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Over-expression of OsAGAP, an ARF-GAP, interferes with auxin influx, vesicle trafficking and root development

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Summary

Development and organogenesis in both dicot and monocot plants are highly dependent on polar auxin transport (PAT), which requires the proper asymmetric localization of both auxin influx and efflux carriers. In the model dicot plant *Arabidopsis thaliana*, the trafficking and localization of auxin efflux facilitators such as PIN-FORMED1 (PIN1) are mediated by GNOM, a guanine-nucleotide exchange factor (GEF) for the ADP-ribosylation factor (ARF) family of small GTPases, but molecular regulators of the auxin influx facilitators remain unknown. Here, we show that over-expression of OsAGAP, an ARF-GTPase-activating protein (ARF-GAP) in rice, impaired PAT and interfered with both primary and lateral root development. The lateral root phenotype could be rescued by the membrane-permeable auxin 1-naphthyl acetic acid, but not by indole 3-acetic acid (IAA) or by 2,4-dichloro-phenoxyacetic acid, which require influx facilitators to enter the cells. OsAGAP-over-expressing plants had alterations in vesicle trafficking and localization of the presumptive *A. thaliana* auxin-influx carrier AUX1, but not in the localization of the auxin efflux facilitators. Together, our data suggest that OsAGAP has a specific role in regulating vesicle trafficking pathways such as the auxin influx pathway, which in turn controls auxin-dependent root growth in plants.

Keywords: rice, root development, polar auxin transport, AUX1, ARF-GAP.

Introduction

Polar auxin transport (PAT) in plant cells is a unique and significant feature of the phytohormone auxin, which controls organogenesis and plant development. Auxin is transported into and out of cells across the plasma membrane, with strict directionality mediated by specialized influx and efflux facilitators (Muday and DeLong, 2001). Mutation of the auxin influx facilitator *AUX1* disrupts facilitator-mediated transport of indole acetic acid (IAA) between source and sink tissues and results in defects in lateral root formation (Marchant *et al.*, 1999, 2002). The PIN-FORMED (*PIN*) gene family encodes important components of the auxin efflux facilitator (Friml and Palme, 2002; Palme and Galweiler, 1999). *pin* mutants show defective auxin transport and reduced lateral root initiation and leaf organogenesis (Chen *et al.*, 1998; Friml *et al.*, 2002; Galweiler *et al.*, 1998; Muller *et al.*, 1998). PAT, mediated by a network involving the AUX1 influx facilitator and the PIN auxin efflux facilitator, controls root growth and patterning in Arabidopsis (Blilou *et al.*, 2005). The polarity of auxin transport is controlled by the asymmetric distribution of auxin transport proteins. Both the influx and efflux facilitators are localized in auxin transport-competent cells in a polar manner (Friml *et al.*, 2002; Galweiler *et al.*, 1998; Muday and Murphy, 2002; Muller *et al.*, 1998; Swarup *et al.*, 2001).

Polar localization of auxin facilitators is established by vesicle trafficking (Grebe *et al.*, 2002; Muday *et al.*, 2003; Steinmann *et al.*, 1999). Brefeldin A (BFA), an inhibitor of vesicle trafficking, interferes with PAT by disrupting the

polar localization of PIN-FORMED1 (PIN1) (Geldner *et al.*, 2001). GNOM, an ADP ribosylation factor–GTP exchange factor (ARF-GEF) known to regulate vesicle trafficking in various organisms (Geldner *et al.*, 2003) is required for polar localization of PIN1 and hence PAT (Geldner *et al.*, 2003; Steinmann *et al.*, 1999). ARF-GEF catalyzes the conversion of ARF-bound GDP to GTP, which is necessary for the efficient delivery of the vesicle to the target membrane (Poon *et al.*, 1999). The auxin influx facilitator AUX1 is asymmetrically localized to the plasma membrane of root protophloem cells in Arabidopsis (Swarup *et al.*, 2001). Recently, it has been reported that AXR4, a novel ER accessory protein, regulates the localization of AUX1 but not PIN proteins in Arabidopsis (Dharmasiri *et al.*, 2006). However, much less is known about the regulation mechanism for localization and activation of auxin influx facilitators in plants.

ARF-GTPase-activating protein (ARF-GAP) contributes to the hydrolysis of GTP-bound ARF, the opposite of the conversion catalyzed by ARF-GEF, which is necessary for the efficient delivery of the vesicle to the target membrane (Jensen *et al.*, 2000; Zhuang *et al.*, 2005). The main structural characteristic of ARF-GAP is the $CX_2CX_{16}CX_2C$ zinc finger protein motif, which is critical for the GTPase-activating activity (Jensen *et al.*, 2000; Zhuang *et al.*, 2005). Such a structure is always arrayed with a distinct C-terminal and is important for regulating ARF-GAP localization and activity (Huber *et al.*, 2002). We previously cloned *OsAGAP* from rice (Zhuang *et al.*, 2005). It encodes a protein including ARF-GAP with a zinc finger and C2 domain and identified as an activating protein for ARF GTPase activity. OsAGAP can rescue the defect of the membrane structure in the yeast double mutant *gcs1*Δ*glo3*Δ, identified as two members of yeast ARF-GAP (Poon *et al.*, 1999). Transgenic Arabidopsis over-expressing *OsAGAP* showed altered patterning phenotypes related to auxin, such as reduced apical dominance, shorter primary roots, an increasing number of longer adventitious roots and defects in gravitropism (Zhuang *et al.*, 2005). In Arabidopsis, a ZAC involving an ARF-GAP zinc finger and C2 domain, which shared homology with OsAGAP, showed promiscuous and specific phospholipid binding (Jensen *et al.*, 2000). Recently, there have been several reports about the function of ARF-GAP in Arabidopsis development. VAN3, with ARF-GAP activity, was identified as involved in leaf development (Koizumi *et al.*, 2005), and SFC, another ARF-GAP, is required for normal vein patterning mediated by the auxin efflux facilitator PIN1 (Sieburth *et al.*, 2006). So far, much less is known about molecular regulators of the auxin influx facilitators, although there are a number of reports on the regulation mechanism for auxin efflux.

Here we show that over-expression of *OsAGAP* disrupts the asymmetric plasma membrane localization of the auxin influx facilitator, reduces auxin influx and inhibits root development in rice. The vesicle trafficking pattern was disrupted by the increased expression of *OsAGAP* in rice.

Results

OsAGAP *transgenic rice plants showed changes in root growth and development and altered efficiency of PAT*

To uncover the mechanism of action of ARF-GTPase as a molecular switch for the regulation of PAT (Geldner *et al.*, 2003; Xu and Scheres, 2005), we isolated an *OsAGAP* gene encoding a zinc finger protein that stimulates the GTPase activity of ARF in rice, the opposite process to ARF-GEF (Zhuang *et al.*, 2005). Transformed *OsAGAP* rice plants showed a single-copy insertion pattern on Southern blotting. RT-PCR and Northern blotting showed that *OsAGAP* was expressed highly in the transgenic lines (Figure 1a–c). During seedling development, transgenic rice over-expressing *OsAGAP* exhibited developmental defects in root growth and development (Figure 1d). The emergence of lateral roots in *OsAGAP*-over-expressing rice seedlings was delayed for approximately 2 days compared to that in the wild-type, and the number of lateral roots in the seedlings of *OsAGAP* transgenic plants was approximately 50% of that of the wild-type at 12 days after germination (Figure 1e). In addition, fewer lateral root primordia were formed in *OsAGAP* transgenic plants (data not shown), which is consistent with the reduced number of emerged lateral roots in *OsAGAP* transgenic plants. The transgenic rice plants also showed reduced growth of primary roots and adventitious roots compared with wild-type plants (Figure 1f). Microscopy revealed a reduced cell size in mature, elongation and meristem zones of transgenic plants compared with wild-type control plants (Figure 1g). Transgenic plants showed greater inhibited root growth in the mature zone (51.2%) than in either the elongation (33.7%) or meristem zones (34.2%). Both the reduced root growth and disrupted patterning of lateral root and adventitious root development suggested a defect in auxin action.

We tested whether the phenotype of *OsAGAP*-over-expressing plants is due to a defect in PAT. First, the responses to either 2,3,5-triiodobenzoic acid (TIBA) or 1-*N*-naphthylphthalamic acid (NPA), inhibitors of PAT, were investigated in transgenic rice plants. As shown in Figure 2(a), root elongation was inhibited by TIBA in wild-type rice. The root elongation in *OsAGAP* transgenic plants was about twice that of the wild-type when grown on media containing 10 or 100 nM TIBA. Similarly, the response to NPA in the transgenic plants was slower than that in the wild-type under 50 or 100 nM (Figure 2b). Thus, the transgenic plants were resistant to both PAT inhibitors TIBA and NPA. In Arabidopsis *agr1* mutants, root growth showed decreased sensitivity to TIBA, which resulted from a defect in efficient delivery of auxin (Chen *et al.*, 1998; Luschnig

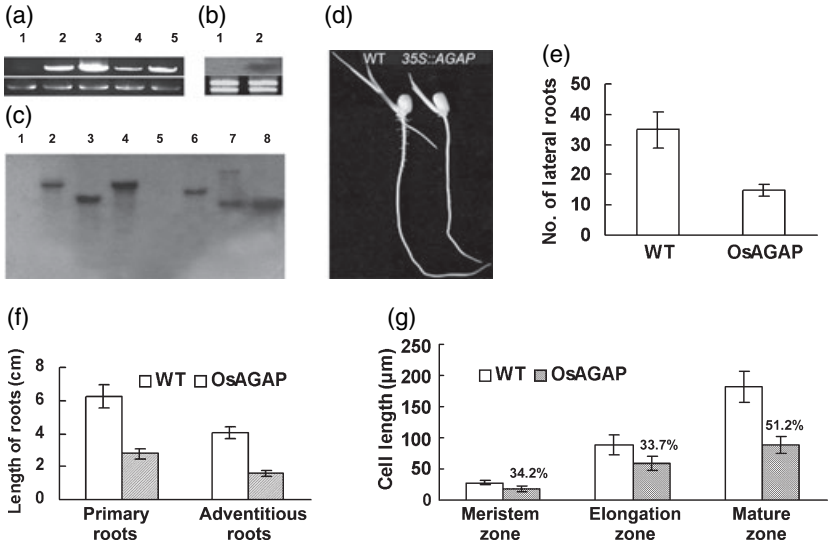

Figure 1. OsAGAP transgenic rice plant phenotypes and their identification at a molecular level.
(a) Products of RT-PCR. Lane 1, wild-type; lanes 2–5, *OsAGAP* transgenic lines with positive staining for β-glucuronidase in four individual *OsAGAP*-over-expressing transgenic rice plants, *otr-1*, *otr-2*, *otr-3* and *otr-4*.
(b) Northern blotting of *OsAGAP* transgenic line. Lane 1, wild-type; lane 2, *otr-2*, an example of transgenic line used in further experiments.
(c) Southern blot analysis of transgenic rice lines. Lanes 1 and 5, wild-type; lanes 2 and 6, *otr2*; lanes 3 and 7, *otr3*; lanes 4 and 8, *otr8*. Genomic DNA was digested by *Eco*RI in lanes 1–4 and by *Hin*dIII in lanes 5–8.
(d) Seedling phenotype of *OsAGAP*-transformed rice plants at 5 days after germination.
(e) Number of lateral roots. WT, wild-type; OsAGAP, over-expressing transgenic rice line. There was a significant difference in phenotype in the transgenic plant populations, as determined repeated-measures analysis of variance (two-sample *t* test; $P < 0.05$).
(f) Length of primary and adventitious roots (5-day-old seedlings).
(g) Cell length at meristem, elongation and mature zones in primary roots. The percentage numbers above the columns are the percentage inhibition compared with wild-type (data were from at least four independent experiments).

et al., 1998). Therefore, the increased resistance of *OsAGAP* transgenic plants to the inhibitors might suggest a defect in PAT. To further confirm this result, analysis of the efficiency of PAT in the roots of transgenic plants revealed that total [^{3}H] IAA transport was significantly reduced as compared with that in the wild-type (Figure 2c). These results imply that OsAGAP may be involved in regulation of PAT in plants.

Effects of over-expression of OsAGAP *on activity and distribution of auxin influx facilitator AUX1 in transgenic plants*

A root elongation bioassay was used to examine the response of *OsAGAP*-over-expressing plants to exogenous auxin analogs that are differentially transported by influx and efflux facilitators (Delbarre *et al.*, 1996). Root elongation in transgenic plants was more resistant to inhibition by IAA and 2,4-D (Figure 2d,e) compared with that in the wild-type. The dose–response curves in both the transgenic and wild-type roots, however, showed the same rate of reduction in root elongation by 1-naphthyl acetic acid (NAA) treatment (Figure 2f). Both IAA and 2,4-dichloro-phenoxyactetic acid (2,4-D) use influx facilitators to facilitate their uptake, while NAA can enter cells via diffusion (Delbarre *et al.*, 1996). The reduced responses of the *OsAGAP* transgenic roots to auxin analogs that require influx facilitators for uptake is consistent with a loss of activity of the auxin influx facilitator (Marchant *et al.*, 1999; Muller *et al.*, 1998). A similar tendency has been seen in Arabidopsis *aux1* mutants, which showed selective auxin resistance and a phenotype of a reduced number of lateral roots (Marchant *et al.*, 1999). This evidence supports the hypothesis that OsAGAP may be involved in regulating PAT through influx facilitator activity.

Auxin transport influences the initiation and development of lateral roots by regulating the hormone distribution between the source and sink tissues (Blakely *et al.*, 1982; Casimiro *et al.*, 2001; Ruegger *et al.*, 1997). The reduced number of lateral roots formed in *OsAGAP* transgenic plants might be a direct consequence of defective auxin transport. It is worth noting that treatment of transgenic and wild-type roots with exogenous NAA (5×10^{-7} M), which could enter cells without the help of influx facilitators, rescued the phenotype of a reduced number of lateral roots (Figure 2g–i). Neither IAA nor 2,4-D could restore the lateral root development in the transgenic plants (data not shown). These results suggest that disrupted auxin influx contributes to the defect of lateral root development in *OsAGAP* transgenic plants.

We next asked whether the localization of influx facilitators in *OsAGAP* transgenic plants was disrupted. Only one

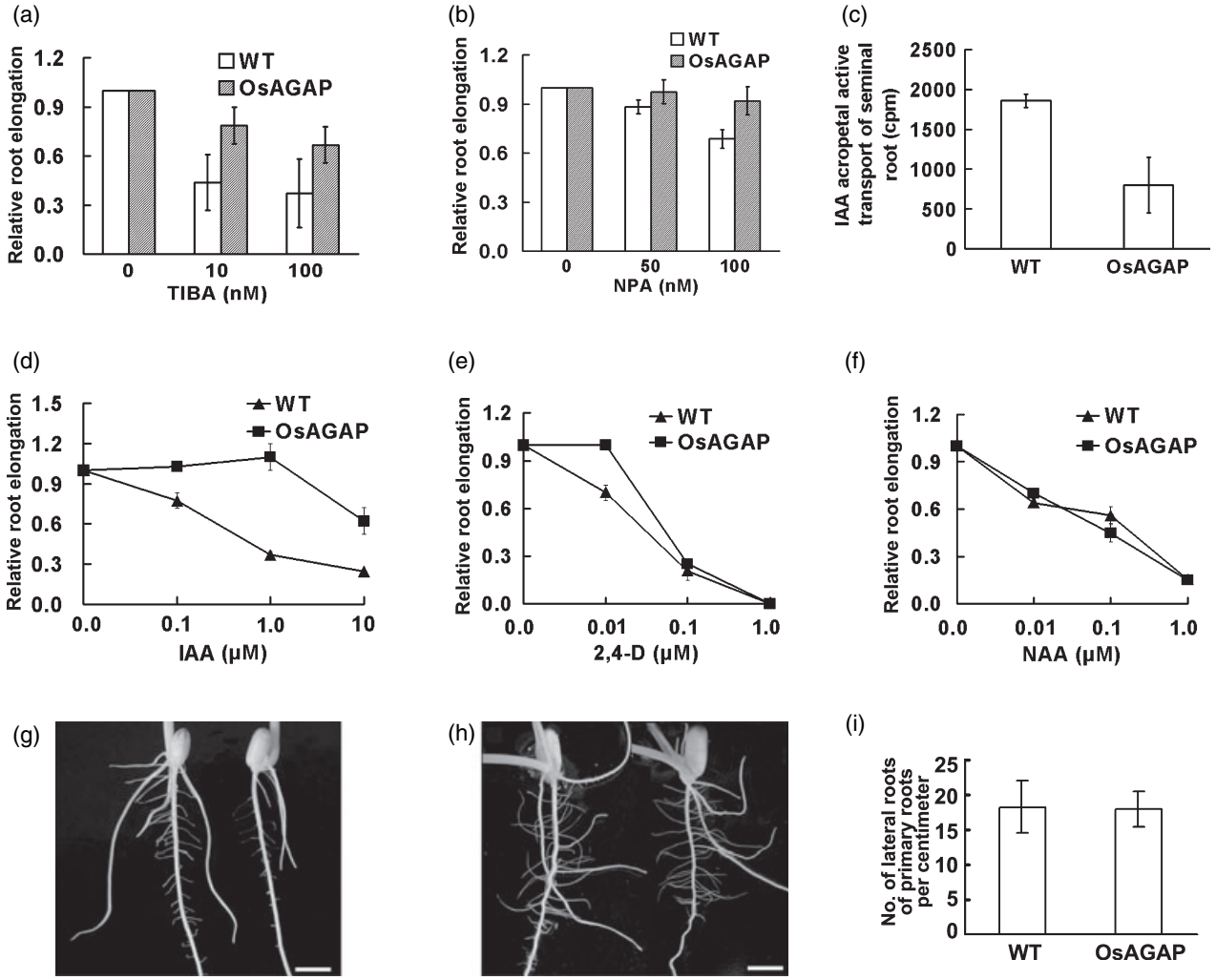

Figure 2. Auxin transport in transgenic rice plants.
(a) Response of root elongation to 3,3,5-triiodobenzoic acid (TIBA) treatment. Relative root elongation was defined as the elongation ratio between roots treated with various TIBA concentrations and non-treated controls.
(b) Response of root elongation to 1-*N*-naphthylphthalamic (NPA) treatment; Relative root elongation was defined as in (a).
(c) Acropetal active auxin transport in wild-type and *otr-2* root apices.
(d)–(f) Sensitivity of root elongation to indole 3-acetic acid (IAA) (d), 2,4-dichloro-phenoxyacetic acid (2,4-D) (e) and 1-naphthyl acetic acid (NAA) (f). The relative root elongation represents the ratio of root elongation after treatment to that before treatment. Each data point represents the average of data from 8–12 plants.
(g) Root phenotype of untreated wild-type rice (left) and an *OsAGAP* transgenic plant (right).
(h) Treatment with NAA (5×10^{-7} M) rescued the root development in an *OsAGAP* transgenic plant (right). The left root is an untreated control. Bar = 5 mm.
(i) Number of lateral roots in primary roots. WT, wild-type; OsAGAP, over-expressing transgenic rice line.
Statistical analysis of data in (a)–(c) indicated the significant difference between the transgenic and the control plant populations as determined by repeated-measures analysis of variance (two-sample *t* test; $P < 0.05$). However, there was no statistically significant difference between wild-type and transgenic lines when both were treated with NAA.

auxin influx facilitator, AUX1, has been identified so far. *OsAGAP* transgenic Arabidopsis showed altered root patterning mediated by auxin (Zhuang *et al.*, 2005). Therefore, we studied the effects of OsAGAP over-expression on the subcellular localization of AUX1 in Arabidopsis. AUX1 showed an asymmetric localization in the root cells of *HA-AUX1* transgenic Arabidopsis plants (Figure 3a). However, in progeny from a cross between 35S*::OsAGAP* transgenic Arabidopsis plants and the *HA-AUX1* transgenic line (Supplementary material; Figure S1), the asymmetric localization of HA-AUX1 in epidermis cells was absent, and immunofluorescence was detected in the cytoplasm (Figure 3b). In contrast, the efflux facilitators PIN1 and PIN2 was still localized at the apical or basal plasma membranes of root cells, respectively, in the progeny from crosses between 35S*::OsAGAP* transgenic Arabidopsis plants and the *PIN1-YFP* transgenic line (Xu *et al.*, 2006) or the *PIN2-GFP* transgenic line (Xu and Scheres, 2005) (Figure 3d,f). PIN1 signal was detected at the basal plasma membrane in vascular tissues and weakly in cortical tissues in wild-type

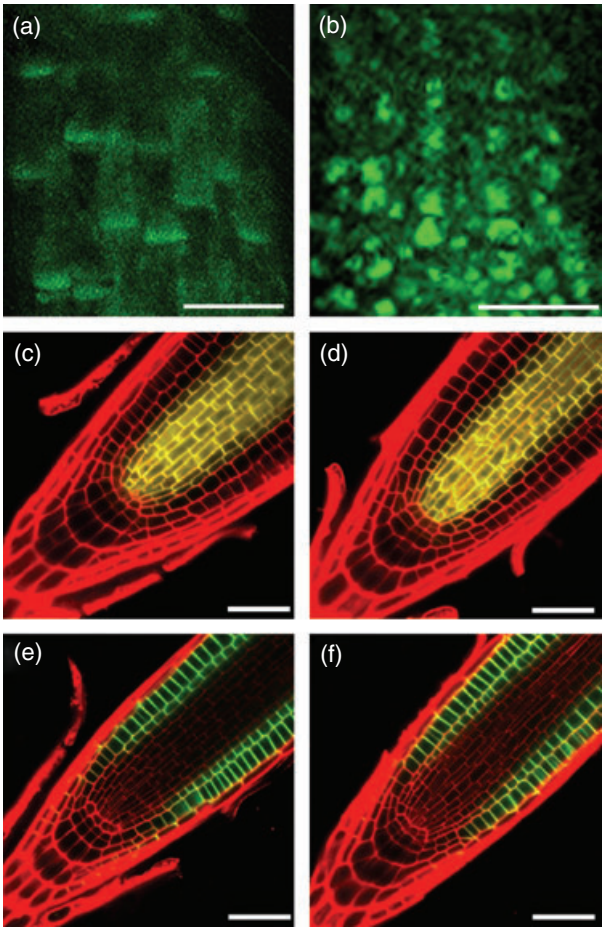

Figure 3. Localization of AUX1, PIN-FORMED1 (PIN1) and PIN2 in transgenic Arabidopsis roots.
(a) Immunolocalization of AUX1 (green) in *HA-AUX1* Arabidopsis.
(b) Immunolocalization of AUX1 in cells in the roots of the progeny of a cross between *OsAGAP* transgenic Arabidopsis and the *HA-AUX1* line.
(c) Localization of PIN1 (yellow) in *PIN1-YFP* Arabidopsis plants.
(d) Localization of PIN1 (yellow) in cells in the roots of the progeny of a cross between *OsAGAP* transgenic Arabidopsis (homozygous) and the *PIN1-YFP* line.
(e) Localization of PIN2 (green) in *PIN2-GFP* Arabidopsis.
(f) Localization of PIN2 (green) in cells in the roots of the progeny of a cross between *OsAGAP* transgenic Arabidopsis and the *PIN2-GFP* line.
Bars = 50 μm.

(Figure 3c), and PIN2 was observed at the apical membrane of epidermal cells and the basal membrane of the cortical cells (Figure 3e). The asymmetric localization patterns of either PIN1 or PIN2 were the same as those in their wild-type parents (Figure 3c–f), which is consistent with a previous study (Xu and Scheres, 2005). These results indicate that over-expression of *OsAGAP* disrupted the asymmetric localization of the auxin influx facilitator in the root cells.

The subcellular localization of OsAGAP was determined by use of a transient transfection assay. Constructs encoding an OsAGAP:GFP fusion protein and GFP protein alone were introduced into onion epidermal cells. The control GFP protein alone was detected throughout the cell, which indicated that the GFP protein remained in the cytosol membrane and nucleus (Figure 4a,b). OsAGAP was localized to the plasma membrane, cytoplasm and nucleus (Figure 4c,d), which is similar to the pattern of free GFP expression. The OsAGAP localization at membrane and cytoplasm is consistent with its role in regulation of AUX1 localization.

Expression pattern of OsAGAP in rice root and the response pattern of vesicle trafficking in transgenic plants

RNA *in situ* hybridization of longitudinal sections of the seminal root tip showed that *OsAGAP* was expressed mainly in the cortex, vascular tissues and meristematic cells under the root cap (Figure 5a,b). In roots, the shoot-derived auxin travels predominantly down the central vascular tissues, and then upon reaching the root tip is distributed back upwards along the root in the epidermis and subtending cortical cells (Jones, 1998). The root tissues showing *OsAGAP* expression overlapped those involved in auxin transport and redistribution. *OsAGAP* mRNA is expressed in tissues related to both acropetal and basipetal auxin transport in roots, and shows some overlap with auxin transport facilitators such as *AUX1* (Swarup *et al.*, 2002). This *OsAGAP* expression pattern is consistent with a possible role in the regulation of auxin transport facilitators. In addition, *OsAGAP* was also expressed in lateral and adventitious roots, as well as young leaves and shoot apical meristem (Figure 5c–h). This is consistent with a role in the regulation of lateral and adventitious root development mediated by auxin.

Polar localization of auxin transport facilitators is regulated by vesicle trafficking (Geldner *et al.*, 2001, 2003), so we investigated vesicle trafficking in the *OsAGAP* transgenic plants. The fluorescent styryl dye FM4-64 can specifically

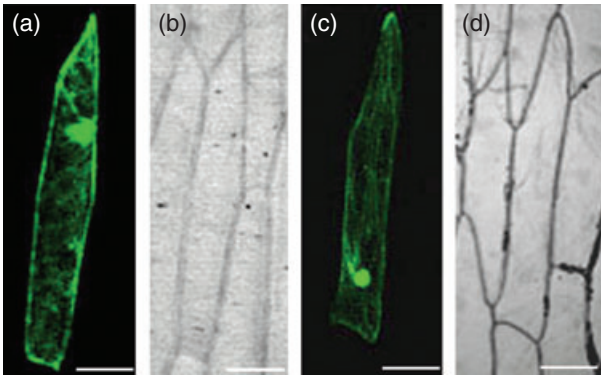

Figure 4. Subcellular localization analysis of OsAGAP using a transient transfection assay.
(a), (c) Green fluorescent protein (a) or OsAGAP:GFP (c) in onion epidermal cells. (b), (d) Corresponding images in bright field.
Bars = 100 μm.

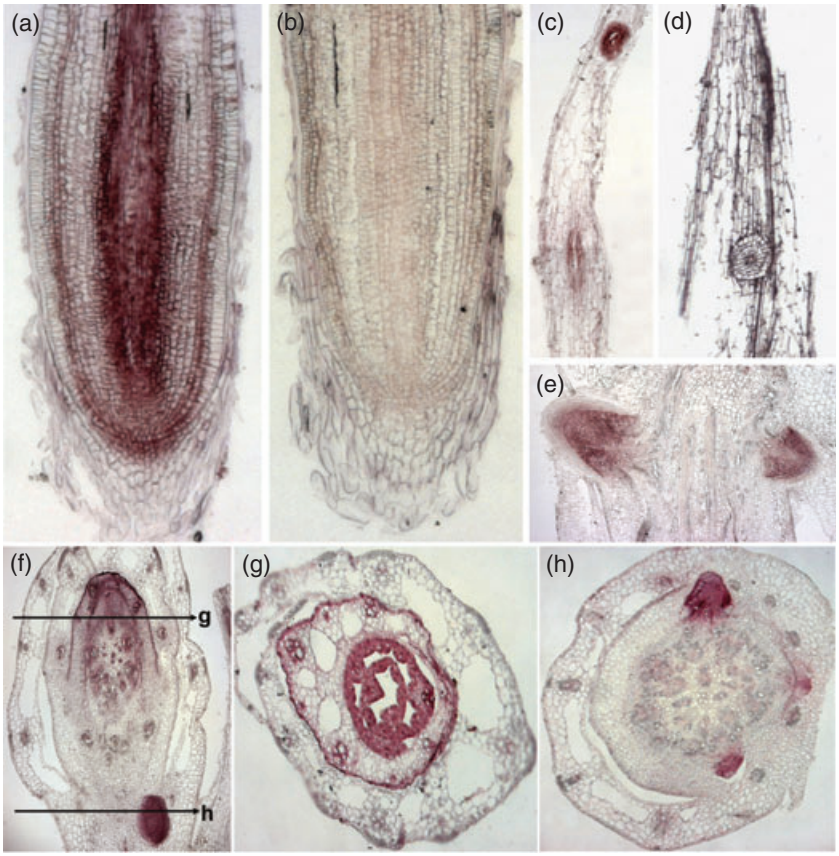

Figure 5. *In situ* localization of the *OsAGAP* transcript in rice.
(a)–(f) Longitudinal sections of root tip (a,b), mature zone (c,d) and adventitious roots morphogenesis (e) and their joint tissues and shoots (f).
(g), (h) Cross-sections at positions g and h in (f), respectively.
(a), (c), (e)–(h) Hybridization with the antisense probe. (b), (d) Hybridization with a sense probe as control. The specific sequence of the *OsAGAP* gene was used as a template for labeling probe in the *in situ* hybridization.

label the pathway of vesicle trafficking in live cells (Emans *et al.*, 2002). Transport vesicles labeled with small dots by FM4-64 were shown to be sporadically distributed in wild-type root cells (Figure 6a). However, the marker fluorescence accumulated into large patches in the *OsAGAP* transgenic root cells (Figure 6b). These abnormal aggregates of fluorescence also appeared in cells of the wild-type under treatment with BFA, an inhibitor of vesicle trafficking (Figure 6c), and are described as 'BFA compartments' (Emans *et al.*, 2002; Geldner *et al.*, 2003). The transgenic plants showed large patches with a few small dots under treatment with BFA (Figure 6d), which indicates a typical phenotype of disrupted vesicle traffic (Emans *et al.*, 2002; Geldner *et al.*, 2003). Electron microscopy revealed more BFA-induced multi-vesicles in cells of the wild-type under BFA treatment than in untreated *OsAGAP* transgenic plants. Furthermore, Golgi stacks in wild-type plants disassembled and the stacks became thinner than in non-treated controls (Figure 6e–g). Cells of the transgenic plants showed multi-vesicles and Golgi stack aggregates, while the typical BFA-induced structure appeared in the treated transgenic plants (Figure 6h–j). The aggregates shown on electron microscopy corresponded to large patches of vesicle trafficking markers in the *OsAGAP*-over-expressing transgenic root cells.

Discussion

OsAGAP*, an ARF-GTPase-activating protein, is involved in regulation of vesicle trafficking*

A number of reports support the hypothesis that ARF-GAPs are involved in vesicle trafficking because they contain catalytic, protein–protein interaction and lipid interaction domains in addition to the ARF-GAP domain (Randazzo and Hirsch, 2004). ARF-GAP1 in yeast responds to membrane curvature by means of a lipid-packing sensor motif with a central sequence of about 40 amino acids (Bigay *et al.*, 2005). ARF-GAP1 in human cells promotes vesicle formation by functioning as a component of the coat protein I (Yang *et al.*, 2002). ZAC, a membrane-associated Arabidopsis protein with an ARF-GAP zinc finger and a C2 domain,

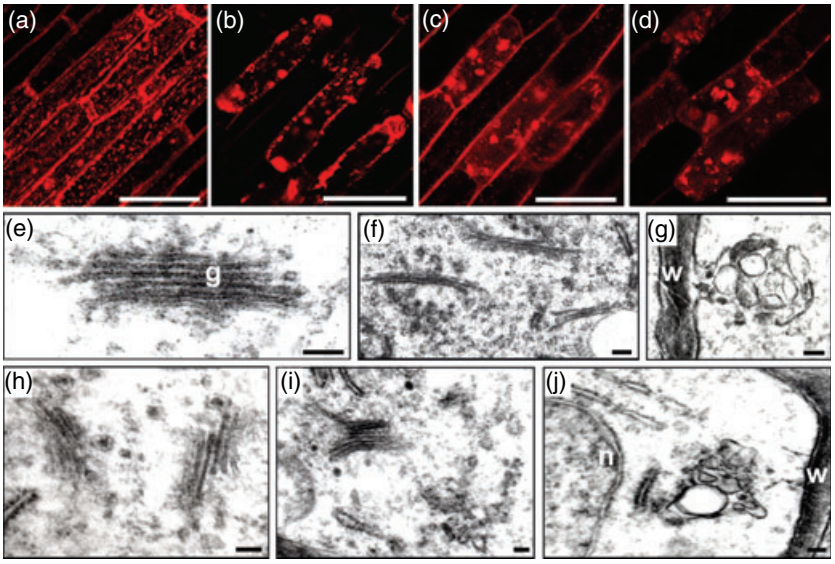

Figure 6. Vesicle transport in transgenic roots and transmission electron microscopy observations.
(a), (b) Wild-type root tips (a) and *otr-2* root tips (b) labeled with FM4-64.
(c), (d) Response of wild-type (c) and *otr-2* root tips (d) labeled with FM4-64 to Brefeldin A (BFA) treatment at 50 μM for 90 min.
(e)–(j) Electronic observation of root cells in wild-type (e–g) and *otr-2* (h–j); (e), (h) non-treatment; (f), (g), (i), (j) treatment with BFA.
g, Golgi stack; n, nucleus; w, cell wall; bars = 20 μm in (a)–(d) and 100 nm in (e)–(j).

sharing 59.4% homology with OsAGAP, has been shown to fractionate with Golgi plasma membrane marker proteins and have affinity to phospholipids in Arabidopsis (Jensen *et al.*, 2000). VAN3 is found in a sub-section of the trans-Golgi transport network in Arabidopsis (Koizumi *et al.*, 2005). *OsAGAP* rescued the defect of vesicular transport in the yeast ARF-GAP double mutant *gcs1*Δ*glo3*Δ (Zhuang *et al.*, 2005). This suggested that OsAGAP might be involved in the regulation of vesicular transport between endoplasmic reticulum and Golgi compartments (Zhuang *et al.*, 2005). In our study, over-expression of OsAGAP in rice resulted in pattern changes in vesicle trafficking (Figure 6). The response pattern of vesicle trafficking to BFA still appeared. The endocytic tracer FM4-64 (Figure 6) showed aggregated Golgi stacks and endosomes, which become surrounded by Golgi stacks in over-expressing transgenic plants, consistent with a previous report (Geldner *et al.*, 2003). This finding of aggregated multi-vesicles and Golgi stacks in the transgenic plants was supported by electron microscopy results (Figure 6). This indicates that vesicle trafficking is involved in AUX1 function in PAT. The endocytosis patterns in the transgenic lines were similar to those resulting from BFA treatment in wild-type plants (Figure 6), suggesting that a BFA-sensitive pathway may be involved in ARF-GAP-mediated vesicle transport. It has been reported that *AUX1* function is required for BFA-mediated cell polarity changes, and BFA may act on AUX1 localization during establishment of trichoblast polarity (Grebe *et al.*, 2002). Our results on *OsAGAP* transgenic rice plants, such as pattern changes in the physiological response to various auxins (Figure 2), vesicle tracking (Figure 6) and primary and lateral root development mediated by auxin (Figure 1g), may support a hypothesis that vesicle trafficking mediated by OsAGAP is involved in root growth and development in rice.

OsAGAP *regulates the asymmetric localization and activity of an auxin influx facilitator during root development*

There have been reports that the effects of auxin on endocytosis and auxin transport are linked in plant development (Geldner *et al.*, 2003; Grebe *et al.*, 2002; Koizumi *et al.*, 2005). Auxin controls PIN abundance and activity at the cell surface by modulating PIN protein trafficking (Paciorek *et al.*, 2005). IAA is transported in cells under the control of influx and efflux facilitators, which are asymmetrically located in the apical and basal plasma membranes of root cells, respectively. 2,4-D is a substrate for an auxin influx facilitator, whereas NAA can enter cells via diffusion. An Arabidopsis mutant of *aux1* was rescued by NAA but not 2,4-D or IAA in terms of lateral root development or cell polarity (Grebe *et al.*, 2002; Marchant *et al.*, 2002). In our studies, the transgenic rice plant lines differed from the wild-type control in their ability to transport various auxins. NAA, but not 2,4-D or IAA, could rescue the patterning of root development (Figure 2). In contrast to the wild-type, the transgenic plants showed insensitive response phenotypes both to IAA and 2,4-D, which require auxin influx machinery for their transport. However, they were as sensitive as the wild-type plants to NAA, which enters cells via diffusion (Figure 2).

OsAGAP did not affect the asymmetric localization of the efflux facilitators PIN1 and PIN2 in the transgenic plants (Figure 3). There are a number of reports that the correct polar localization of PIN1 is regulated by ARF-GEF (Geldner *et al.*, 2003; Grebe *et al.*, 2002; Koizumi *et al.*, 2005). In contrast, *OsAGAP* over-expression results in impaired asymmetric localization of the auxin influx facilitators AUX1, reduced efficiency of PAT and disturbed vesicle trafficking, which resulted in morphological defects in root growth and development related to defective auxin transport. RNAi knockdown transgenic lines showed a complementary phenotype to over-expression transgenic lines in terms of length of root, but an inconspicuous response to various auxins or PAT inhibitors in terms of root growth and development as compared with the wild-type (data not shown). There were no significant differences at a statistical level between the knockdown transgenic lines and wild-type in the number of lateral roots and response of root elongation to the various auxins, as well as the efficiency of PAT. These results may be explained by a functional redundancy of ARF-GAP in the regulation of root growth and development, and an unknown complicated mechanism. The altered PAT and the inhibitors' sensitivity to *OsAGAP* over-expression, as well as impaired asymmetrical localization of AUX1 due to increased expression of *OsAGAP*, suggest that this gene acts upstream of the events of PAT control during normal development in rice. Therefore, the ARF-GAP functions in control of PAT as a regulator of the auxin influx facilitator in plants.

ARF-GAP and ARF-GEF (Geldner *et al.*, 2003; Steinmann *et al.*, 1999) might cooperate to regulate PAT by mediating the proper localization of influx facilitators and efflux facilitators, respectively. In *gnom* mutants of Arabidopsis, PIN1 localization appeared to be disorganized, with no coordinated polar localization (Geldner *et al.*, 2003; Steinmann *et al.*, 1999). Furthermore, in plants harboring a fully functional GNOM variant, PIN1 localization and auxin transport were no longer sensitive to BFA (Geldner *et al.*, 2003). The presumptive auxin influx facilitator AUX1 was identified as another important component in the auxin transport pathway regulated by vesicle trafficking (Grebe *et al.*, 2002). Asymmetric distribution of AUX1 is altered by induced expression of *OsAGAP* (Figure 3) and is directly affected by BFA (Grebe *et al.*, 2002). ARF-GAPs are responsible for the inactivation of ARFs by catalyzing hydrolysis of GTP to GDP (Zhuang *et al.*, 2005), while GEF activates ARFs (Poon *et al.*, 1999). This evidence may support a loop mechanism model for the modulation of auxin transport in plant cells, with PAT being regulated by ARF as a molecular switch in vesicle trafficking, with GEF as a positive regulator for auxin efflux facilitators and GAP as a negative factor for the AUX1 influx facilitator. Our results demonstrate that OsAGAP is involved in the auxin-dependent root growth, and suggest that plant ARF-GAPs regulate specific vesicle trafficking pathways such as the auxin influx pathway.

Experimental procedures

Vector construction and plant transformation

The full-length cDNA of *OsAGAP* was cloned into the *Kpn*I and *Sac*I sites in the sense orientation in the pUN1301 expression vector driven by a ubiquitin promoter. The *uidA* gene encoding β-glucuronidase (GUS) driven by a CaMV 35S promoter was present as a marker in the construct. The construct was transformed into embryonic calli induced from seeds of *Oryza sativa* cv. Zhonghua 10 by *Agrobacterium tumefaciens* EHA105 as described previously (Ge *et al.*, 2004; Xu *et al.*, 2005). Regenerated transgenic seedlings were grown in a greenhouse under a 12 h light, 28°C, 85% relative humidity regime.

Southern and Northern blot assays

Genomic DNA isolated from transgenic 2-week-old rice seedlings was digested with *Eco*RI or *Hin*dIII, fractioned electrophoretically on a 0.7% agarose gel, and blotted onto a nylon membrane. Hybridization was as described previously (Ge *et al.*, 2000). The membrane was pre-hybridized at 65°C for 2 h and then hybridized in the same solution containing [α-^{32}P]-dCTP-labeled probe (China Isotope Company, Beijing City, Beijing, China) for 20 h at 65°C.

Northern blotting was performed as described previously (Ge *et al.*, 2000). A total of 30 μg of RNA was loaded on each lane. A probe of *OsAGAP* cDNA labeled with [α–^{32}P]-dCTP was synthesized for hybridization. After hybridization for 20 h at 68°C, the membrane was washed once with 2× SSC plus 0.1% SDS at 68°C for 20 min, then with 1× SSC plus 0.1% SDS at 37°C for 30 min. The membrane was exposed to X-ray film (Kodak, Rochester, New York, USA) at −70°C for 3–7 days.

Auxin and inhibitor treatment, and imaging of root cell size

Seeds from transformed plants were surface-sterilized and washed with sterilized distilled water four times. The sterilized seeds were germinated on half-strength Murashige–Skoog (MS) agar at pH 5.8 containing various auxins [IAA, 2,4-D, 1-NAA], TIBA or NPA at 28°C. After seeds had been cultured for 5 days, root elongation was measured and analyzed.

Imaging of root cells was as described previously (Wang *et al.*, 2006). Root tips were stained with 100 μg ml^{-1} propidium iodide (PI) solution and observed under a confocal microscope (Zeiss, Oberkochen, Germany).

Polar auxin transport assays

Polar auxin transport assays were performed with 4-day-old seminal roots of wild-type and *OsAGAP*-over-expressing seedlings as described previously (Scarpella *et al.*, 2000, 2002). Seminal root of 2 cm length was excised from the tip, and the basal part (approximately 5 mm) was immerged in half-strength MS solution containing 10^{-7} M [^{3}H]-IAA (pH 5.8) in the presence or absence of 10^{-6} M NPA. Roots were incubated for 3 h in the dark at room temperature. After incubation, the roots were cut into three segments: top part (10 mm), middle part (5 mm) and basal part (5 mm). The segments were incubated separately in scintillation liquid. Radioactivity in the

segments was counted after overnight incubation. In the presence of NPA, the ratio between the radioactivity accumulated in the middle and basal segments was the diffusion factor (DF). In the absence of NPA, the product of the DF and the radioactivity measured in the basal part was the amount of diffused [^{3}H]-IAA in the middle segment. Then the auxin active transport was calculated as the difference between the measured radioactivity in the middle segment and the diffused [^{3}H]-IAA in the middle segment.

Immunofluorescence analysis of HA-tagged AUX1

A female transgenic Arabidopsis parent homozygous for *HA-AUX1* (tested by kanamycin resistance of all progeny) was crossed with a 35S*::OsAGAP* transgenic male parent (Zhuang *et al.*, 2005). The F_1 progeny were distinguished by PCR with specific primers from the *OsAGAP* sequence (5′-CCAGCCAGGAGAAATCCA-3′ and 5′-ACAAATGAACCAAGTTAACA-3′), and the identification of HA-tagged AUX1 was bypassed because of the homozygous female parent. HA-tagged AUX1 immunolocalization was performed as described by Swarup *et al.* (2001). Fixed seedlings of 5-day-old Arabidopsis were incubated with anti-HA antibody (1:200) (Clontech, Mountain View, CA, USA) and then with antirabbit FITC-conjugated secondary antibody (1:10) (Biodee, Beijing, China). To visualize the AUX1-specific signals (green) in different epidermal cells, confocal images of epidermal root cells were overlayed.

The progeny of the cross between over-expressing *OsAGAP* transgenic Arabidopsis (homozygous) and *PIN1-YFP* (or *PIN2-GFP*) transgenic lines was used in the experiments. A female transgenic Arabidopsis parent homozygous for 35S*::OsAGAP* whose progeny were identified by kanamycin resistance was crossed to a *PIN1-YFP* (or *PIN2-GFP*) transgenic male parent. The F_1 progenies were distinguished by fluorescence of YFP (or GFP), and the identification of *OsAGAP* was bypassed because of the homozygous female parent.

For confocal microscopy images, a Leica SP2 confocal microscope (Wetzlar, Germany) was used. PI (20 mg ml^{-1}) in distilled water was used to stain the cell walls of living root cells (red signal).

Transient subcellular localization

The ORF cDNA of *OsAGAP* was ligated with *Xba*I and *Kpn*I double-digested pGFP221 to create pGFP-OsAGAP, in which the cDNA region covering the ORF of *OsAGAP* is fused in-frame to the N-terminus of the green fluorescent protein (GFP), under the control of the CaMV 35S promoter. Cells in the epidermal layers of onion bulbs were transformed by particle bombardment as previously described (Han *et al.*, 2005), using gold particles (Bio-Rad, Hercules, CA, USA) was coated with pGFP-221 as control plasmid DNA or with pGFP-OsAGAP. Twenty-four hours after bombardment, GFP fluorescence in onion epidermal cells was visualized under a Nikon fluorescence microscope (Kawasaki, Kanagawa, Japan).

RNA in situ *hybridization*

In situ hybridization of rice with digoxigenin-labeled probes was performed as described by Xu *et al.* (2001). A specific sequence of *OsAGAP* was amplified as probe using the primers 5′-CTCTCAAACTGCAAGCGT-3′ and 5′-CAGCTCCTGCACCT-3′ in PCR. The fragment was cloned into the T-easy vector (Madison, WI, USA) for labeling. The sense and antisense probes were synthesized with a linear plasmid according to DIG RNA labeling kit manual (Roche, Mannheim, Germany). Tissues (1 cm) were fixed overnight (12 h) in RNase-free FAA. Samples were dehydrated in a graded ethanol series and embedded in Paraplast Plus (Sigma, St Louis, MO, USA) as described previously (Xu *et al.*, 2001).

FM4-64 fluorescence analysis in live roots

Fluorescence dyeing with FM4-64 (Molecular Probes, Eugene, OR, USA) of 6-day-old seedling root tips was performed as described previously (Ueda *et al.*, 2001). Rice seedlings were incubated with 5 μg ml^{-1} FM4-64 for 10 min on ice, washed twice, and observed under a confocal microscope (Leica-TCS SP2) at 514 nm, and images were taken.

Transmission electron microscopy

The general procedure for conventional thin sectioning of chemically fixed root samples was essentially as described previously (Byers and Goetsch, 1975; Johnston *et al.*, 1991) Root tips germinated on MS medium for 4 days were dissected and fixed in 2.5% glutaraldehyde in 100 mM phosphate buffer, pH 7.2, for 2 h at room temperature. The fixed roots were embedded in Spurr's resin (Sigma). Ultra-thin sections were mounted on Forvar-coated grids (Sigma) and stained with 2% aqueous uranyl acetate and lead citrate, and observed under an electron microscope (JEOL 1210; Tokyo, Japan) at 80 Kv (Wang *et al.*, 2004).

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Identification of the progeny of crosses between the transgenic *OsAGAP* Arabidopsis line and the transgenic *HA-AUX1* line.

Figure S2. Effect of IAA on subcellular localization analysis of OsAGAP using a transient transfection assay.

This material is available as part of the online article from http://www.blackwell-synergy.com.

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