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OsAGAP, an *ARF-GAP* from rice, regulates root development mediated by auxin in *Arabidopsis*

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ABSTRACT

Arf (ADP-ribosylation factor) proteins, which mediate vesicular transport, have little or no intrinsic GTPase activity. They rely on the action of GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) for their function. In the present study the *OsAGAP* gene in rice, which encoded a protein with predicted structure similar to ArfGAP, was identified. The purified OsAGAP-GST fusion protein was able to stimulate the GTPase activity of rice Arf. Furthermore, OsAGAP can rescue the defect of vesicular transport in the yeast *gcs1*Δ*glo3*Δ double-mutant cells. Transgenic *Arabidopsis* with *OsAGAP* constitutively expression showed reduced apical dominance, shorter primary roots, increasing number of longer adventitious roots. Many of the phenotypes can be phenocopied by treatment of exogenous indoleacetic acid level (IAA) in wild-type plants. Determination of whole-plant IAA level showed that there is a sharp increase of free IAA in *OsAGAP* transgenic *Arabidopsis* seedlings. In addition, removal of the 4-day-old shoot apex could inhibit the adventitious root formation in the transgenic seedlings. These results suggest OsAGAP, an *ARF-GAP* of rice, may be involved in the mediation of plant root development by regulating auxin level.

Key-words: *Arabidopsis*; ArfGAP; auxin; decapitation; indoleacetic acid level; vesicular transport.

INTRODUCTION

The Arfs (ADP-ribosylation factors) represent a subfamily of GTP-binding proteins, within the Ras superfamily (Chavrier & Goud 1999; Randazzo *et al*. 2000). Arfs are considered inactive in their GDP bound state and active when binding GTP. This cycling between GTP bound and GDP bound states is catalysed by GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), respectively (Donaldson & Jackson 2000). ArfGAPs are responsible for the inactivation of Arfs by catalysing hydrolysis of GTP to GDP, while GEFs contribute to the opposite conversion (Furman *et al*. 2002; Nie *et al*. 2002; Yanagisawa *et al*. 2002). Arfs participate in the formation of transport vesicles from donor compartments and the selection of transmembrane protein cargo for incorporation into the vesicles (Poon *et al*. 1999, 2001; Yang *et al*. 2002). As other small GTP-binding proteins, Arfs have no or little intrinsic GTPase activity. They rely on the help of GAPs and GEFs to exhibit their activity (Spang 2002).

A series of ArfGAPs have been reported, such as GCS1, GLO3, Age2, SAT in yeast, AGAP1, DEF-1/ASAP1, ARAPs in mammal cells, ZAC in *Arabidopsis* (Dogic *et al*. 1999; Jensen *et al*. 2000; Poon *et al*. 2001; Furman *et al*. 2002; Krugmann *et al*. 2002; Miura *et al*. 2002; Nie *et al*. 2002, 2003; Oda *et al*. 2003). The main characteristic of ArfGAPs is the $CX_2CX_{16}CX_2C$ zinc finger motif which is critical for the GTPase-activating activity (Poon *et al*. 1999; Jensen *et al*. 2000). ArfGAPs have been demonstrated to play important roles in the membrane trafficking and actin cytoskeleton. They control the generation of transport vesicles, proper packaging of cargos and the efficient delivery of vesicles to the target membrane (Springer, Spang & Schekman 1999). In yeasts, GCS1, GLO3 and AGE2 have an overlapping essential function at the endoplasmic reticulum (ER)-Golgi stage of vesicular transport. Their deletion would lead to growth arrest, impaired invertase transport and endocytosis that are characteristic of defective secretory pathway (Poon *et al*. 1999, 2001). In plants, ZAC as an ArfGAP, isolated from *Arabidopsis*, was studied only in the expression patterns. It was mainly expressed in the flowering tissue, rosettes and roots. ZAC protein has been shown to fractionate with Golgi and plasma membrane marker proteins and demonstrate affinity to phospholipids (Jensen *et al*. 2000). Little, however, is known about its physiological role.

Auxin plays a critical role in the regulation of plant growth and development (Davis 1995), such as phototropism, gravitropism (Rashotte *et al*. 2000; Parry *et al*. 2001), apical dominance, lateral root development (Celenza, Grisafi & Fink 1995; Laskowski *et al*. 1995), embryogenesis (Liu, Xu & Chua 1993). The auxin level of tissues have been reported to modulate the root formation, based on indirect evidence from physiological and genetic studies. Auxin is synthesized within young apical tissues (shoot apex, young leaves and developing seeds), then conveyed to the basal target tissues by employing polar transport (Normanly, Cohen & Fink 1991; Lomax & Rubery 1995). Cellular auxin levels can be regulated by several processes such as synthe-

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sis, degradation, or transport and *sur1* mutants developed an increased number of adventitious roots and lateral roots. Elevated indoleacetic acid (IAA) level was responsible for the 'super root' phenotype (Boerjan *et al*. 1995) and altered auxin response mutants, *axr3*, generated an increased number of adventitious rooting (Leyser, Pickett & Dharmasiri 1996). There are also a group of *alf* mutants which have significant defects in root development (Celenza *et al*. 1995). *alf1–1* made an increased number of lateral roots and adventitious roots, whereas *alf4–1* failed in lateral root formation. Analysis of these mutants showed IAA is required for initiation and promotion of cell division in root development (Celenza *et al*. 1995).

In the present study *OsAGAP*, an *ArfGAP* from rice, was characterized by heterologous expression in *Arabidopsis*. Phenotypes of transgenic *Arabidopsis* corresponded to those of auxin effects. In addition, the free IAA level in *OsAGAP* transgenic plants was elevated by about 50-fold. Molecular and physiological analysis suggested that *OsAGAP* promoted the root formation mediated by auxin.

MATERIALS AND METHODS

Sequence amplification and reverse transcriptase-polymerase chain reaction analysis

RNA of *Oryza Sativa* cv. Zhonghua 10 was prepared according to a protocol from Invitrogen Co. (Carlsbad, CA, USA). Fresh tissue was ground in liquid nitrogen. The resulting powder was incubated in TRIZOL reagent (Invitrogen Co.) and then precipitated with isopropyl alcohol. RNA pellet was washed with 75% ethanol, air-dried for 5–10 min and dissolved in RNase-free water. cDNA of *OsAGAP* was cloned using an reverse transcriptase (RT)-polymerase chain reaction (PCR) kit from Promega Corporation (Madison, WI, USA) with primers CCA GCC AGG AGA AAT CCA and ACA AAT GAA CCA AGT TAA CA based on sequence information of our unique cDNA database. Identification of transgenic seedlings was performed with the same primers and RT-PCR protocol.

Fusion protein preparation and GTPase activity assay

A cDNA of rice *ARF* gene (GenBank accession number D17760) was amplified using RT-PCR strategy. Oligo nucleotides with *Eco*RI-linker (AGG AAT TCA GGA TCC TCA TGG TCG G) and ones with *Sal*I-linker (ATG TCG ACA GCC TTG TTG GCA ATG T) were used as primers. Both full-length cDNA of *OsAGAP* and *Arf* were cloned into pGEX-4T-1, respectively. The constructions were transformed into *Escherichia coli* strain BL21 and grown at 37 °C. The GST fusion proteins were purified by glutathione affinity chromatography as described in the Bulk and RediPack GST purification kit from Pharmacia (New York, NY, USA). GTPase activity assays were performed with Enzcheck™ Phosphate Assay Kit (Eugene, OR, USA). The rate of the phosphate released in the reaction was monitored as the GTPase activity. The reaction mixture included 1 μM Arf-GST fusion protein, 0.2 mM GTP, 0.2 mL 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG), 10 μL purine nucleotide phosphorylase, and HEPES buffer. It was incubated at 22 °C for 20 min and then 250 nM OsAGAP-GST protein was added to the reaction and absorbance at 360 nm was monitored immediately. The GTPase activity of reaction mix without OsAGAP-GST fusion protein was considered as a reaction control (Wu, Li & Yang 2000)

Yeast transformation and transmission electron microscopy observation

The yeast cells were cultured as described by Poon (Poon *et al*. 1999). The open reading frame (ORF) of *OsAGAP* was inserted into the *Aha*II and *Bam*HI sites of vector YEP352 driven by GCS1 promoter, in the sense and antisense orientations, respectively. The sense construct was transformed into yeast *gcs1*Δ*glo3*Δ double-mutant cells for complementation according to the protocol from Clontech Co. (Palo Alto, CA, USA). and the antisense construct was introduced into wild-type yeast cells. After incubation in the glucose medium for 10 h, cells were centrifuged for 5 min at 1000 *g* and fixed in glutaraldehyde. The fixed cells were processed for electron microscopic analysis as described (Byers & Goetsch 1975; Johnston, Prendergast & Singer 1991).

Vector construction and *Arabidopsis* transformation

The cDNA of *OsAGAP* was cloned into *Xba*I and *Bam*HI sites in the sense orientation in the pBI121 GUS expression vector. The construct was transformed into *Arabidopsis* with *Agrobacterium tumefaciens* GV3101 by vacuum filtration (Bechtold, Ellis & Pelletire 1993). Seeds from the transformed plants were surfaced sterilized with 10% NaClO (hypochlorite sodium) and selected by growth onto 1/2 MS agar (3% sucrose and 0.3% gelrite, PH 5.8) plates with 50 mg L^{-1} kanamycin. Plates with *Arabidopsis* seeds were stored in darkness at 4 °C for 48 h and then grown at 21 °C with a 16-h photoperiod.

Scanning electron microscopy observation

The plant materials were pre-treated according to a method described by Chen, Wang & Huang (2000). The shoot apex was fixed in FAA (50% ethanol, 5% acetic acid, 37% formaldehyde) at 4 °C for 2 h and then dehydrated through a graded alcohol series of 70, 85 and 95% ethanol, changed to 100% ethanol twice. After mounted on scanning electron microscope (SEM) stubs, the organs were dissected using glass needles. The mounted specimens were sputter-coated with gold and palladium (4 : 1) and examined with a SEM (Hitachi S-2460; Tokyo, Japan).

IAA quantification

The IAA level of 3-week-old whole plants was determined by high-performance liquid chromatography (HPLC) analysis. Fresh materials were carefully weighed and ground in liquid nitrogen. The resulting powder was extracted in methanol. The extract was subjected to HPLC (Novapak C18-HPLC column, 0.4 cm × 15 cm) analysis. The fraction was eluted with 15%CH_3CN–25%CH_3OH–60%H_2O (maintained at pH 3.5 with H_3PO_4) at a speed of 0.7 mL every minute and detected at UV 254 nm.

Gravitropic response analysis

Seeds were germinated as described above. The plates with germinated seeds were placed vertically to allow the roots to grow uprightly. Ten days later the plates were then turned through 90°. Angles of roots bending downward were measured and analysed every 2 h.

Decapitation of *Arabidopsis* seedlings

Arabidopsis seedlings was decapitated by removal of the shoot apex at 4 and 7 d old, respectively, and continued to cultivate for another 5 d. Root development was monitored and photographed.

RESULTS

Characterization of *OsAGAP* gene from rice

The cDNA encoding zinc finger proteins were screened using the conserved domain in our unique rice cDNA database (Lan *et al*. 2004). A cDNA encoding the $CX_2CX_{16}CX_2C$ type zinc finger protein was discovered which consisted of 1328 nucleotides. Sequence analysis in GenBank (http://www.ncbi.nlm.nih.gov) revealed genomic DNA corresponding to the cDNA contained five exons and four introns. All of the introns were located in the ORF and none in the uncoding region.

Its predicted ORF encoded a 320 amino acid peptide with a calculated molecular mass of 32.4 kDa. The putative protein was constituted by zinc finger domain and C2 domain (Fig. 1a). The $CX_2CX_{16}CX_2C$ zinc finger domain was characteristic of ArfGAPs (Arf GTPase activating protein) (Poon *et al*. 1999; Jensen *et al*. 2000). Thus the gene corresponding to the cDNA was termed as *OsAGAP*. As the polygenetic tree showed, the OsAGAP protein displayed the highest homology with predicted proteins in *Arabidopsis*, most of which encoded zinc finger domain protein or putative GTPase activating protein (Fig. 1b). Among them, only ZAC, an ArfGAP in *Arabidopsis*, has been studied (Jensen *et al*. 2000). OsAGAP shared 59.35% homology with ZAC in the protein sequence. Furthermore, both proteins are composed of the zinc finger and C2 domain, which was distinct from ArfGAPs of other organisms (Poon *et al*. 1996; Jensen *et al*. 2000; Vitale, Moss & Vaughan 2001).

Identification of GTPase activating activity of OsAGAP protein

In *Arabidopsis*, ARF1 was used to test the GTPase activity of ZAC (Jensen *et al*. 2000). A similar approach was taken with *ARF (*accession number D17760) in rice which shared 97.79% homology with *Arabidopsis ARF1* in protein sequence. Then, GST fusion proteins of OsAGAP and ARF were expressed in *E. coli* cells, respectively. After induction by isopropyl β-D-thiogalactoside (IPTG), recombinant proteins with either 69 or 47 kDa, appeared in the cell-soluble fraction of *E. coli* cells, respectively (Fig. 2a, lane 3 and 5). The two recombinant proteins were further purified by affinity chromatography (Fig. 2a, lane 4 and 6). Stimulation of GTPase activity by OsAGAP was detected as the rate of phosphate released from the reaction mixture. As OsAGAP-GST fusion protein was added, an increasing amount of phosphate was observed to be released from the reaction rapidly. In contrast, the blank reaction showed little response to stimulation of OsAGAP (Fig. 2b). These results suggest that OsAGAP could stimulate the GTPase activity of ARF *in vitro*.

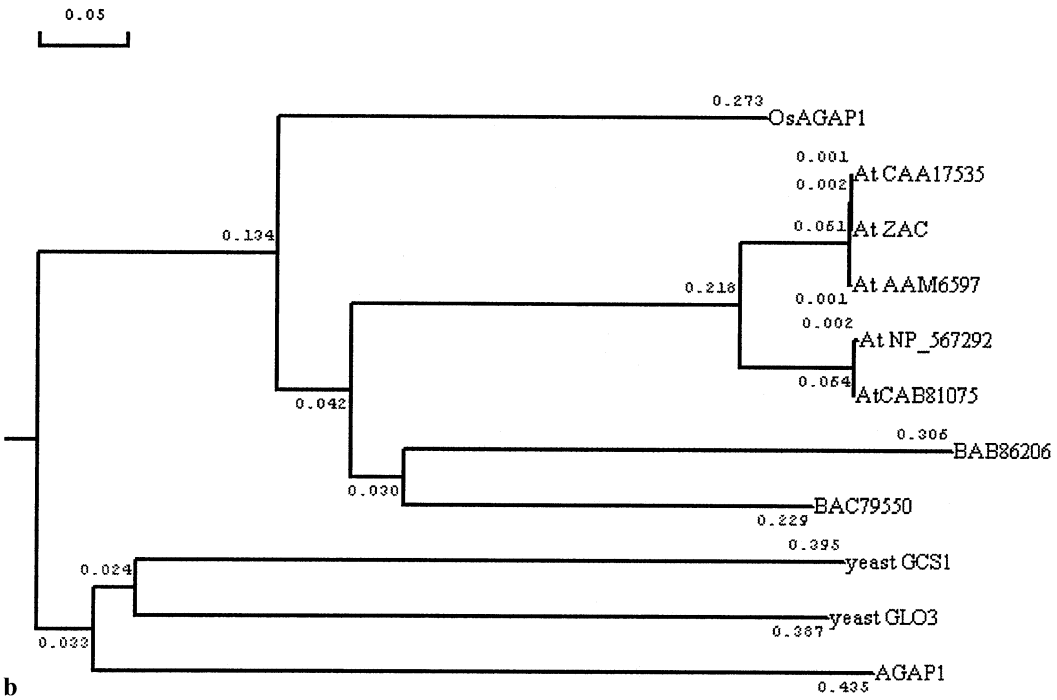
GCS1 and GLO3 were identified as GAPs for Arf protein in yeast (Ireland *et al*. 1994; Poon *et al*. 1996). The *gcs1*Δ*glo3*Δ double-mutant cells ceased cell division when transferred to glucose-containing medium. When observed using the electron microscope, the mutant cells transferred to glucose medium contained highly elaborated endoplasmic reticulum (ER). This accumulation of ER is an indication of disrupted vesicular transport between the ER and Golgi membranes (Novick, Field & Schekman 1980; Poon *et al*. 1999). As a test of its ability to restore vesicular transport, *OsAGAP* was transformed into yeast *gcs1*D*glo3*D double-mutant cells. As shown in Fig. 3a–c, double-mutant cells expressing *OsAGAP* appeared morphologically indistinguishable from wild-type cells displaying a morphology typical of proliferating cells. On the other hand, wild-type yeast cells harbouring antisense *OsAGAP* displayed obvious membranous structures which resembled *gcs1*Δ*glo3*Δ double-mutant cells (Fig. 3d). So *OsAGAP* was able to rescue the defect of yeast *ARF-GAP* knockout mutant. The results showed that OsAGAP could serve as an ARF-GAP for yeast vesicular transport between the Golgi and ER.

Identification and characterization of *OsAGAP* transgenic *Arabidopsis*

Little physiological function of ArfGAPs is known although there are many reports on their biochemical role. Full-length cDNA of *OsAGAP* driven by CaMV 35S promoter was transformed into *Arabidopsis* to evaluate the significance of ArfGAPs in plant. Twelve transgenic lines were generated and were confirmed with inserts from *OsAGAP* by PCR and Southern-blot analysis (data not shown). RT-PCR was used to test the transcription of *OsAGAP* in transgenic *Arabidopsis*. Corresponding to the transcript of *OsAGAP*, a single band of 1.3 kb was identified in the transgenic lines. In contrast, it was absent in wild type

1 MSGRRDDSAK **MAKLKELLHR SENCICADCS APDPKWASAN IGVFICLKCS GVHRSLGTHI**
61 **SKVLSVTLDQ WSDNEIDNVI EVGGNSHANA IYEAFLPQNH SKPHPDSTQE EREKFIRSKY**
121 **ELQEFLEPSL RIVSHQSSDS** GKHAGSASHS VSSKSEVGMI EFIGILNVKV KGGTNLAIRD
181 MSSSDPYVVL TLGQQKAQTS VIKANLNPVW NEELKLSVPQ QYGPLKLQAF DHDMLSKDDL
241 MGEAEIDLQP MINAAAAFGD PGLLGDRQIG RWLKSGDNAL VRDSAVVVPG GEVRQELALK
301 LQFTESGEVE LEMQWFPLNM

a



b

Figure 1. Analysis of *OsAGAP* gene from rice. (a) Deduced amino acid sequence of the OsAGAP protein. Zinc finger domain is in bold characters. The characters boxed indicate the four 'Cys' composed of zinc finger structure. C2 domain is underlined. (b) Polygenetic relationship of ArfGAPs from different organisms. The numbers in the polygenetic tree indicate the branch lengths. The species and corresponding references are as follows. AtAAM65970 is a putative GTPase activating protein in *Arabidopsis*; AtZAC (accession number NP_849416) is an Arf GTPase activating protein in *Arabidopsis*; AtCAA17535 and AtCAB81075 are putative proteins from *Arabidopsis*; AtNP_567292 is a zinc finger domain and C2 domain protein isolated from *Arabidopsis*; Both of BAB86206 and BAC79550 are zinc finger domain and C2 domain proteins in rice. AGAP1 (accession number NP_055729) is an Arf GTPase activating protein in mammal cells. GCS1 (accession number S47006) and GLO3 (accession number CAA56046) are ArfGAPs in yeast cells.

(Fig. 4). The result showed *OsAGAP* was expressed constitutively in the transgenic *Arabidopsis*.

As shown in Fig. 5a, *OsAGAP* transgenic *Arabidopsis* displayed epinastic cotyledons during the seedling stage. Adult plants exhibited reduced apical dominance with longer lateral shoots and more inflorescence stems (Fig. 5b & c). In addition to the sticking stems, multiple new buds appeared in the cluster of rosette leaves which were not able to develop into mature inflorescence (Fig. 5d). Primordia in the leaf axils were observed in the transgenic plants, although none occurred in the wild plants (Fig. 5e & f). Both the epinastic cotyledon and the reduced apical dominance was reminiscent of auxin effects. Furthermore, the transgenic plants developed smaller flowers and had poorer fertility. Under the dissecting microscope, it was found that filaments of the transgenic lines did not elongate enough to reach the stigma (Fig. 5g).

Seeds of *OsAGAP* transgenic lines were germinated on 1/2 MS medium under the light. The transgenic seedlings grew a shorter primary root than that of wild type (Fig. 5a). Two kinds of root phenotypes were discovered in *OsAGAP* transgenic line1 (*OsAGAP-1*) and line2 (*OsAGAP-2*). An increased number of prolonged adventitious roots were developed in *OsAGAP-1* transgenic seedlings compared to wild type (Fig. 6a & d) and longer adventitious and lateral roots occurred in *OsAGAP-2* seedlings (Fig. 6a & e). The phenotype of the transgenic roots could be phenocopied by treatment of IAA (10^{-7}–10^{-6} M) in wild-type plants (data not shown). This result suggested that abnormal roots of *OsAGAP* transgenic seedlings may be related to IAA level.

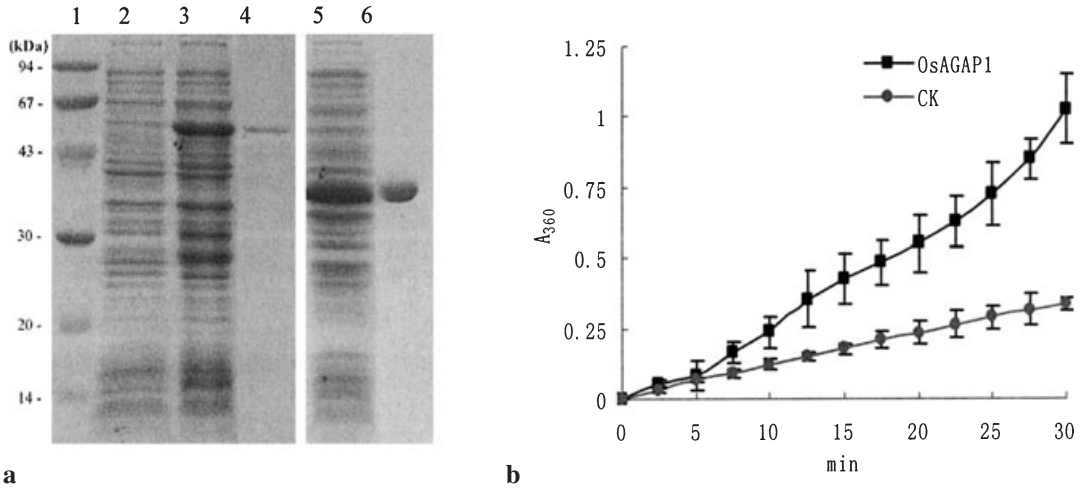


Figure 2. Protein expression and GTPase activating activity assay of OsAGAP. (a) Expression and purification of OsAGAP-GST and Arf-GST recombinant protein. Lane 1, molecular-mass markers. Lane 2, total proteins fraction of *E. coli* BL21 strain cells. Lane 3, total proteins of BL21 cells containing pGST-OsAGAP-4T-1 plasmid. Lane 4, OsAGAP-GST fusion protein purified by glutathione-Sepharose 4B chromatography. Lane 5, total proteins of BL21 cells containing pGST-Arf-4T-1 plasmid. Lane 6, Arf-GST purified fusion protein. (b) Dynamic curve of the GTPase activating activity of OsAGAP. In the control reaction (dot symbols), GTP and Arf-GST recombinant protein were involved, without OsAGAP-GST fusion protein. It represented the background of the GTPase activity assay. The GTPase activating activity of OsAGAP is indicated by solid diamonds.

The gravitropic response was studied with the wild-type and *OsAGAP* transgenic roots. The transgenic roots showed slower and smaller gravitropic curvature than wild type during the time-course study (Fig. 7b). On average, roots of wild type showed an angular turn of 47° after 6 h gravity stimulation, whereas an angle of 28° appeared in the transgenic roots (Fig. 7a & b). The result suggested that *OsAGAP* transgenic seedlings were more insensitive to gravity stimulation.

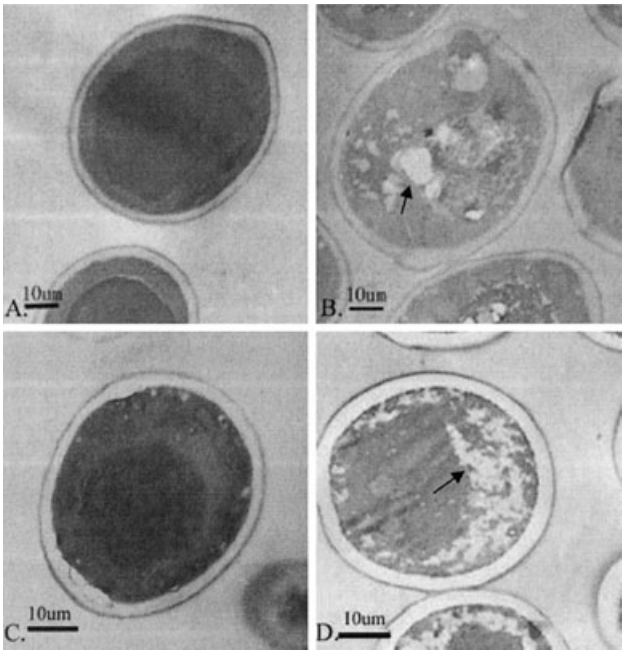


Figure 3. TEM observation of yeast cells. (a) Wild-type yeast cells; (b) *gcs1*Δ*glo3*Δ double-mutant cells; (c). *gcs1*Δ*glo3*Δ double-mutant cells transformed with *OsAGAP*; (d) wild-type cells transformed with antisense *OsAGAP*. Arrows in (b) and (d) indicate the elaborated membrane structures in yeast cells.

Physiological analysis of *OsAGAP* transgenic *Arabidopsis*

Effects of 3,3,5-triiodobenzoic acid (TIBA) on *OsAGAP* transgenic plants were investigated. Roots in both of transgenic and wild-type plants were reduced by 45% under treatment of 10^{-7} M TIBA (Fig. 8b). That's to say, the transgenic plants showed the same sensitivity to TIBA treatment as the wild-type plants. Furthermore, as Fig. 8a shows, the number of adventitious roots was greatly decreased in *OsAGAP-1* and longer adventitious and lateral roots disappeared in *OsAGAP-2* plants.

Exogenous IAA was used to treat the transgenic plants and wild type *Arabidopsis*. The growth of roots in wild type was greatly inhibited by IAA (5×10^{-7} M). Roots of trans-

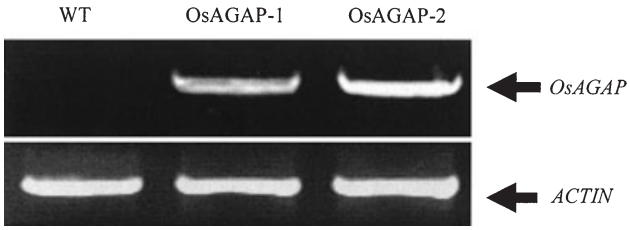


Figure 4. Reverse transcription polymerase chain reaction (RT-PCR) analysis for *OsAGAP* transcript in transgenic *Arabidopsis*. Actin was amplified as a loading control. Lane 1, wild-type *Arabidopsis*. lanes 2, 3, independent *OsAGAP* transgenic lines.

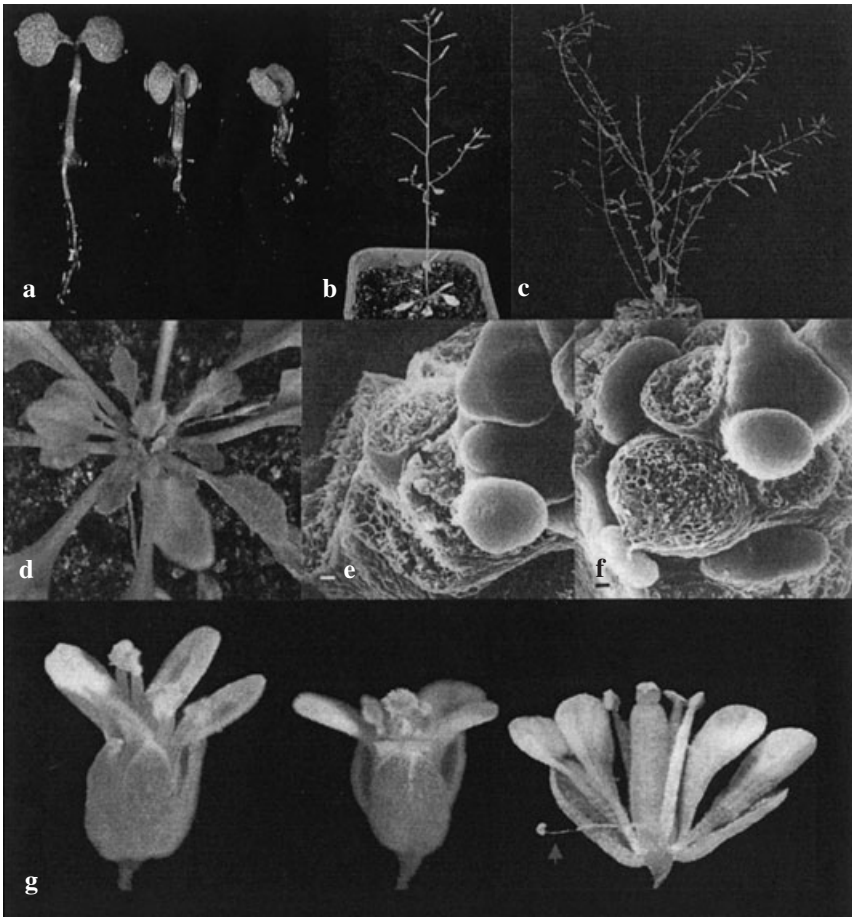


Figure 5. Phenotype of *OsAGAP* transgenic *Arabidopsis* .(a) Seedlings grown for 6 d under light. From left: wild-type, *OsAGAP*-1, *OsAGAP*-2. (b, c) Adult plants of wild-type (b) and *OsAGAP* transgenic seedlings (c). (d) The acervate new shoots around the bolting stem. Red arrows indicate the new buds embedded in the rosette leaves. (e, f) SEM observation of shoot meristem and lateral meristems in wild type (e) and *OsAGAP* transgenic plants (f). Arrows in (e) indicate the area of leaf axils. Arrows in (f) show the primordia in the leaf axils. (g) Comparison of wild-type and transgenic flowers. From left: wild-type, *OsAGAP*-1, *OsAGAP*-2. Arrow shows the defective stamen.

genic seedlings, however, showed no additional response (Fig. 8b). In addition, the transgenic seedlings also displayed decreased sensitivity to 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene biosynthesis. The growth of *OsAGAP* transgenic root was not inhibited as much as wild type by ACC (10^{-7} M) (Fig. 8b), whereas, root growth inhibition by ABA and promotion by GA were not affected in the transgenic plants (data not shown).

Whole plants of *OsAGAP* transgenic and wild-type seedlings were harvested and subjected to IAA quantification. As shown in Table 1, free IAA level in transgenic seedlings with different lines was 65 or 48.5 nM g^{-1} FW (corresponding to 250 or 194 nM/100 seedlings) which was mor than 50-fold the level of wild-type plants (1 nM g^{-1}.FW or 5 nM/100 seedlings). So the increased level of endogenous free IAA might contribute to the phenotypes of *OsAGAP* transgenic seedlings. To investigate this possibility, the source of IAA synthesis was removed by excision of shoot apex. As the IAA pulse always reaches the root between 5 and 7 d after germination (Bhalerao *et al*. 2002), we decapitated 4- and and 7-day-old seedlings, respectively, and observed the development of roots. The excised seedlings were continued in cultivation until the intact plants had developed lateral and adventitious roots. In the case of seedlings decapitated at 4 d, no adventitious and lateral roots were formed in either of the decapitated *OsAGAP-1* and *OsAGAP-2* transgenic seedlings (Fig. 6b). In contrast, transgenic seedlings excised at 7 d developed extended adventitious roots, and wild-type decapitated plants fully developed lateral roots (Fig. 6c). The result suggested the formation of excess adventitious roots in the transgenic *Arabidopsis* was induced by the high level of shoot-derived IAA.

Table 1. HPLC quantification of free IAA in wild-type and *OsAGAP* transgenic *Arabidopsis*

	Free IAA	
	nM g^{-1} FW	nM per100 seedlings
Wild type	1	5
OsAGAP-1	65	250
OsAGAP-2	48.5	194

Measurements were performed as described in Methods. For each series of measurements, 50 individual plants were analysed.

DISCUSSION

OsAGAP functions as an ArfGAP

OsAGAP was identified as a GTPase activating protein for Arf proteins in rice by two approaches (Figs 2 & 3). OsAGAP shared the conservative zinc finger domain with other members of ArfGAP family. The $CX_2CX_{16}CX_2C$ type

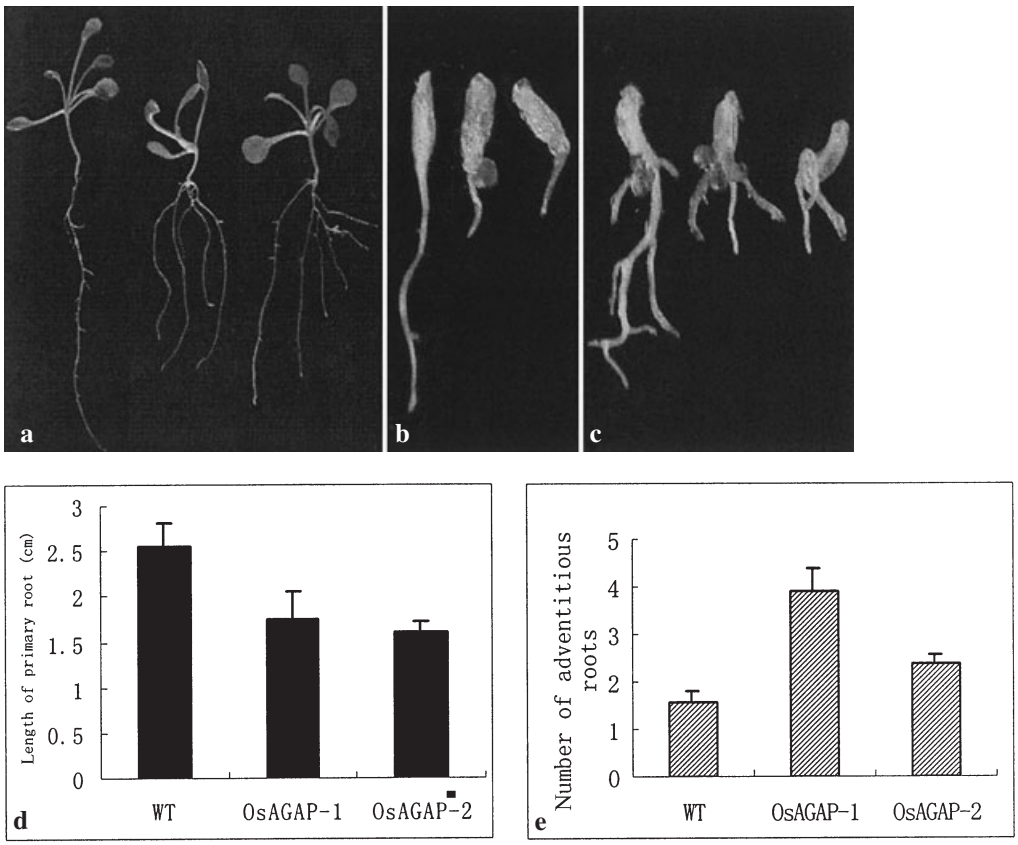


Figure 6. Roots of transgenic *Arabidopsis* seedlings. (a) Phenotype of transgenic *Arabidopsis* roots. From left: wild-type, *OsAGAP*-1, *OsAGAP*-2. (b, c) Roots of seedlings decapitated at 4 d (b) and 7 d old (c). From left: wild-type, *OsAGAP*-1, *OsAGP*-2. (d, e) Statistics of the length of primary roots (d) and the number of adventitious roots (e) in transgenic seedlings.

domain was always arrayed with distinct C-terminal domains in different family members which were expected to be important for regulating ArfGAPs localization and activity (Antonny *et al*. 1997; Huber *et al*. 2002). Distinct from ArfGAPs in other organisms, OsAGAP protein was constituted by zinc finger and C2 domain. The C2 domain is typically found in multi-domain proteins involved in signal transduction or membrane trafficking (Sutton *et al*. 1995; Kopka *et al*. 1998; Zhang, Rizo & Sudhof 1998). Many of them could bind negatively charged phospholipids (Jensen *et al*. 2000). Although both the zinc finger and C2 domain had been found separately in proteins involved in membrane trafficking or signal transduction, they were rarely combined together (Jensen *et al*. 2000). The domain combination was only ever found in *Arabidopsis* (Jensen *et al*. 2000) and rice (Fig. 1).

ArfGAPs have been reported to be responsible for the vesicular transport by regulating the activity of Arfs (Boman & Kahn 1995). GCS1 and GLO3 were two ArfGAPs isolated from yeast, which were able to stimulate the GTPase activity of the yeast ARF1 protein (Poon *et al*. 1996, 1999). Depletion of both *GCS1* and *GLO3* resulted in the accumulation of membrane structures which indicated the disruption of vesicular transport between the ER and Golgi membranes. As yeast ArfGAPs, OsAGAP was able to rescue the defect of membrane structure in the *gcs1*Δ*glo3*Δ double-mutant cells (Fig. 3). The results indicated OsAGAP was also involved in the regulation of vesicular transport between ER and Golgi compartments which was consistent with ArfGAPs from other species.

OsAGAP is responsible for the root development mediated by auxin

Phenotypes of *OsAGAP* transgenic seedlings, such as epinastic cotyledons, reduced apical dominance, defect in gravitropism, were typical of auxin effect (Fig. 5). *OsAGAP* transgenic plants generated shorter roots with increasing numbers of adventitious and longer lateral roots, which could be phenocopied by treatment of wild-type *Arabidopsis* with exogenous auxin. Auxin has been shown to inhibit root elongation, stimulate adventitious rooting, mediate gravitropism (Katsumi, Chiba & Fukuyama 1969; Evans 1985; Feldman 1985). These phenotypes provided an evidence for a primary defect in auxin action of *OsAGAP* transgenic seedlings.

sur1, an auxin overproducing mutant, developed excess adventitious and lateral roots. The level of free IAA in the *sur1* mutants was increased up to four-fold (Boerjan *et al*. 1995). Tobacco transformed with *rolB* also developed

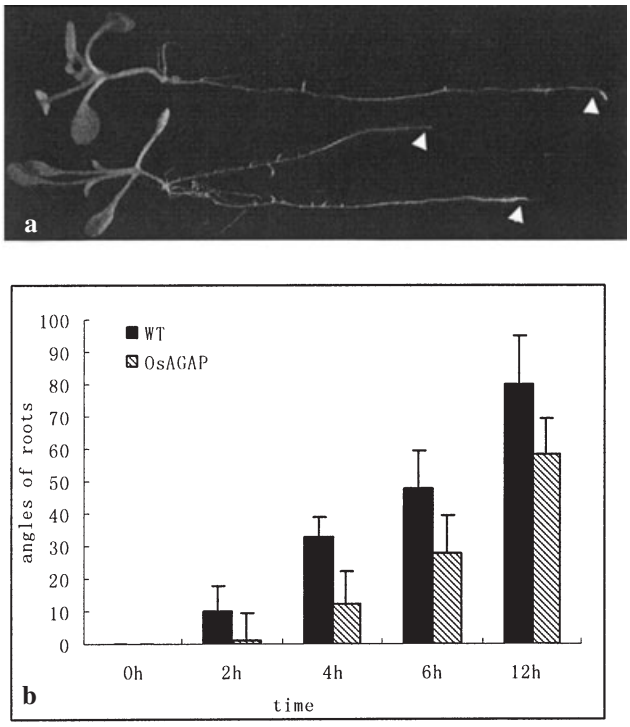


Figure 7. Gravitropic response of *OsAGAP* transgenic roots. (a) Root response after 6 h gravi-stimulation. From top: wild-type, transgenic roots. Arrows showed the turned root tips. (b) Time-course study of the gravitropic response in transgenic roots.

numerous roots, but neither free IAA nor conjugated IAA level was modified in the transformed plants (Nilsson, Crozier & Schmulling 1993; Delbarre, Muller & Imhoff 1994). It was the greatly increased sensitivity to IAA that contributed to the hyperplasia of roots in transgenic tobacco (Maurel, Leblanc & Barber-Brygoo 1993). Furthermore, *OsAGAP* transgenic plants shared much similarity with *axr3* plants, such as an increasing number of adventitious roots, defect of gravitropism, resistance to IAA and ACC, no defect in the response to ABA (Leyser *et al.* 1996). *AXR3* gene was involved in modulating the level of response to auxin. In *axr3* mutants, the amplitude of response to auxin was much greater such that the plants over-reacted to the auxin stimulus (Leyser *et al.* 1996). It could be inferred that the increasing number of adventitious roots could result from enhanced auxin action.

Increased auxin activity in *OsAGAP* transgenic *Arabidopsis* may be attributed to several possible reasons, such as local auxin accumulation as a result of defective auxin transport, or increased sensitivity to auxin, or an elevated auxin level. First, the transgenic plants were as sensitive as wild type in response to the treatment of TIBA (Fig. 8a). It showed that there is no defect with auxin transport in the transgenic plants. Second, the sensitivity to exogenous IAA in transgenic plants had not been changed (Fig. 8b). Third, *OsAGAP* transgenic roots could be phenocopied by wild-type seedlings under the treatment of exogenous auxin. Fourth, IAA quantification affirmed the elevated level of free auxin in the transgenic *Arabidopsis* (Table 1). In addition, the development of adventitious roots in the transgenic seedlings could be inhibited by removal of the shoot apex at 4 d old, before shoot-derived auxin reached the root (Fig. 6b & c). Thus, it could be concluded that the phenotype of *OsAGAP* transgenic plants might result from a high level of shoot-derived endogenous auxin.

As proposed by Kaldeway (1984), if the amount of IAA to be transported from the apical part of the hypocotyl to its base exceeds the capacity of auxin transport, IAA will accumulate in the lower regions of the hypocotyl where it will exert its effects. This is the so-called 'barrier effect'. The theory may be used to explain the increasing amount of adventitious root development in *OsAGAP* transgenic seedlings. The greatly increased endogenous IAA production of *OsAGAP* transgenic seedlings will result in an accumulation and spillover of IAA from the transport system. The spillover would firstly occur at the basal part of the hypocotyl as a barrier effect (Boerjan *et al.* 1995). Then, the adventitious root formation at the joint of hypocotyl and primary root can be the result of an increased level of IAA, or a longer exposure to IAA in these cells. In addition, new primordia were found in the leaf axils of transgenic *Arabidopsis*, which might also be a result of IAA accumulation (Fig. 5c & d). TIBA is able to inhibit the basipetal transport of IAA from the shoot apex to the hypocotyl. It is conceivable that the accumulation of IAA at the base of hypocotyl may be relieved by TIBA. So, *OsAGAP* transgenic roots would lose long adventitious roots and resemble the wild type under the treatment of 10^{-7} M TIBA. The elevated IAA level can also explain the slower response to gravitropism in transgenic plants. Overproduction of IAA may saturate the auxin carriers. As a result, the ability to mediate auxin redistribution was decreased in the transgenic plants.

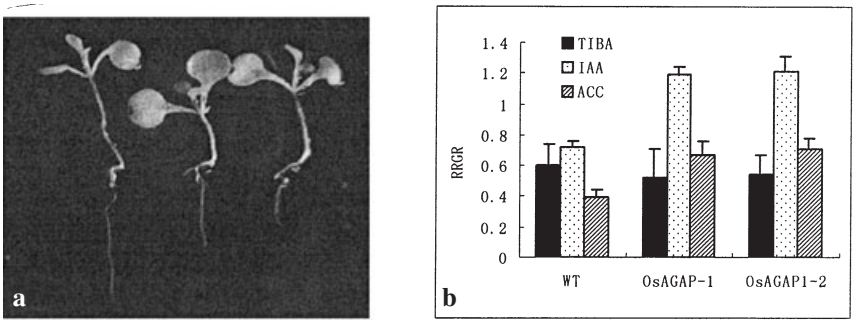


Figure 8. Analysis of *OsAGAP* transgenic roots treated with TIBA and hormones. (a) Morphology of wild type and *OsAGAP* transgenic seedlings. From left: wild type, *OsAGAP-1*, *OsAGAP-2*. (b). Effects of TIBA (10^{-7} M), IAA (5×10^{-7} M) and ACC (10^{-7} M) on the primary root growth rates of wild type and *OsAGAP* transgenic seedlings. RRGR (relative root growth ratio) represents the ratio of root elongation after treatment by TIBA, IAA and ACC to those before any treatment.

Roots may take more time or even unable to regulate the asymmetric redistribution of IAA in response to gravity. So *OsAGAP* transgenic roots showed an insensitive response to gravitropism (Fig. 7). Ethylene has been reported to be related to auxin transport and sensitivity to endogenous auxin in plants (Visser *et al*. 1996; Chen *et al*. 1998; Luschnig *et al*. 1998). The resistance to ACC in *OsAGAP* transgenic plant also confirmed the interaction between these two hormones.

ArfGAPs play a role in the mediation of auxin effects

Only a small amount of work on ArfGAPs has been reported in plants and so far, one ArfGAP, namely ZAC, has been investigated in plants. ZAC showed spatial expression in all tissues of the plants (Jensen, Hangarter & Estelle 1998). However, little of its detailed physiological function is known. OsAGAP has been demonstrated to be an ArfGAP in rice by biochemical and cell biological characters (Figs 2 & 3). *OsAGAP* transgenic *Arabidopsis* showed phenotypes typical of enhanced auxin action (Figs 5 & 6). Furthermore, IAA quantification confirmed a sharp increase of endogenous free IAA in transgenic plants (Table 1). It can be inferred that *OsAGAP* may mediate plant growth and development by regulating auxin level.

Both ArfGAPs and ArfGEFs exert their function by regulating the conversion between the Arf-GTP and Arf-GDP bound state. GNOM is an ArfGEF in *Arabidopsis* which has been shown to mediate auxin polar transport by regulating the polar localization of auxin transport carriers (Steinmann *et al*. 1999; Geldner *et al*. 2003). The result on *OsAGAP* suggests ArfGAPs in plants may be involved in the regulation of root development mediated by auxin.

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REFERENCES

Antonny B., Huber I., Paris S., Chabre M. & Cassel D. (1997) Activation of ADP-ribosylation factor 1 GTPase-activating protein by phosphatidylcholine -derived diacylglycerols. *Journal of Biological Chemistry* **272,** 30848–30851.

Bechtold N., Ellis J. & Pelletire G. (1993) In planta *Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis* plants. *C R Academy of Science Paris, Life Science* **316,** 1194–1199.

Bhalerao R.P., Eklof J., Ljung K., Marchant A., Bennett M. & Sandberg G. (2002) Shoot-derived auxin is essential for early lateral root emergence in *Arabidopsis* seedlings. *Plant Journal* **29,** 325–332.

Boerjan W., Cervera M.T., Delarue M., Beeckman T., Dewitte W., Bellini C., Caboche M., Van Onckelen H., Van Montagu M. & Inze D. (1995) Superroot, a recessive mutation in *Arabidopsis*, confers auxin overproduction. *Plant Cell* **7,** 1405–1419.

Boman A.L. & Kahn R.A. (1995) Arf proteins: the membrane traffic police? *Trends in Biochemistry and Science* **20,** 147–150.

Byers B. & Goetsch L. (1975) Behavior of spindles and spindle plaques in the cell cycle and conjugation of *Saccharomyces cerevisiae*. *Journal of Bacteriology* **124,** 511–523.

Celenza J.L. Jr, Grisafi P.L. & Fink G.R. (1995) A pathway for lateral root formation in *Arabidopsis* thaliana. *Genes and Development* **9,** 2131–2142.

Chavrier P. & Goud B. (1999) The role of ARF and Rab GTPases in membrane transport. *Current Opinions in Cell Biology* **11,** 466–475.

Chen R., Hilson P., Sedbrook J., Rosen E., Caspar T. & Masson P.H. (1998) The *Arabidopsis thaliana* AGRAVITROPIC 1 gene encodes a component of the polar-auxin-transport efflux carrier. *Proceedings of the National Academy of Sciences of the USA* **95,** 15112–15117.

Chen C.B., Wang S.P. & Huang H. (2000) LEUNIG *has multiple functions in gynoecium development in* Arabidopsis. *Genesis* **26,** 42–54.

Davis P.J. (1995) *Plant Hormones*. Kluwer Academic Publisher, Dordrecht, The Netherlands.

Delbarre A., Muller P. & Imhoff V. (1994) The rolB gene of agrobacterium rhizogenes does not increase the sensitivity of tobacco protoplasts by modifying the intracellular auxin concentration. *Plant Physiology* **105,** 563–569.

Dogic D., Chassey B., Pick E., Cassel D., Lefkir Y., Hennecke S., Cosson P. & Letourneur F. (1999) The ADP-ribosylation factor GTPase-activating protein Glo3p is involved in ER retrieval. *European Journal of Cell Biology* **78,** 305–310.

Donaldson J.G. & Jackson C.L. (2000) Regulators and effectors of the ARF GTPases. *Current Opinions in Cell Biology* **12,** 475–482.

Evans M.L. (1985) The action of auxin on plant cell elongation. *Critical Reviews in Plant Science* **2,** 317–365.

Feldman L.J. (1985) Root gravitropism. *Physiologia Plantarum* **65,** 341–344.

Furman C., Short S.M., Subramanian R.R., Zetter B.R. & Roberts T.M. (2002) DEF-1/ASAP1 is a GTPase-activating protein (GAP) for ARF1 that enhances cell motility through a GAP-dependent mechanism. *Journal of Biological Chemistry* **277,** 7962–7969.

Geldner N., Anders N., Wolters H., Keicher J., Kornberger W., Muller P., Delbarre A., Ueda T., Nakano A. & Jurgens G. (2003) The *Arabidopsis* GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. *Cell* **112,** 219–230.

Huber I., Cukierman E., Rotman M. & Cassel D. (2002) ARF GTPase-activating protein 1. *Methods in Molecular Biology* **189,** 199–206.

Ireland L.S., Johnston G.C., Drebot M.A., Dhillon N. & Demaggio A.J. (1994) A member of a novel family of yeast 'Zn-finger' proteins mediates the transition from stationary phase to cell proliferation. *EMBO Journal* **13,** 3812–3821.

Jensen P.J., Hangarter R.P. & Estelle M. (1998) Auxin transport is required for hypocotyl elongation in light-grown but not dark-grown *Arabidopsis*. *Plant Physiology* **116,** 455–462.

Jensen R.B., Lykke-Andersen K., Frandsen G.I., Nielsen H.B., Haseloff J., Jespersen H.M., Mundy J. & Skriver K. (2000) Promiscuous and specific phospholipid binding by domains in ZAC, a membrane- associated *Arabidopsis* protein with an ARF GAP zinc finger and a C2 domain. *Plant Molecular Biology* **44,** 799–814.

Johnston G.C., Prendergast J.A. & Singer R.A. (1991) The *Saccharomyces cerevisiae* MYO2 gene encodes an essential myosin

for vectorial transport of vesicles. *Journal of Cell Biology* **113,** 539–551.

Kaldeway H. (1984) *Transport and Other Modes of Movement of Hormones (mainly auxin)*. Springer Verlag, Berlin, Germany.

Katsumi M., Chiba Y. & Fukuyama M. (1969) The roles of the cotyledons and auxin in the adventitious root formation of hypocotyl cuttings of light-grown cucumber seedlings. *Physiologia Plantarum* **22,** 993–1000.

Kopka J., Pical C., Hetherington A.M. & Muller-Rober B. (1998) Ca2+/phospholipid-binding (C2) domain in multiple plant proteins: novel components of the calcium-sensing apparatus. *Plant Molecular Biology* **36,** 627–637.

Krugmann S., Anderson K.E., Ridley S.H., *et al.* (2002) Identification of ARAP3, a novel PI3K effector regulating both Arf and Rho GTPases, by selective capture on phosphoinositide affinity matrices. *Molecular Cell* **9,** 95–108.

Lan L.F., Chen W., Lai Y., *et al.* (2004) Monitoring of gene expression profiles and isolation of candidate genes involved in pollination and fertilization in rice (*Oryza sativa* L.) using a 10K cDNA microarray. *Plant Molecular Biology* **54,** 471–487.

Laskowski M.J., Williams M.E., Nusbaum H.C. & Sussex I.M. (1995) Formation of lateral root meristems is a two-stage process. *Development* **121,** 3303–3310.

Leyser H.M., Pickett F.B. & Dharmasiri S. (1996) Mutation in the *AXR3* gene of *Arabidopsis* result in altered auxin response including ectopic expression from the *SAUR-AC1* promoter. *Plant Journal* **10,** 403–413.

Liu C., Xu Z. & Chua N.H. (1993) Auxin polar transport is essential for the establishment of bilateral symmetry during early plant embryogenesis. *Plant Cell* **5,** 621–630.

Lomax T.L.M. & Rubery P.H. (1995) auxin transport. In *Plant Hormones – Physiology, Biochemistry and Molecular Biology* (ed. P.J. Davies), pp. 00–00. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Luschnig C., Gaxiola R.A., Grisafi P. & Fink G.R. (1998) EIR1, a root-specific protein involved in auxin transport, is required for gravitropism in *Arabidopsis* thaliana. *Genes and Development* **12,** 2175–2187.

Maurel C., Leblanc N. & Barbier-Brygoo H. (1993) Alterations of auxin perception in rolB- transformed tobacco protoplasts: Time course of rolB mRNA expression and increase in auxin sensitivity reveal multiple control by auxin. *Plant Physiology* **105,** 1209–1215.

Miura K., Jacques K.M., Stauffer S., Kubosaki A., Zhu K., Hirsch D.S., Resau J., Zheng Y. & Randazzo P.A. (2002) ARAP1: a point of convergence for Arf and Rho signaling. *Molecular Cell* **9,** 109–119.

Nie Z., Boehm M., Boja E.S., Vass W.C., Bonifacino J.S., Fales H.M. & Randazzo P.A. (2003) Specific regulation of the adaptor protein complex AP-3 by the Arf GAP AGAP1. *Developmental Cell* **5,** 513–521.

Nie Z., Stanley K.T., Stauffer S., Jacques K.M., Hirsch D.S., Takei J. & Randazzo P.A. (2002) AGAP1, an endosome-associated, phosphoinositide-dependent ADP-ribosylation factor GTPase - activating protein that affects actin cytoskeleton. *Journal of Biological Chemistry* **277,** 48965–48975.

Nilsson O., Crozier A. & Schmulling T. (1993) Indole-3-acetic acid homeostasis in transgenic tobacco plants expressing the Agrobacterium rhizogenes rolB gene. *Plant Journal* **3,** 681–689.

Normanly J., Cohen J.D. & Fink G.R. (1991) *Arabidopsis thaliana* auxotrophs reveal a tryptophan-independent biosynthetic pathway for indole-3 -acetic acid. *Proceedings of the National Academy of Science of the USA* **90,** 10355–10359.

Novick P., Field C. & Schekman R. (1980) Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* **21,** 205–215.

Oda A., Wada I., Miura K., *et al.* (2003) CrkL directs ASAP1 to peripheral focal adhesions. *Journal of Biological Chemistry* **278,** 6456–6460.

Parry G., Delbarre A., Marchant A., Swarup R., Napier R.C. Perrot-Rechenmann & Bennett M.J. (2001) Novel auxin transport inhibitors phenocopy the auxin influx carrier mutation aux1. *Plant Journal* **25,** 399–406.

Poon P.P., Cassel D., Huber I., Singer R.A. & Johnston G.C. (2001) Expression, analysis, and properties of yeast ADP-ribosylation factor (ARF) GTPase activating proteins (GAPs) Gcs1 and Glo3. *Methods in Enzymology* **329,** 317–324.

Poon P.P., Cassel D., Spang A., Rotman M., Pick E., Singer R.A. & Johnston G.C. (1999) Retrograde transport from the yeast Golgi is mediated by two ARF GAP proteins with overlapping function. *EMBO Journal* **18,** 555–564.

Poon P.P., Wang X., Rotman M., Huber I., Cukierman E., Cassel D., Singer R.A. & Johnston G.C. (1996) Saccharomyces cerevisiae Gcs1 is an ADP-ribosylation factor GTPase-activating protein. *Proceedings of the National Academy of Science of the USA* **93,** 10074–10077.

Randazzo P.A., Andrade J., Miura K., Brown M.T., Long Y.Q., Stauffer S., Roller P. & Cooper J.A. (2000) The Arf GTPase-activating protein ASAP1 regulates the actin cytoskeleton. *Proceedings of the National Academy of Science of the USA* **97,** 4011–4016.

Rashotte A.M., Brady S.R., Reed R.C., Ante S.J. & Muday G.K. (2000) Basipetal auxin transport is required for gravitropism in roots of *Arabidopsis*. *Plant Physiology* **122,** 481–490.

Spang A. (2002) ARF1 regulatory factors and COPI vesicle formation. *Current Opinions in Cell Biology* **14,** 423–427.

Springer S., Spang A. & Schekman R. (1999) A primer on vesicle budding. *Cell* **97,** 145–148.

Steinmann T., Geldner N., Grebe M., Mangold S., Jackson C.L., Paris S., Galweiler L., Palme K. & Jurgens G. (1999) Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. *Science* **286,** 316–318.

Sutton R.B., Davletov B.A., Berghuis A.M., Sudhof T.C. & Sprang S.R. (1995) Structure of the first C2 domain of synaptotagmin I: a novel Ca2+/phospholipid-binding fold. *Cell* **80,** 929–938.

Visser E., Cohen J.D., Barendse G., Blom C. & Voesenek L. (1996) An ethylene-mediated increase in sensitivity to auxin induces adventitious root formation in flooded *Rumex palustris* Sm. *Plant Physiology* **112,** 1687–1692.

Vitale N., Moss J. & Vaughan M. (2001) Purification and properties of ARD1, an ADP-ribosylation factor (ARF)-related protein with GTPase-activating domain. *Methods in Enzymology* **329,** 324–334.

Wu G., Li H. & Yang Z. (2000) *Arabidopsis* RopGAPs are a novel family of rho GTPase-activating proteins that require the Cdc42/Rac-interactive binding motif for rop-specific GTPase stimulation. *Plant Physiology* **124,** 1625–1636.

Yanagisawa L.L., Marchena J., Xie Z., Li X., Poon P.P., Singer R.A., Johnston G.C., Randazzo P.A. & Bankaitis V.A. (2002) Activity of specific lipid-regulated ADP-ribosylation factor GTPase activating proteins is required for Sec14p-dependent Golgi secretory function in yeast. *Molecular Biology of Cells* **13,** 2193–2206.

Yang J.S., Lee S.Y., Gao M., Bourgoin S., Randazzo P.A.R., Premont T. & Hsu V.W. (2002) ARFGAP1 promotes the formation of COPI vesicles, suggesting function as a component of the coat. *Journal of Cell Biology* **159,** 69–78.

Zhang X., Rizo J. & Sudhof T.C. (1998) Mechanism of phospholipid binding by the C2A-domain of synaptotagmin I. *Biochemistry* **37,** 12395–12403.

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