Appl Biol Chem (2016) 59(2):163–171
DOI 10.1007/s13765-015-0143-6

Online ISSN 2468-0842
Print ISSN 2468-0834

ARTICLE

Rice small GTPase Rab11 is required for intracellular trafficking from the *trans*-Golgi-network to the plasma membrane and/or prevacuolar compartments

Hee Rang Yun[1] · Yeong Gil Rim[2] · Jae Bok Heo[1]

Received: 1 October 2015 / Accepted: 1 November 2015 / Published online: 19 January 2016

Abstract Members of the Rab family of small GTPases play important roles in membrane trafficking along the exocytic and endocytic pathways, their function being dependent on their localization. Here, we show the vital roles of the *Oryza sativa Rab11* (*OsRab11*) in intracellular trafficking using a dominant-negative mutant approach based on a protoplast-trafficking assay. GTP-binding and GTP hydrolysis assays revealed that OsRab11 is a small GTP-binding protein complementing △*ypt32/ypt31*. Green fluorescent protein (GFP)-OsRab11 has been localized to both the *trans*-Golgi-network (TGN) and the endosomes/prevacuolar compartments (PVC) in Arabidopsis protoplasts. The protoplast transformation with the dominant-negative mutant *OsRab11(S28N)* revealed that the trafficking of plasma membrane marker proteins [H^+-ATPase-GFP and Ca^{2+}-ATPase8-GFP (ACA8-GFP)] and central vacuole marker proteins [Arabidopsis aleurain-like protein (AALP-GFP) and sporamin (Spo-GFP)] was inhibited. Moreover, overexpression of Arabidopsis AtRha1 did not recover the trafficking inhibition of marker proteins from the central vacuole. These results strongly indicate that OsRab11 localizes to either the TGN or the PVC, and plays a significant role in the intracellular trafficking from the TGN to the PM and/or to the PVC *in planta.*

Keywords Golgi · Membrane trafficking · Rab protein · Plasma membrane · Vacuole

Introduction

The importance of intracellular vesicular trafficking has been documented extensively in eukaryotic cells (Rothman and Wieland 1996; Jahn and Sudhof 1999; Guo et al. 2000). Membrane trafficking includes many processes such as vesicle formation (budding), motility, docking, and fusion all of which are controlled by several protein factors (Derby and Gleeson 2007; Lowe and Barr 2007). For example, the Rab proteins (members of the Rab GTPase family) are major regulators of vesicular trafficking (Fukuda 2008).

The Rab protein family is the largest family within the Ras superfamily of monomeric small GTPases (Novick and Zerial 1997). To date, more than 60 members of the Rab have been identified in several organisms (Zuk and Elferink 1999; Allan et al. 2000). These proteins are localized at different organelles and they are pivotal in the vesicular trafficking pathway and in other signaling pathways (Chen et al. 1993; Dugan et al. 1995; Batoko et al. 2000). For instance, the human Rab1 regulates the protein transport of calcium-sensing receptors from the Endoplasmic Reticulum (ER) to the Golgi (Zhuang et al. 2010). The tobacco pollen-predominant *Rab2* plays a role in vesicle trafficking between the ER and the Golgi body in elongating pollen tubes and, thus, contributes to pollen tube growth (Cheung et al. 2002). The Arabidopsis Rha1 (AtRha1, a *Rab5* homolog) localizes to the PVC and it is involved in the trafficking of soluble cargo from the PVC to the central vacuole (Sohn et al. 2003; Lee et al. 2004). The rice OsRab7 is localized to the vacuolar membrane and it is

Electronic supplementary material The online version of this article (doi:10.1007/s13765-015-0143-6) contains supplementary material, which is available to authorized users.

✉ Jae Bok Heo
jbheo72@dau.ac.kr

[1] Department of Molecular Biotechnology, Dong-A University, Busan 604-714, Republic of Korea

[2] Systems & Synthetic Agrobiotech Center, Gyeongsang National University, Jinju 660-701, Republic of Korea

thought to be involved in the vesicular trafficking to the vacuole (Nahm et al. 2003). More recently, another rice Rab protein, OsRab11 has been identified as a protein involved in the regulation of the JA-mediated signaling pathway (Hong et al. 2013). Hong et al. (2013) reported that OsRab11 interacts with the 12-oxo-phytodienoate-10,11-reductase8 (OPR8) increasing the NADPH oxidation activity of this protein. Plants overexpressing OsRab11 show increased resistance to pathogens due to a greater expression of JA-responsive genes (Hong et al. 2013). In addition, it has also been shown that the rice GTPase-activating protein 1 (GAP1) is a positive regulator of the OsRab11 protein and, together, they play a role in vesicular trafficking from the TGN to the PM or to the central vacuole (Heo et al. 2005). Despite all these vital roles, the detailed function of OsRab11 remains to be elucidated.

In this study, we describe the basic GTP-binding and GTP hydrolysis activities of the recombinant OsRab11 protein and investigate the biological role of OsRab11 in intracellular vesicular trafficking using an Arabidopsis protoplast-based trafficking assay. We conclude that OsRab11 plays a vital role in the vesicular trafficking from the TGN to the PM and/or to the PVC.

Materials and methods

Strains, growth conditions

The genotypes of the yeast strains *S. cerevisiae* YTH3 (*MATa/MATα LEU2/leu2 ura3/ura3 ypt32*::*URA3 HIS/his3 ypt31*::*HIS3 TRP1/trp1)* was used for the complementation assay (Benli et al. 1996). The strain was transformed with recombinant pYES2 plasmid containing T7-OsRab11 cDNA under control of the *gal1* promoter. Haploid progeny were produced by streaked on YPG(gal) medium (1.25 % yeast extract, 2 % peptone, 0.1 % galactose, and 2 % agar) for 5 days.

Purification of GST-fused OsRab11 in *E. coli*

The *OsRab11* cDNA was subcloned into the pGEX-2T vector which produces GST-fusion protein. The recombinant construct was transformed into the *E. coli* BL21strain. Transformed cells were inoculated into 5 mL of LB medium containing 50 μg/mL Ampicillin. The cultures were grown at 28 °C overnight and then poured into 500 mL of fresh LB medium. When an A600 of 0.9 was reached, isopropylthio-ß-galactoside was added to the cultures to a final concentration of 1 mM and the incubation was continued for another 3 h at 28 °C for induction. GST-fusion proteins were purified using glutathione agarose (Sigma.USA) according to each manufacturer's instructions.

GTP-binding assay

The [^{35}S] GTPγS binding activity of full-length OsRab11 protein was measured by the rapid filtration technique (Seo et al. 1997). 0.5 μg of the purified recombinant proteins was incubated at 30 °C in 200 μL of 50 mM Tris–Cl buffer, pH 8.0, containing 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM NaN_3, 0.2 μM [^{35}S]GTPγS, and 10 mM $MgCl_2$. The reaction was stopped by addition of 2 mL of ice-cold buffer (20 mM Tris–Cl, pH 8.0, 100 mM NaCl, and 25 mM $MgCl_2$). Aliquots were taken at indicated times, vacuum-filtered onto nitrocellulose membrane (Millipore, USA) which had been rinsed with 3 mL of ice-cold buffer, and membrane-bound material was washed rapidly three times with 3 mL of ice-cold buffer. The washed membranes were dried and quantified for radioactivity by liquid scintillation counting.

GTP hydrolysis assay

GTPase assays were performed using PEI-cellulose TLC plates and was carried out according to the method of Seo et al. (1997) with a slight modification. Briefly, the reaction was performed at 30 °C in a buffer containing 20 mM Na·HEPES, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM NaN_3, 33 nM [α-32P]GTP, and 0.5 μg of OsRab11 protein. Aliquots of 10 μL were sampled at appropriate time intervals and the same volume of 0.5 M EDTA (pH 8.0) was added to stop the reaction. Samples (2 μL) were spotted onto PEI-cellulose TLC plates, which were then developed in 0.5 M KH2PO4, pH 3.4 solution. After drying, the plate was exposed to an X-ray film.

Transient expression assay

OsRab11 cDNA was fused upstream of the green fluorescent protein (GFP) cDNA under the control of the CaMV 35S promoter (pUC::GFP). Transient expression of GFP- and RFP-fused constructs in *Arabidopsis* protoplasts was performed according to the method described by Sohn et al. (2003). Briefly, recombinant plasmids were introduced by polyethylene glycol-mediated transformation into *Arabidopsis* protoplasts that had been prepared from leaf tissues. Expression of the fusion constructs was monitored after transformation, and observed by fluorescence microscopy (Olympus AX70 TR, Olympus. JAPAN).

Immunohistochemistry

Immunohistochemistry assay was referred to Sohn et al. 2003 (Sohn et al. 2003). Briefly, protoplasts coexpressing GFP-OsRab11 and AtPEP12a-Hemagglutinin (HA) were

placed onto poly-L-lysine-coated slide glasses and fixed with 2 % paraformaldehyde in a fixing buffer (10 mM HEPES pH7.5, 125 mM NaCl, 125 mM $CaCl_2$, 2.5 mM maltose, 5 mM KCl) for 1 h at room temperature. The fixed cells were incubated with rat monoclonal anti-HA (Roche, Switzerland) at 4 °C overnight and washed with TSW buffer five times. Subsequently, the cells were incubated with TRITC-conjugated goat anti-rat IgG (Zymed) as the second antibody.

Generation of OsRab11(S28N) mutant construct

The mutant OsRab11(S28N) was constructed by PCR using the following primers: forward primer 5′-CCGGCG TCGGCAAG**AAC**AACCTCCTCTCCCGCT-3′and reverse primer 5′-AGCGGGAGAGGAGGTT**GTT**CTTGCCGACG CCGG-3′. The T7 epitope tag was added to the N-terminus of OsRab11(S28N) mutant. *Xba*I/*Xho*I digestion fragments of the sequence were ligated into the same restriction sites of the pUC vector which has 35S cauliflower mosaic virus promoter. HA epitope tag was added to the N-terminus of OsRab11 wild type and Rha1 and *Xba*I/*Xho*I digestion fragments of these sequences were ligated into the same vector.

Western blot analysis

To prepare cell extracts from protoplasts, protoplasts expressing T7-OsRab11(S28N), HA-OsRab11 or HA-Rha1 were lysed by repeated freeze–thaw cycles and then centrifuged at 6500×*g* for 5 min at 4 °C in a microcentrifuge to remove cell debris (Sohn et al. 2003). The total protein extracts were separated on a 12 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel, transferred onto a nitrocellulose membrane using a semi-dry electro-blotter, and incubated with each of the indicated antibodies.

Results

OsRab11 is a small GTP-binding protein homologous to the yeast Ypt31p

A large number of Rab GTPases have been identified in plant cells. Most of these proteins play important roles in plant development and in the control of environmental stresses (Cheung et al. 2002; Woollard and Moore 2008; Hong et al. 2013). In a recent study, we showed that OsRab11 is involved together with OPR8 in the JA-mediated defense signaling pathway (Hong et al. 2013). Here, we further characterize the intracellular trafficking function of OsRab11 as a small GTPase. First, to determine whether OsRab11 is a true GTP-binding protein, the biochemical properties of OsRab11 were assayed. A purified recombinant GST-fusion protein was used to measure both the GTP-binding capacity and the GTPase activity of OsRab11 (Fig. 1B, C). During the GTP-binding test, the amount of [γ-^{35}S]GTP bound to the GST-OsRab11 protein was suddenly increased when the cofactor Mg^{2+} was present in the reaction buffer (Fig. 1B). After this initial sudden increase, the amount of [γ-^{35}S]GTP bound to OsRab11 was stabilized (Fig. 1B). Next, poly(ethyleneimine)-cellulose thin-layer chromatography (PEI-cellulose TLC) (Seo et al. 1997) was used to determine the GTPase activity of OsRab11. In 2 h, more than 50 % of the [α-^{32}P]GTP-labeled OsRab11 was converted to [α-^{32}P]GDP-labeled OsRab11 protein by the recombinant OsRab11 protein (Fig. 1C), indicating that OsRab11 is indeed a small GTP-binding protein.

Finally, to address whether OsRab11 is involved in vesicular trafficking from the TGN to the PM or to the central vacuole, a complementation assay was also performed using yeast △*ypt31/ypt32* mutant cells which partially missort vacuolar proteinase carboxypeptidase Y (CPY) (Luo and Gallwitz, 2003). Usually, CPY is synthesized at the endoplasmic reticulum (ER) and then transported to the vacuole through the Golgi body (Tsukada et al. 1999). Thus, the CPY synthesis process produces three CPY forms: a 67-kDa ER core-glycosylated form (p1CPY), a 69-kDa Golgi-modified form (p2CPY), and a 61-kDa mature form (mCPY) found in the vacuole (Tsukada et al. 1999). Unlike W303 wild-type yeast cells, the Golgi-glycosylated p2CPY is partially missorted in the △*ypt31/ypt32* null mutant cells (Fig. 1D lane 2). To determine whether OsRab11 could rescue the CPY missorting in the △*ypt31/ypt32* null mutant cells, we used T7 tagged-OsRab11 to transform △*ypt31/ypt32* null mutant cells and observed the secretion forms of CPY by Western blotting. As expected, the missorted unprocessed Golgi form (p2CPY) disappeared in the transformed cells suggesting that OsRab11 complements the yeast Ypt31p function and, that consequently, it is involved in vacuolar protein transport (Fig. 1D lane 3).

Subcellular localization of the OsRab11 protein

The Rab proteins are localized to different organelles and, consequently, they are involved in different steps of the intracellular trafficking (Chavrier et al. 1990; Morimoto et al. 1991). Therefore, observation of OsRab11 localization in plant cell is important to address the involvement of OsRab11 in vesicular trafficking. To confirm the subcellular localization of OsRab11, a protoplast transient assay was performed. Arabidopsis protoplasts were transformed with free GFP or GFP fused to OsRab11(GFP-OsRab11) and the GFP signal was observed by fluorescence

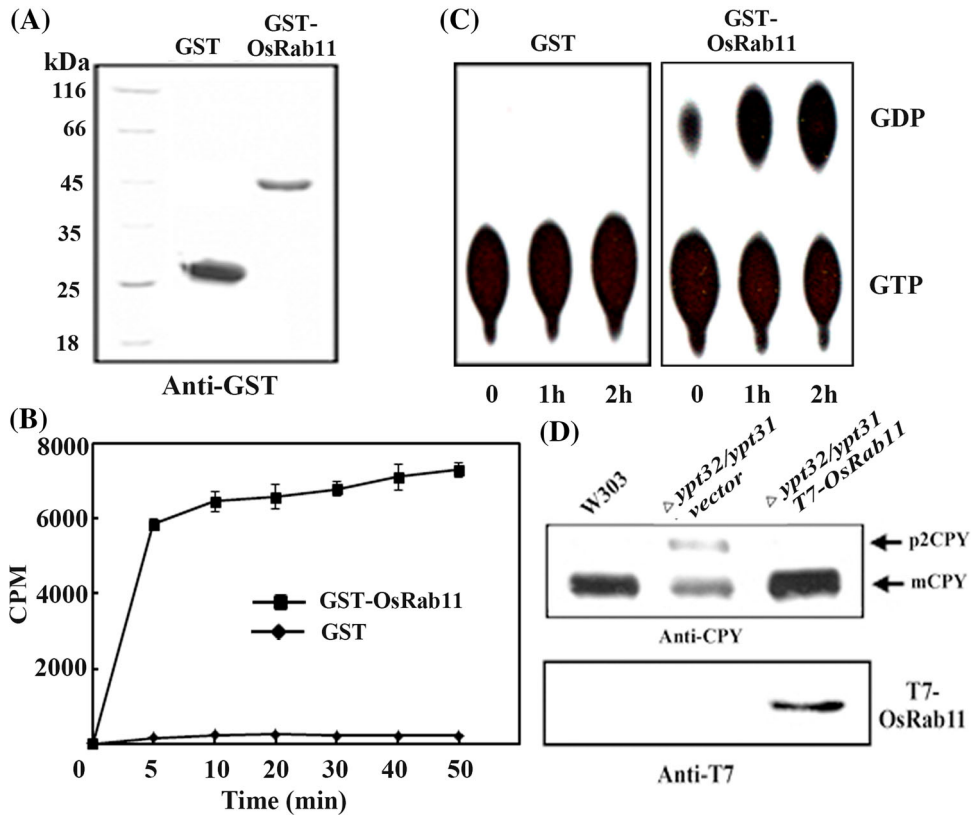

Fig. 1 Purification and biochemical activities of OsRab11. (**A**) Purified GST and GST-fused OsRab11 proteins were separated on 12 % SDS-PAGE gel. (**B**) Time course of [γ-^{35}S]GTP binding to OsRab11. 1 μg of GST or GST-OsRab11 protein were incubated with 200 nM[γ-^{35}S]GTP at 30 °C in the presence of Mg^{2+}. Bound [γ-^{35}S]GTP levels were determined by rapid filtration assay. (**C**) GTP hydrolysis activity of OsRab11. 100 uL reaction mixtures containing 20 pmol of [α-^{32}P]GTP-loaded OsRab11 proteins were incubated for zero, 1 h and 2 h separately. Each 2 uL of removed samples was separated by TLC. (**D**) Complementation test of △*ypt32/ypt31* mutant cells using OsRab11. Cells were grown at 25 °C to mid-log phase and shifted to 37 °C for 1 h in YPG(Gal) media before lysis. Western blot analysis of CPY was performed with total protein extracts from each cell

microscopy. Free GFP was observed in the cytosol and nucleus, whereas protoplasts transformed with GFP-OsRab11 exhibited cytosol staining and punctate staining patterns consistent with a Golgi or a PVC localization (Heo et al. 2005). To examine the exact localization of the punctate staining signal of GFP-OsRab11, GFP-OsRab11 was co-transformed with AtVti1a-RFP, a TGN marker (Heo et al. 2005) in Arabidopsis protoplasts. Although the green and red punctate staining signals from GFP-OsRab11 and AtVtila-RFP overlapped well, there were still many punctate signals from GFP-OsRab11 which did not overlap with RFP signals (Fig. 2B). Considering that most Rab proteins have been shown to play roles in vesicle budding and vesicle fusion at both donor and target compartments (Chavrier et al. 1990; Allan et al. 2000; Prekeris et al. 2000), the punctate staining signals of GFP-OsRab11 were further assessed using AtPEP12p, a PVC marker (da Silva et al. 1997; Bassham and Raikhel 1998). AtPEP12p was tagged with the HA epitope to detect its expression with the anti-HA antibody. We investigated the localization of AtPEP12p-HA in Arabidopsis protoplasts co-transformed with *GFP-OsRab11* and *AtPEP12p-HA*. AtPEP12p-HA was detected by immunohistochemistry using anti-HA antibody. Red punctate signals of AtPEP12p-HA closely overlapped GFP-OsRab11 signals (Fig. 2C). Also, few of GFP signals of OsRab11 did not colocalize with AtPEP12p-HA. Taken together, these results strongly support a vital role of OsRab11 in the intracellular vesicular trafficking at the TGN and/or PVC.

OsRab11(S28N) inhibits the PM trafficking of reporter proteins

OsGAP1 is a GTPase-activating protein which is specifically required for OsRab11 function. Consequently, the trafficking of PM and central vacuolar marker proteins is inhibited in *OsGAP1* mutants, whereas overexpression of OsRab11 relieved this inhibition (Heo et al. 2005). This raised the possibility of OsRab11 being involved in the vesicular trafficking of proteins from the PM and the

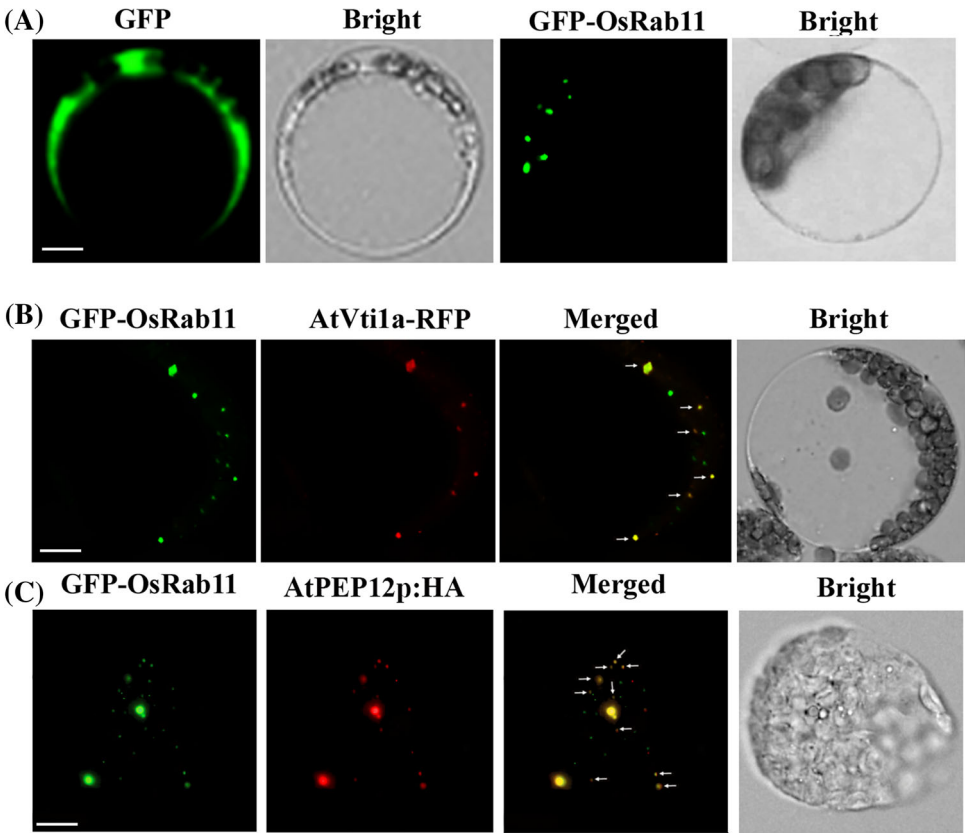

Fig. 2 Subcellular localization of GFP-OsRab11. (**A**) The localization patterns of GFP-OsRab11 in protoplasts. Protoplasts were transformed with GFP-OsRab11. Cells were incubated for 36 h, and then observed by fluorescent microscopy. (**B**) Co-localization of GFP-OsRab11 with AtVti1a-RFP. GFP-OsRab11 was co-transformed with AtVti1a-RFP in protoplasts and the signals of GFP-OsRab11 was examined. *Yellow color* indicates the overlap between *green* and *red* fluorescent signals. (**C**) Protoplasts transformed with GFP-OsRab11 and AtPEP12p-HA were fixed and stained with anti-HA antibody. GFP signals of GFP-OsRab11 were observed directly. The *white-colored arrows* in (**B**) and (**C**) indicate the overlapped GFP and RFP signals. *Bar* = 10 μm

central vacuole. To confirm this, an Arabidopsis protoplast-based trafficking assay was carried out using a dominant-negative mutant [*OsRab11(S28N)*] (Hong et al. 2013) and two PM reporter proteins (H^+-ATPase-GFP and ACA8-GFP). Most cells transformed with *H^+-ATPase-GFP* alone showed a GFP signal in the PM; however, in the presence of T7-OsRab11(S28N), more than half of the cells showed signals in the cytosol (Figs. 3A, 3C). To validate the inhibition of PM protein trafficking in the presence of T7-OsRab11(S28N), a second PM reporter protein (*ACA8-GFP)* was co-transformed together with T7-OsRab11 (S28N) in Arabidopsis protoplasts. As expected, PM localization of ACA8-GFP was severely decreased in cells transformed with both ACA8-GFP and T7-OsRab11 (S28N) when compared to cells transformed only with ACA8-GFP (Fig. 3B, C). These results show that the dominant-negative mutant *OsRab11(S28N)* inhibits the trafficking of both H^+-ATPase-GFP and ACA8-GFP reporter proteins to the PM.

OsRab11(S28N) inhibits the vacuolar trafficking of reporter proteins

As previously mentioned, we assumed that OsRab11 is involved in vacuolar protein trafficking as well as in PM protein trafficking. To test whether this is indeed the case, the vacuolar proteins AALP-GFP and Spo-GFP were selected as reporter proteins and co-transformed with T7-OsRab11(S28N) in Arabidopsis protoplasts. Most cells transformed with either AALP-GFP or Spo-GFP alone showed a staining pattern coinciding in more than 75 % of the area with the central vacuole with only little ER or non-vacuole staining (Fig. 4A–C). However, the majority of cells co-transformed with both vacuole reporter proteins and T7-OsRab11(S28N) showed a cytosol staining pattern with only little ER or non-vacuole staining, and only less than 20 % of the stained area coincided with the central vacuole (Fig. 4A–C). This result suggests that *OsRab11(S28N)* inhibits the targeting of both AALP-GFP and Spo-GFP to the central vacuole probably by competing with endogenous wild-type Rab11 protein. To confirm this, wild-type HA-OsRab11 was co-transformed with T7-OsRab11(S28N) in Arabidopsis protoplasts expressing the PM or the central vacuole reporter proteins (Fig. 5). In the transformation with only Rab11 (S28N), the frequency of the protoplasts showing PM staining pattern was about less than 38 %, whereas the frequency in the co-transformation with HA-Rab11 increased to about 60 % (Fig. 5A, C). Similarly, in the transformation with only Rab11 (S28N), the frequency of the protoplasts showing vacuole staining pattern was about less than 20 %, whereas the frequency in the co-transformation with HA-Rab11 increased to about

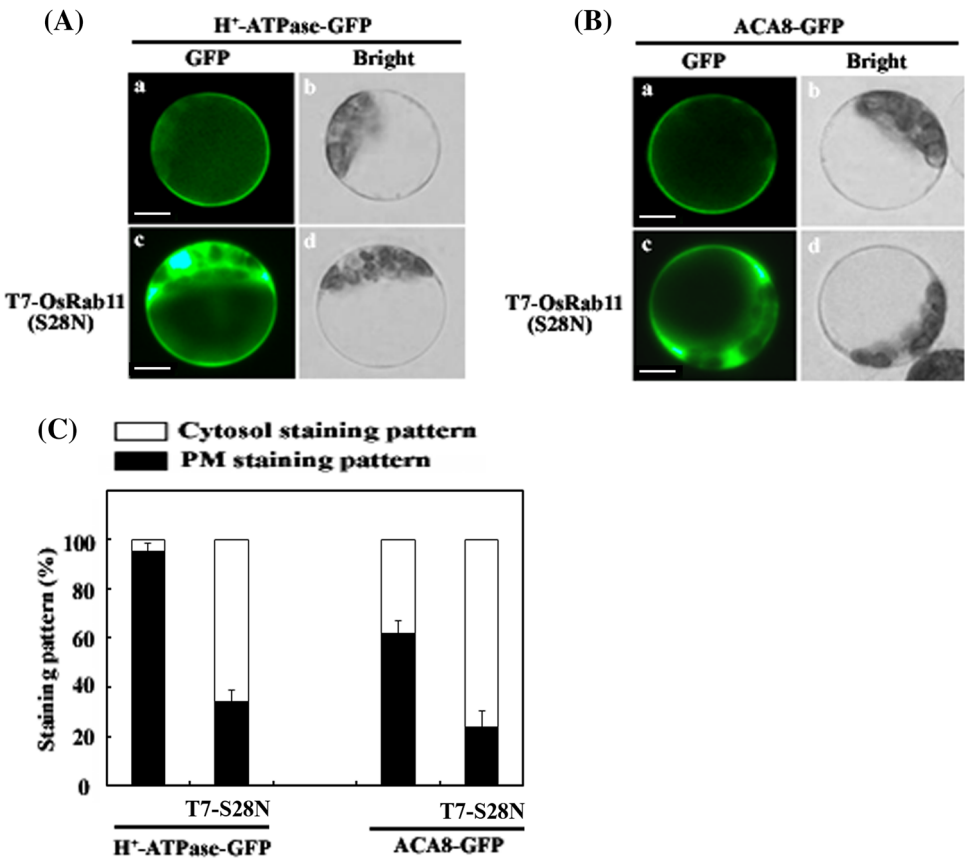

Fig. 3 OsRa11(S28N) inhibits the trafficking of H^+-ATPase-GFP and ACA8-GFP to the plasma membrane. (**A**) Protoplasts were transformed with H^+-ATPase-GFP alone (*a*, *b*) or with T7-OsRab11(S28N) (*c*, *d*), and (**B**) with ACA8-GFP alone (*a*, *b*) or with T7-OsRab11(S28N) (*c*, *d*). Cells were incubation for 36 h, and then observed by fluorescent microscopy. Two types of protoplasts with GFP signals at the PM and the cytosol stains were observed. *Bar* = 10 μm. (**C**) Trafficking efficiency of H^+-ATPase-GFP and ACA8-GFP to the PM. The whole population of transformed protoplasts was divided into two groups based on GFP signal localization: protoplasts with the PM staining and cytosol staining pattern. A minimum of 100 transformed protoplasts in each group was counted

55 % (Fig. 5A, B), providing further support for a role of OsRab11 in the intracellular trafficking from the TGN to the PM and/or to the central vacuole.

Most central vacuole proteins synthesized at the ER are transported to the Golgi apparatus and moved to the TGN after the maturation process (Sohn et al. 2003). Matured vacuole proteins at the TGN are finally transported to the central vacuole through PVC/endosome. As shown above, the vacuolar trafficking inhibition by *OsRab11(S28N)* is very similar to that of the *AtRha1(S24N)* mutant (Sohn et al. 2003). AtRha1 participates in the intracellular trafficking of the cargo from the PVC to the Vacuole. The trafficking inhibition of vacuole proteins by T7-OsRab11(S28N) was not rescued by the introduction of HA-AtRha1 (Fig. 5B and 5D). Therefore, these results support that OsRab11 participates in the trafficking of the cargo from the TGN to the PVC.

Discussion

It has previously been reported that plant Rab proteins are major components in intracellular membrane trafficking and, consequently, they are involved in a variety of signal transduction pathways such as defense, stress signaling pathways, and plant development (Vernoud et al. 2003; Grosshans et al. 2006; Agarwal et al. 2008). In this study, we further characterize one of these proteins, OsRab11, and show its relevance in intracellular trafficking.

Searching for OsRab11 homology in Arabidopsis database (https://www.arabidopsis.org/cgi-bin/Blast/TAIRblast.pl) showed high homology to Rab11/RabA proteins such as RabA1a, RabA1b, RabA1c, and RabA1d with 79, 83, 85, and 86 % identity and 92, 93, 94, and 94 % similarity (Supplemental Fig. 1), and in yeast database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed high homology to Rab/Ypt31 with 65 % identity and 80 % similarity and to Rab/Ypt32 with 60 % identity and 74 % similarity (Supplemental Fig. 2), suggesting that OsRab11 is a close homolog of the Arabidopsis Rab11/RabA proteins and the yeast Ypt31/32 proteins which regulate an early step of the exocytic pathway in yeast (Jedd et al. 1997). Ypt31p plays a role in the fusion of recycling vesicles at the trans-Golgi compartment (Jedd et al. 1997) and depletion of Ypt31p has shown to inhibit invertase secretion and vacuolar protein maturation (Benli et al. 1996). In our study, we found that the missorting of vacuolar proteinase CPY (Fig. 1D) in yeast △*ypt31/ypt32* null mutant cells is rescued by OsRab11 complementation, supporting a role of OsRab11

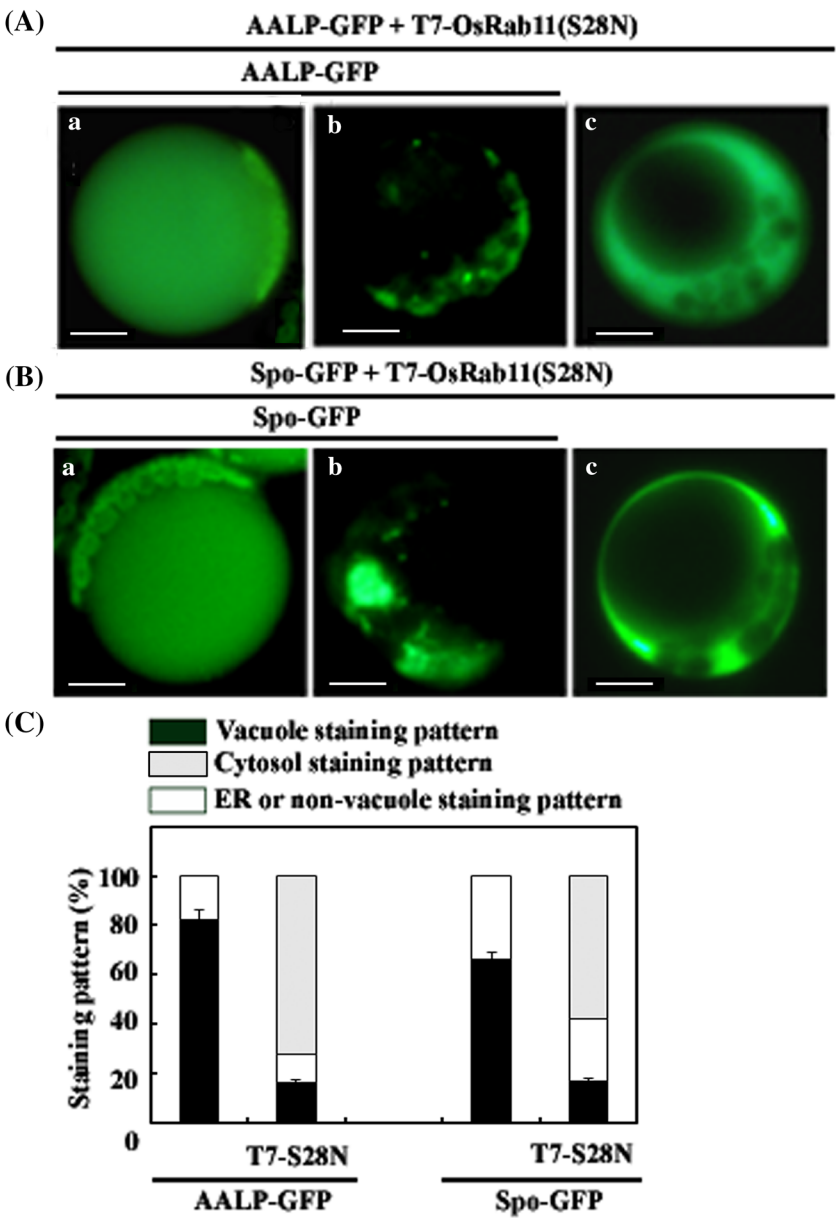

Fig. 4 OsRa11(S28N) inhibits the trafficking of AALP-GFP and Spo-GFP to the central vacuole. (**A**) Protoplasts were transformed with AALP-GFP alone or with T7-OsRab11(S28N), (**B**) and with Spo-GFP alone or with T7-OsRab11(S28N). Cells were incubated for 36 h, and then observed by fluorescent microscopy. Three types of GFP signal pattern were observed, namely a central vacuole pattern (*a*), an ER or non-vacuole pattern (*b*), and a cytosol pattern (*c*). *Bar* = 10 μm. (**C**) The efficiency of AALP-GFP or Spo-GFP trafficking to the central vacuole. The GFP signal patterns in a minimum of 70 randomly selected transformed protoplasts were assessed and classified as central vacuole, ER-non-vacuole, or cytosol staining patterns. The trafficking efficiency of these proteins was calculated as described in Fig. 3(**C**)

in intracellular secretion pathway and/or vacuolar trafficking similar to that of Ypt31/32.

The vacuolar trafficking pathway is also well characterized in Arabidopsis and AtRha1 has been identified as an important component in this pathway using the dominant-negative mutant approach (Sohn et al. 2003). Dominant-negative Rab proteins have shown to prevent the activity of the endogenous wild-type proteins by competing for their effector proteins (Tisdale et al. 1992). For example, the dominant-negative mutant *AtRha1(S24N)* strongly inhibits the vacuolar trafficking of marker proteins (Sohn et al. 2003). This result is very similar to the inhibition of vacuolar trafficking caused by the *OsRab11(S28N)* mutant (Fig. 5). Sohn et al. (2003) also reported that vacuolar cargo proteins are likely to be transported to the central vacuole through the PVC. AtRha1 is thought to be localized to the PVC and maybe tonoplast (Sohn et al. 2003). Whereas GFP-OsRab11 localizes to the TGN and/or to the PVC when expressed transiently in Arabidopsis protoplasts (Fig. 2). In cells expressing *OsRab11*(*S28N*) the vacuolar cargo proteins were mainly accumulated in the cytosol and to a lesser degree on the ER or non-vacuole region (Fig. 4), this is in contrast to what was observed in *AtRha1(S24N)* expressing cells and, thus, it suggests that OsRab11 may not play a role in the trafficking of vacuolar cargo protein from the PVC to the central vacuole. As an alternative, OsRab11 might be involved in the transport of vacuolar cargo proteins from the TGN to the PVC. In order to test

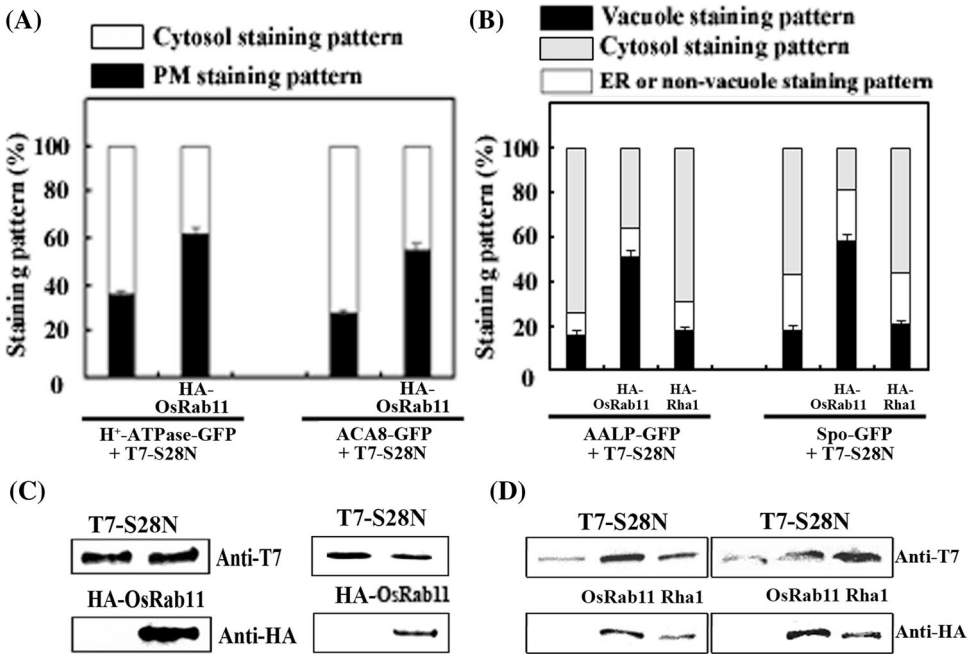

Fig. 5 The overexpression of OsRab11 rescues the inhibitory effect of the OsRab11(S28N) mutant. (**A**) Protoplasts expressing T7-OsRab11(S28N) were co-transformed with HA-OsRab11 along with H^+-ATPase-GFP or ACA8-GFP. (**B**) Protoplasts expressing T7-OsRab11(S28N) were co-transformed with HA-OsRab11 or HA-Rha1 along with AALP-GFP or Spo-GFP. After incubating the cells for 36 h, the targeting efficiencies of the reporter proteins were calculated as described in Fig. 3(**C**). (**C**, **D**) In vivo expression levels of T7-OsRab11(S28N), HA-OsRab11, or HA-Rha1. Total protein extracts were prepared from protoplasts transformed with various constructs and analyzed by protein gel-blot analysis using monoclonal anti-T7 and HA

this hypothesis, Arabidopsis protoplasts were co-transformed with wild-type AtRha1 and *OsRab11(S28N)*. In these protoplasts, the trafficking inhibition of vacuolar cargo protein caused by *OsRab11(S28N)* was not recovered by wild-type AtRha1, suggesting that OsRab11 and AtRha1 might be involved in different stages of the transport of the vacuolar cargo proteins.

Taken together, the biochemical and the in vivo functional characteristics of OsRab11 show that this protein is a rice homolog of the Rab11/Ypt31 subfamily, and it is required for the intracellular vesicular trafficking from the TGN to the PM and/or to the vacuole.

Recently, we found that transgenic plants expressing OsRab11(S28N) is very insensitive to environmental stresses due to trafficking inhibition of proteins involved in each stresses (data not shown). It will be interesting to investigate the relationship between trafficking by Rab protein and plant response to environmental stresses. Nevertheless, in order to improve our understanding of the roles played by OsRab11 protein in rice, further characterization of its regulatory factors is required.

Acknowledgments This work was supported by a grant from the Next-Generation BioGreen 21 Program (SSAC, Grant#: PJ0111062015) and by "Cooperative Research Program for Agriculture Science & Technology development (Project No. PJ009759)" Rural Development Administration, Republic of Korea and by the National Research Foundation of Korea (NRF) Grant funded by Korean Government (NRF-2014R1A1A4A01007558).

Compliance with ethical standards

Conflict of interest All authors have no conflict of interest.

References

Agarwal PK, Agarwal P, Jain P, Jha B, Reddy MK, Sopory SK (2008) Constitutive overexpression of a stress-inducible small GTP-binding protein PgRab7 from Pennisetum glaucum enhances abiotic stress tolerance in transgenic tobacco. Plant Cell Rep 27:105–115

Allan BB, Weissman J, Aridor M, Moyer B, Chen CD, Yoo JS, Balch WE (2000) Stage-specific assays to study biosynthetic cargo selection and role of SNAREs in export from the endoplasmic reticulum and delivery to the Golgi. Methods 20:411–416

Bassham DC, Raikhel NV (1998) An Arabidopsis VPS45p homolog implicated in protein transport to the vacuole. Plant Physiol 117:407–415

Batoko H, Zheng HQ, Hawes C, Moore I (2000) A rab1 GTPase is required for transport between the endoplasmic reticulum and golgi apparatus and for normal golgi movement in plants. Plant Cell 12:2201–2218

Benli M, Doring F, Robinson DG, Yang X, Gallwitz D (1996) Two GTPase isoforms, Ypt31p and Ypt32p, are essential for Golgi function in yeast. EMBO J 15:6460–6475

Chavrier P, Vingron M, Sander C, Simons K, Zerial M (1990) Molecular cloning of YPT1/SEC4-related cDNAs from an epithelial cell line. Mol Cell Biol 10:6578–6585

Chen YT, Holcomb C, Moore HP (1993) Expression and localization of two low molecular weight GTP-binding proteins, Rab8 and Rab10, by epitope tag. Proc Natl Acad Sci USA 90:6508–6512

Cheung AY, Chen CY, Glaven RH, de Graaf BH, Vidali L, Hepler PK, Wu HM (2002) Rab2 GTPase regulates vesicle trafficking between the endoplasmic reticulum and the Golgi bodies and is important to pollen tube growth. Plant Cell 14:945–962

da Silva Conceicao A, Marty-Mazars D, Bassham DC, Sanderfoot AA, Marty F, Raikhel NV (1997) The syntaxin homolog AtPEP12p resides on a late post-Golgi compartment in plants. Plant Cell 9:571–582

Derby MC, Gleeson PA (2007) New insights into membrane trafficking and protein sorting. Int Rev Cytol 261:47–116

Dugan JM, deWit C, McConlogue L, Maltese WA (1995) The Ras-related GTP-binding protein, Rab1B, regulates early steps in exocytic transport and processing of beta-amyloid precursor protein. J Biol Chem 270:10982–10989

Fukuda M (2008) Regulation of secretory vesicle traffic by Rab small GTPases. Cell Mol Life Sci 65:2801–2813

Grosshans BL, Ortiz D, Novick P (2006) Rabs and their effectors: achieving specificity in membrane traffic. Proc Natl Acad Sci USA 103:11821–11827

Guo W, Sacher M, Barrowman J, Ferro-Novick S, Novick P (2000) Protein complexes in transport vesicle targeting. Trends Cell Biol 10:251–255

Heo JB, Rho HS, Kim SW, Hwang SM, Kwon HJ, Nahm MY, Bang WY, Bahk JD (2005) OsGAP1 functions as a positive regulator of OsRab11-mediated TGN to PM or vacuole trafficking. Plant Cell Physiol 46:2005–2018

Hong MJ, Lee Y, Son YS, Im CH, Yi YB, Rim YG, Bahk JD, Heo JB (2013) Rice Rab11 is required for JA-mediated defense signaling. Biochem Biophys Res Commun 434:797–802

Jahn R, Sudhof TC (1999) Membrane fusion and exocytosis. Annu Rev Biochem 68:863–911

Jedd G, Mulholland J, Segev N (1997) Two new Ypt GTPases are required for exit from the yeast trans-Golgi compartment. J Cell Biol 137:563–580

Lee GJ, Sohn EJ, Lee MH, Hwang I (2004) The Arabidopsis rab5 homologs rha1 and ara7 localize to the prevacuolar compartment. Plant Cell Physiol 45:1211–1220

Lowe M, Barr FA (2007) Inheritance and biogenesis of organelles in the secretory pathway. Nat Rev Mol Cell Biol 8:429–439

Luo Z, Gallwitz D (2003) Biochemical and genetic evidence for the involvement of yeast Ypt6-GTPase in protein retrieval to different Golgi compartments. J Biol Chem 278:791–799

Morimoto BH, Chuang CC, Koshland DE Jr (1991) Molecular cloning of a member of a new class of low-molecular-weight GTP-binding proteins. Genes Dev 5:2386–2391

Nahm MY, Kim SW, Yun D, Lee SY, Cho MJ, Bahk JD (2003) Molecular and biochemical analyses of OsRab7, a rice Rab7 homolog. Plant Cell Physiol 44:1341–1349

Novick P, Zerial M (1997) The diversity of Rab proteins in vesicle transport. Curr Opin Cell Biol 9:496–504

Prekeris R, Klumperman J, Scheller RH (2000) A Rab11/Rip11 protein complex regulates apical membrane trafficking via recycling endosomes. Mol Cell 6:1437–1448

Rothman JE, Wieland FT (1996) Protein sorting by transport vesicles. Science 272:227–234

Seo HS, Choi CH, Kim HY, Jeong JY, Lee SY, Cho MJ, Bahk JD (1997) Guanine-nucleotide binding and hydrolyzing kinetics of ORrab2, a rice small GTP-binding protein expressed in *Escherichia coli*. Eur J Biochemistry/FEBS 249:293–300

Sohn EJ, Kim ES, Zhao M, Kim SJ, Kim H, Kim YW, Lee YJ, Hillmer S, Sohn U, Jiang L, Hwang I (2003) Rha1, an Arabidopsis Rab5 homolog, plays a critical role in the vacuolar trafficking of soluble cargo proteins. Plant Cell 15:1057–1070

Tisdale EJ, Bourne JR, Khosravi-Far R, Der CJ, Balch WE (1992) GTP-binding mutants of rab1 and rab2 are potent inhibitors of vesicular transport from the endoplasmic reticulum to the Golgi complex. J Cell Biol 119:749–761

Tsukada M, Will E, Gallwitz D (1999) Structural and functional analysis of a novel coiled-coil protein involved in Ypt6 GTPase-regulated protein transport in yeast. Mol Biol Cell 10:63–75

Vernoud V, Horton AC, Yang Z, Nielsen E (2003) Analysis of the small GTPase gene superfamily of Arabidopsis. Plant Physiol 131:1191–1208

Woollard AA, Moore I (2008) The functions of Rab GTPases in plant membrane traffic. Curr Opin Plant Biol 11:610–619

Zhuang X, Adipietro KA, Datta S, Northup JK, Ray K (2010) Rab1 small GTP-binding protein regulates cell surface trafficking of the human calcium-sensing receptor. Endocrinology 151:5114–5123

Zuk PA, Elferink LA (1999) Rab15 mediates an early endocytic event in Chinese hamster ovary cells. J Biol Chem 274:22303–22312