glycol (PEG) was as described by Asai et al. (2000) and Bart et al. (2006), with some modification. Rice seeds were first sterilized, grown on 1/2 MS medium under light for 3 d, and then transferred to the dark for 7–10 d. Sheath and stem tissues were cut into 0.5 mm pieces with the use of sharp razors. Tissues were immediately incubated in enzyme solution (0.6 M mannitol, 10 mM MES [pH 5.7], 1.5% cellulase RS, 0.75% macerozyme R10, 0.1% bovine serum albumin [BSA], 1 mM $CaCl_2$ and 5 mM β-mercaptoethanol) for 4 h in the dark with gentle shaking (40 rpm). After incubation, one volume of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES [pH 5.7]) was added. Protoplasts were passed through a 35 μm nylon mesh filter. The solution was centrifuged for 5 min at 300 *g* to pellet the protoplasts, and then washed again with W5. Cells at 2×10^6 cells/mL were re-suspended in Mmg solution (0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES [pH 5.7]) for PEG-mediated transformation and kept on ice for 30 min. For transformation, 40% PEG (0.2 M mannitol, 100 mM $CaCl_2$, 40% v/v PEG4000 [Fluka, #81240]) was added to the protoplasts for 15 min. Cells were washed 1× with 10 volumes of W5 and then

re-suspended in W5. All of the experiments were performed at room temperature. Cells were then incubated at 28 °C in the dark overnight.

FM4–64 uptake and microfilament staining

FM4–64 (Molecular Probes) staining of 5-d-old seedling *Arabidopsis* and rice root tips was as described previously (Zhuang et al. 2006). The seedlings were incubated with 1 μg/mL FM4–64 for 10 min on ice, washed twice, and observed under a confocal microscope (Zeiss LSM510 META) at 514 nm, and images were photographed. Signal intensity was quantified by use of Image J (http://rsb.info.nih.gov/ij) which was performed as Lee et al. (2008).

Following the procedure of Van Gestel et al. (2001), 5-d-old rice seedling roots grown under dark were incubated with phalloidin-FITC (0.1 μM, Beyotime, China) in actin stabilizing buffer (100 mM 1,4-Piperazinediethanesulfonic acid (PIPES), 10 mM ethylene glycol tetraacetic acid (EGTA), 5 mM $MgSO_4$, pH 6.8) containing 1% glycerol for 3 h, and images were photographed by use of a Leica TCS SP5 confocal laser-scanning microscope.

Live-cell imaging and FRAP analysis

Live-cell imaging and FRAP analysis involved use of a confocal microscope (Zeiss LSM510 META) equipped with a HeNe laser (543 nm) and an argon laser (488 nm, 514 nm). In some images, regions of interest were drawn. For the FRAP experiment, a region of interest was selected for photobleaching by the 488 nm laser line at 100% emission strength. The florescence recovery images were collected with 25% laser power at 4 min intervals for 1 h. Time-lapse data were analyzed by use of Image J software.

Acknowledgements

The authors thank Drs. Malcolm Bennett (University of Nottingham) for AUX1-YFP seeds; Shanjin Huang (IBCAS) for ABD2-GFP seeds and jasplakinolide; Liwen Jiang (Chinese University of Hong Kong) for plasmid of pGONST1-YFP. We also thank Dr. Jianmin Zhou (NIBS) for help on the transformation system and Drs. Jie Le and Yiyan Zheng (IBCAS) for technical help. This work was supported by the Innovative Program of the Chinese Academy of Sciences (KSCX2-YW-N-041) and the National Natural Science Foundation of China (30670197).

Received 10 May 2011 Accepted 24 May 2011

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(Co-Editor: Chun-Ming Liu)

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Immunofluorescence staining of microtubules. Microtubules are not affected in wild-type **(A)** and OsAGAP-overexpressed **(B)** transgenic rice root cells as detected by anti-tubulin antibody. Bars = 10 μm.

Figure S2. Fluorescence recovery after photobleaching (FRAP) analysis of AUX1 dynamics without drug treatment. **(A–D)** AUX1-YFP incidence at the apical plasma membrane of epidermal cells recovers within 30 min after photobleaching. **(E)** Color-coded signal intensity of **D** (from blue, low signal, to red, high signal). Arrows indicate that the recovery of AUX1-YFP at the apical plasma membrane (PM) is not due to diffusion from the other side of the plasma membrane. Bars = 10 μm. Box indicates the bleached area.

Figure S3. Fluorescence recovery after photobleaching (FRAP) analysis of PIN2 dynamics. **(A,B)** PIN2-GFP recovery in the root epidermal cells of wild-type **(A)** and OsAGAP-overexpressed **(B)** *Arabidopsis* treated with dimethylsulfoxide (DMSO) for 3 h. **(C)** PIN2-GFP recovery in the root epidermal cells of wild-type *Arabidopsis* treated with 20 μM Lat **B** for 3 h. **(D)** Fluorescence recovery rate of PIN2-GFP. Similar results were obtained in three different FRAP experiments. Control is fluorescence quantification of Figure S3A(⧫), OsAGAP is Figure S3B (■), and Lat B is Figure S3C (▲) Images in the right columns indicate the color-coded signal intensity of images after photobleaching for 60 min. Bars = 10 μm. **Figure 5**. Videos 1, 2 and 3 show the dynamics of FM4–64-labled early endosomes in the root epidermal cells of **Figure 5A**, a to c, respectively. Time interval = 2.18 s. **Figure 7**; Video1 to 4 show FRAP analysis of AUX1-YFP recovery in the root epidermal cells of **Figure 7A–D**, respectively. Arrow indicates the bleached area.

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