学位論文

Studies on activation mechanism of two types of RAB5 in *Arabidopsis thaliana*

（植物における２つの RAB5 グループの活性化機構の研究）

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Abstract

Recent studies have revealed that endocytosis plays important roles in various plant functions, whose underlying molecular mechanisms and significance in development still remain largely unknown. Rab5, a subfamily of Rab GTPases, regulates various endosomal functions as a molecular switch. In animal cells, Rab5 is known to regulate not only endosomal fusion but also signaling through endosomes. Rab GTPases are activated by specific guanine nucleotide exchange factors (GEFs), which accelerate exchange of GDP for GTP. The Vps9 domain, a catalytic core for activation of Rab5, is conserved in all Rab5 GEFs identified thus far. I have already demonstrated that *A. thaliana* VPS9a activates all of three *A. thaliana* Rab5 members (ARA6, ARA7, and RHA1). VPS9a consists of the N-terminal conserved the Vps9 domain and the C-terminal region with no similarity to known functional domains. I found that truncation of the C-terminal region of VPS9a resulted in increased GEF activity toward ARA6. VPS9a forms a homo-oligomer, which is mediated by CTR. Truncate mutants exhibited subcellular localization distinct from full-length VPS9a, suggesting that CTR have effect on subcellular localization.

In addition, in order to elucidate the molecular basis of plant-unique membrane trafficking system, I analyzed the functions of other plant VPS9 homologues in *Marchantia polymoropha*, as a model of livewort. I found that MpVPS9 activates and interacts with both of MpRAB5 and

MpARA6. MpARA6 is sensitive to MpVPS9 like AtARA6. AtVPS9a activates MpARA6 and MpRAB5 to a similar extent to MpVPS9. I also demonstrated that MpVPS9 is RAB5 GEF.

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Abbreviations

BFA	Brefeldin A
CTR	C-terminal region
GAP	GTPase activating protein
GDI	GTPase dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GMP-PNP	guanosine 5'-[(β, γ)-imido] triphosphate
GST	glutathione S-trasferase
GTP	guanosine triphosphate
IPTG	isopropylthio-β-galactoside
mRFP	monomeric red fluorescent protein
MVE	multivesicular endosome
ST	sianyl transferase
TGN	*trans*-Golgi network
Wm	Wortmannin

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General introduction

RAB GTPases regulate membrane trafficking

In eukaryotic cells, the correct transport of proteins is essential for fundamental cellular activities such as the maintenance of organelle functions, response to the environment and intracellular communication. Membrane trafficking between organelles is a major transport system and has an important role in eukaryotic cells.

Membrane trafficking events consist of following steps: the budding of vesicles/tubules from a donor organelle, the delivery of transport carriers and the tethering and fusion of carriers to a target membrane. RAB GTPases are involved in the last step. RAB GTPases comprise the largest class within the Ras superfamily of small GTPases, and are evolutionarily conserved in eukaryotes. RAB GTPases generally act as specific regulators of membrane trafficking events and determine organelle identities.

RAB GTPases are molecular switches that act through a conformational change between the GTP- (active) and GDP- (inactive) bound forms (Figure 1). RAB GTPases are positively and negatively, regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively. GAPs accelerate the intrinsic rate of GTP hydrolysis, while GEFs stimulate the release of GDP, allowing GTP to bind. The activation of RAB GTPases is

catalyzed by specific GEFs.

RAB5 GTPases and the post-Golgi trafficking pathway in plants

Plant endocytosis has essential roles not only for basic cellular functions but also for growth and development, auxin transport (Geldner et al 2001; Paciorek et al 2005; Dhonukshe et al 2008), immune responses (Robatzek et al 2006; Choi et al 2013), cytokinesis (Dhonukshe et al 2006; Boutté et al 2010), pollen tube growth (Sousa et al 2008; Zhao et al 2010) and self-incompatibility (Ivanov and Gaude 2009). Although recent studies have revealed that endocytosis is essential for a variety of plant functions, the underlying molecular mechanisms in plant development still remain largely unknown.

The post-Golgi trafficking network in plants is different from that in animals. The *trans*-Golgi network (TGN) acts as a sorting platform for secreted and vacuolar/lysosomal proteins in eukaryotic cells. In addition to this function, the TGN also functions as "early endosome" in plant cells (Dettmer et al 2006; Lam et al 2007; Chow et al 2008; Viotti et al 2010; Choi et al 2013) (Figure 2). RAB5 are localized on the early endosomes in animal cells, while plant RAB5s are localized to the multivesicular endosomes (MVEs), which function as "late" endosomes (Haas et al 2007; Lam et al 2007; Viotti et al 2010; Choi et al 2013).

Recent comparative genomics studies have indicated that each eukaryotic lineage is equipped

with a unique set of RAB GTPases (Dacks and Field 2007; Dacks et al 2008; Mackiewicz and Wyroba 2009); this was also demonstrated to be true for the land plant lineage. Since the plant lineage lacks several RAB GTPase subgroups that are conserved in animals, several RAB members have undergone unique diversification during plant evolution (Rutherford and Moore 2002).

RAB5 is one of the most extensively studied members of the RAB GTPase family. The RAB5 subfamily is a key regulator of the endocytic pathway and is localized to the endosomes. In *A. thaliana,* there are three RAB5 members. ARA7 and RHA1 share high similarity with mammalian RAB5s and ARA6 is the plant-unique RAB5 member (Ueda et al 2001; Ueda et al 2004) (Figure 3). ARA6 has unique structural features, such as the lack of the conserved C-terminal Cys motif and the presence of an additional N-terminus stretch that contains myristoylation and palmitoylation sites. ARA6 is conserved in plants from green algae to land plants (Ebine et al 2011; Hoepflinger et al 2013). ARA7 and ARA6 are localized to endocytic compartments, though ARA6 is localized to a population of endosomes distinct from those of conventional RAB5s (Ueda et al 2004). Conventional RAB5s regulate the pathway from endosomes to the vacuole, while ARA6 regulates the pathway from endosomes to the plasma membrane (Ebine et al 2011).

A. thaliana ara6 mutations exhibited no detectable abnormal phenotypes under laboratory

conditions (Ebine et al 2011). However, *ara6* mutations suppressed *syp22-1/vam3* (a mutant of endosomal/vacuolar Qa-SNARE) which exhibited abnormal phenotypes such as semi-dwarfism and late flowering, while *ara7* and *rha1* mutations enhanced *syp22-1/vam3*(Ebine et al 2011). These results also indicate that plant-unique ARA6 and conventional RAB5 (ARA7 and RHA1) have different functions.

In the active state, RAB GTPases interact with specific effector molecules, to trigger downstream reactions. In plants, plant-specific RABA4b/RAB11, RABA4d/RAB11, and RABE1d/RAB8 effectors have been identified (Preuss et al 2006; Camacho et al 2009; Ellinger et al 2014). Cui and colleagues also demonstrated that the Mon1-CCZ-1 complex is a RAB5 effector (Cui et al 2014). However, homologous proteins of animal RAB5 effector proteins, such as early endosommal antigen 1 (EEA1) and Rabaptin-5, are not conserved in plants. Recent studies in our laboratory have also identified some plant-specific RAB5 effectors (unpublished works by E. Ito and H. Sakurai).

About my thesis

Although accumulating evidence suggests to an important role for endocytosis in plants, the molecular mechanism is still not well understood. RAB5 GTPases are activated by RAB5 GEFs, and the Vps9 domain is essential for RAB5 activation. *A. thaliana* has two Vps9

domain-containing proteins, VPS9a and VPS9b. These proteins show high amino acid sequence similarity. However, VPS9b is specifically expressed in pollen and the embryo sac (T. Goh, unpublished), while VPS9a is expressed in all tissues and mainly functions during *A. thaliana* development (Goh et al 2007).

VPS9a consists of a conserved the Vps9 domain at the N-terminus and a C-terminal region with no similarity to known functional domains or motifs. During my master course, I found that D185 and Y225 in VPS9a have important roles for the GEF activity and the interactions with ARA7 and ARA6 (Figure 5). These results suggest that the Vps9 domain of VPS9a is the conserved region which act as catalytic core, and has a common activation mechanism in eukaryotes (Figure 6). Additionally, from the aligned sequences, the C-terminal region of VPS9a (CTR) contains a plant-specific high similarity region (PSR) that is widely conserved from algae to land plants. Thus, I investigated how plant RAB5 GEFs regulate two RAB5 group members.

In Chapter 1, I mainly demonstrated that the CTR is involved in homo-oligomerization and ARA6 regulation. In Chapter 2, I identified that a Vps9 domain-containing protein in *Marchantia polymorpha* also interacts with and has GEF activity toward both of RAB5 group members.

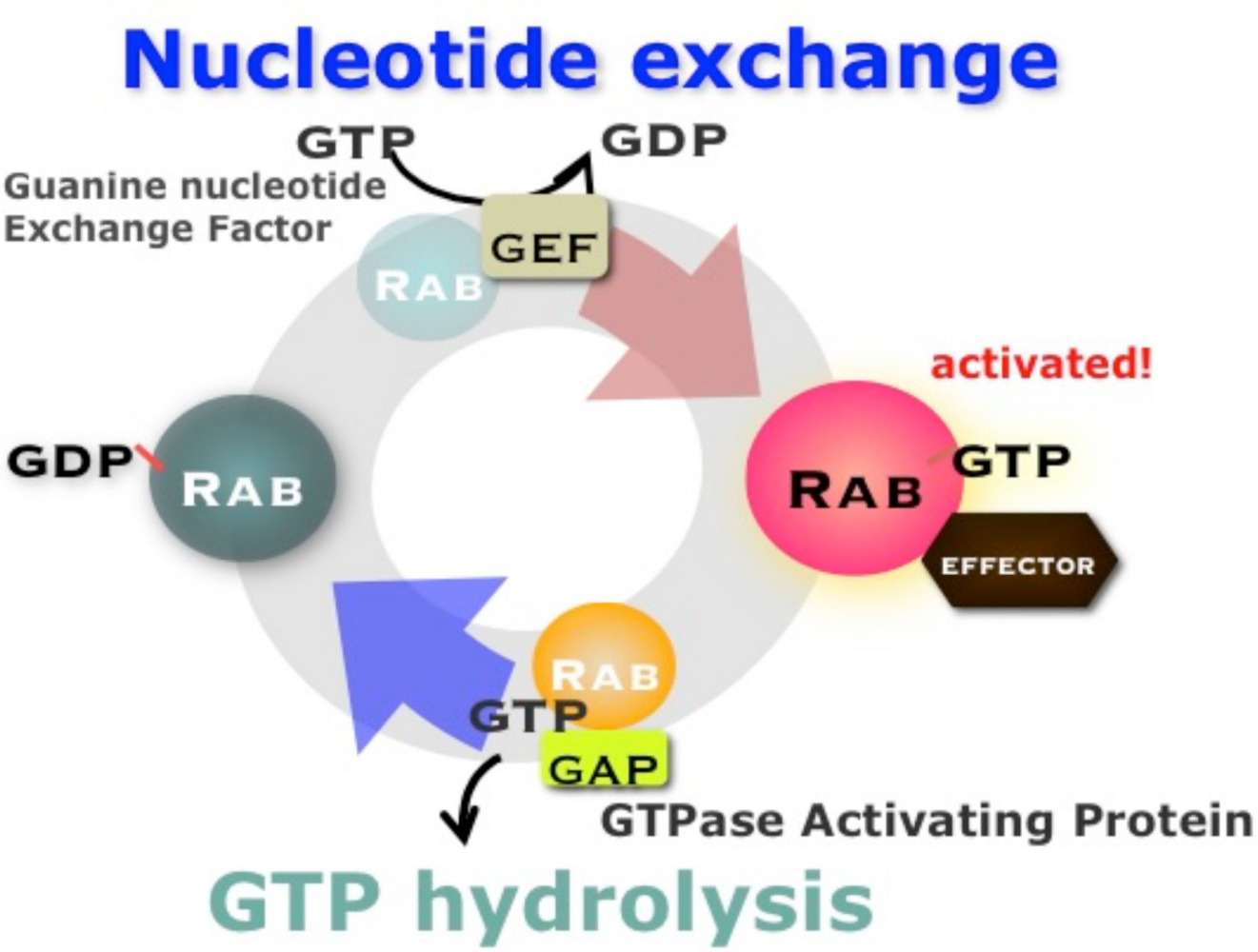


Figure 1. RAB5 GTPase is activated by GEF.

RAB is a small GTPase, and acts as a molecular switch. Activation of RAB is mediated by the guanine nucleotide exchange factor (GEF), which stimulates the release of GDP, allowing GTP to bind. Effector proteins interacts specifically with active form of RABs.

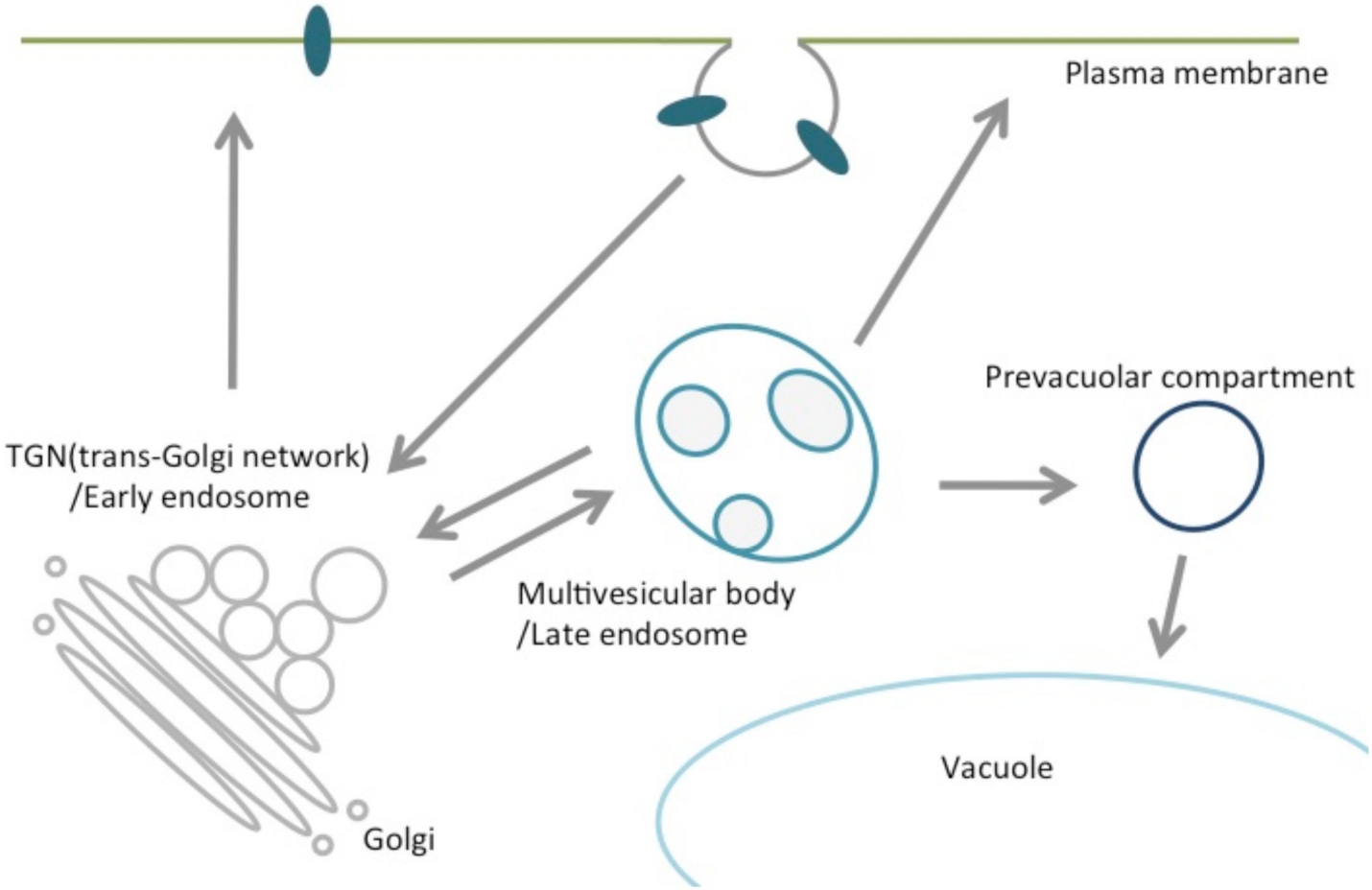


Figure 2. Membrane traffic pathway in plant cells.

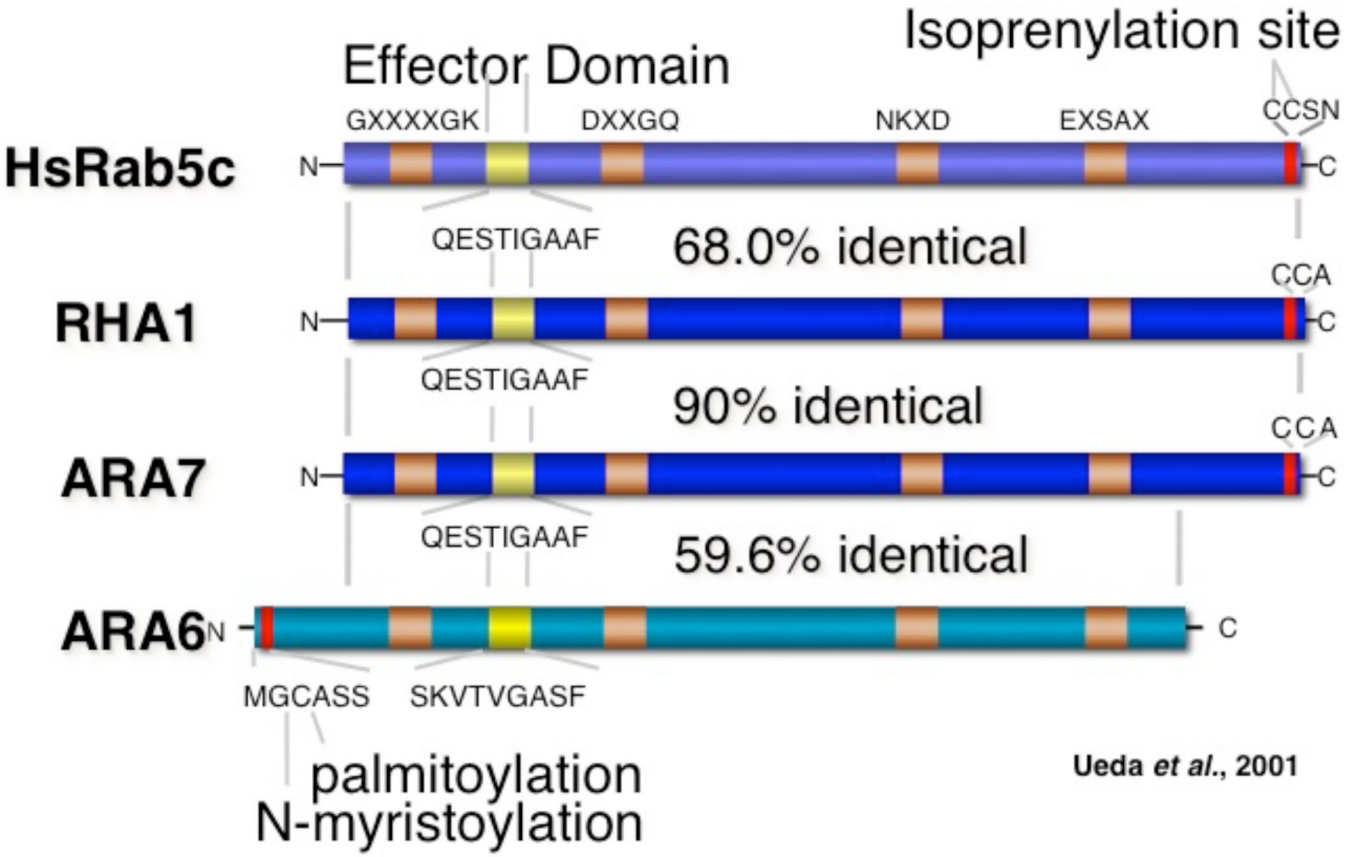


Figure 3. RAB5 members in *A.thaliana*

Arabidopsis thaliana has three RAB5 members; conventional RAB5, ARA7 and RHA1, and plant-unique ARA6. ARA6 lacks the C-terminal hyperviable region and cycteine motif essential for membrane binding correct localization in conventional RAB5, instead, insted of N-terminus stretch with N-myristoylation and palmitoylation sites.

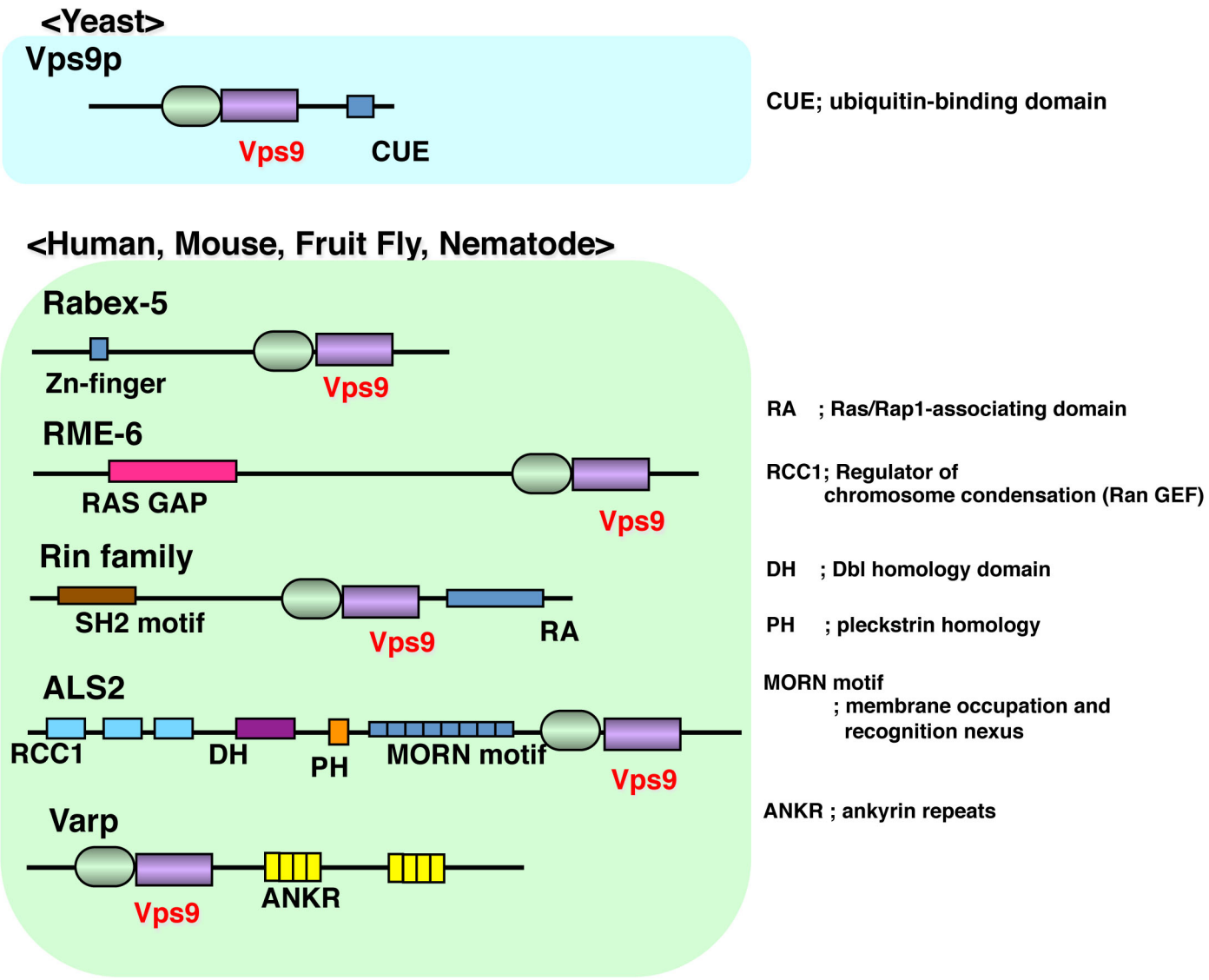


Figure 4. RAB5 GEFs contain Vps9 domain and many known domains and motifs

Vps9 domain, a catalytic core for activation of RAB5, is conserved in all RAB5 GEFs identified thus far.

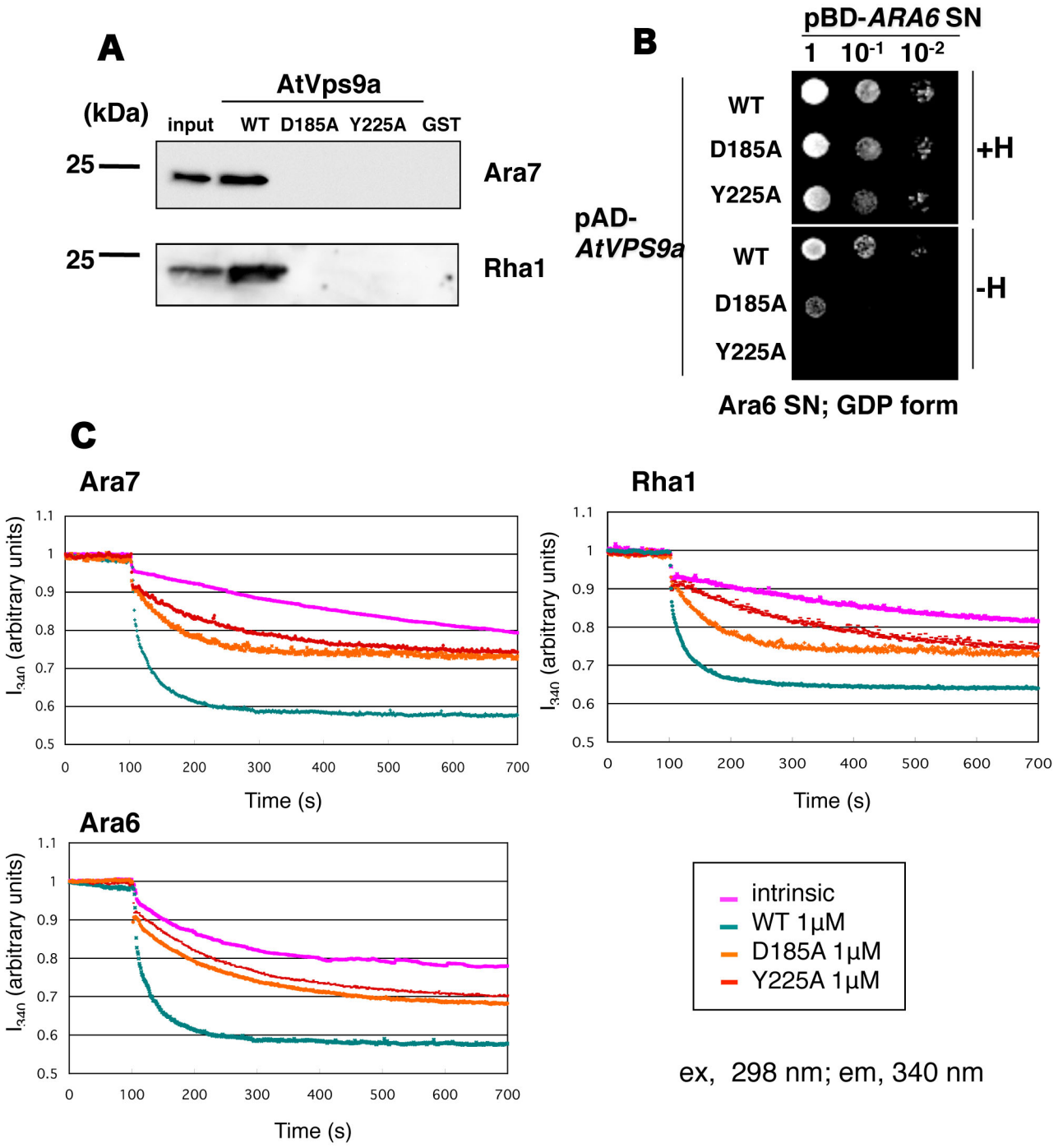


Figure 5. D185 and Y225 are important for interaction with RAB5s and GEF activity

(A) In vitro pull-down assay between full-length (WT) or truncated (S304*) VPS9a and ARA7 or RHA1.

(B) Interactions between RAB5 members and VPS9a detected by the yeast two-hybrid assay. The transformants were spotted onto selection plates and incubated at 30 °C for 5 days. Interactions between two proteins were tested using the *HIS3* gene.

(C) In vitro GEF assay of D185A and Y225A.

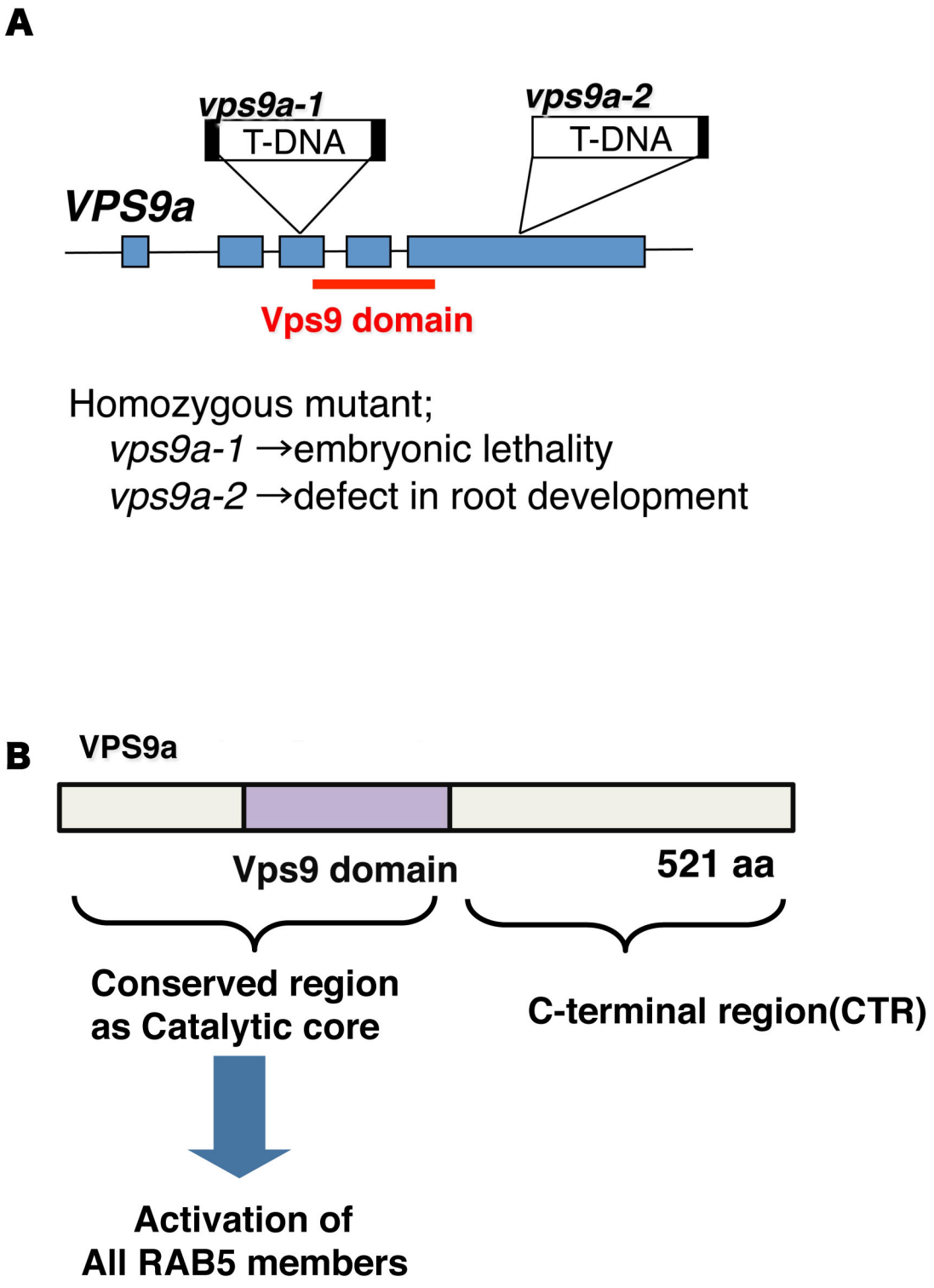


Figure 6. VPS9aRAB5 GEFs contain Vps9 domain and many known domains and motifs

(A) Schematic representation of VPS9a gene and positions of T-DNA insertion.

(B) VPS9a consists of two parts: the N-terminal conserved Vps9 domain and the C-terminal region with nosimilarity to known functional domains.

CHAPTER 1

Functional analyses of the plant-specific C-terminal region of VPS9a: the activating factor for RAB5 in *Arabidopsis thaliana*

Abstracts

Recent studies demonstrated that endosomal transport played important roles in various plant functions. The RAB GTPase regulates the tethering and fusion steps of vesicle trafficking to target membranes in each trafficking pathway by acting as a molecular switch. RAB GTPase activation is catalyzed by specific guanine nucleotide exchange factors (GEFs) that promote the exchange of GDP on the RAB GTPase with GTP. RAB5 is a key regulator of endosomal trafficking and is uniquely diversified in plants; the plant-unique RAB5 group ARA6 was acquired in addition to conventional RAB5 during evolution. In *Arabidopsis thaliana*, conventional RAB5, ARA7 and RHA1 regulate the endosomal/vacuolar trafficking pathways, whereas ARA6 acts in the pathway from the endosome to the plasma membrane. Despite their distinct functions, all RAB5 members are activated by the common GEF VPS9a. VPS9a consists of an N-terminal conserved domain and C-terminal region (CTR) with no similarity to known functional domains. In this study, I investigated the function of the CTR by generating truncated versions of VPS9a and found that it was specifically responsible for ARA6 regulation; moreover, the CTR was required for the oligomerization and correct localization of VPS9a. The oligomerization of VPS9a was mediated by a distinctive region consisting of 36 amino acids in the CTR that was conserved in plant RAB5 GEFs. Thus the VPS9a CTR plays an important role in the regulation of the two RAB5 groups in plants.

Introduction

In eukaryotic cells, proteins synthesized at the endoplasmic reticulum (ER) need to be correctly delivered to their destinations in order to fulfill their functions. The correct delivery of proteins is also essential for the proper functions of organelles, which actively exchange proteins and lipids with one another. This exchange is responsible for communication between organelles and signal transduction across the endomembrane system (Platta and Stenmark 2011; Bravo-Sagua et al 2014). These processes are fulfilled by the membrane trafficking system mediated by small membrane vesicles or tubules. Membrane trafficking is a fundamental system in eukaryotic cells that underlies diverse eukaryotic functions ranging from housekeeping events responsible for cell viability to higher-order functions of complex biological systems (Dacks and Field 2007). Several key regulators of membrane trafficking are evolutionarily conserved, including the RAB GTPase. The RAB GTPase is a small GTPase that belongs to the Ras superfamily and acts as a molecular switch by cycling between its GTP-bound active and GDP-bound inactive states. Activation of the RAB GTPase is catalyzed by specific guanine nucleotide exchange factors (GEFs) that promote the exchange of GDP on RAB GTPases with GTP. Activated RAB GTPases interact with specific sets of effector proteins; this interaction triggers various downstream functions, including the tethering of transport vesicles to target compartments. A large number of RAB effector proteins have been

identified to date, including tethering components, lipid kinases, and proteins implicated in signal transduction, cytoskeletal dynamics, and cytokinesis, indicating a wide range of functions of RAB GTPases in complex eukaryotic functions (Schwartz et al 2007; Stenmark 2009). Active RAB GTPases are inactivated to shut off the downstream effects by hydrolyzing GTP to GDP, which is supported by GTPase activating proteins (GAPs) (Stenmark 2009).

Recent comparative genomics studies indicated that each eukaryotic lineage was equipped with a unique set of RAB GTPases (Dacks and Field 2007; Dacks et al 2008; Mackiewicz and Wyroba 2009); this was also demonstrated to be true for the land plant lineage. While the plant lineage lacks several subgroups of RAB GTPases that are conserved in animals, several RAB members have undergone unique diversification during plant evolution (Rutherford and Moore 2002). For example, animals have only two or three RAB11 members that mainly mediate membrane trafficking around the recycling endosome (Ullrich et al 1996; Green et al 1997; Ren et al 1998). In contrast, 26 of 57 RAB GTPases encoded in the genome of *Arabidopsis thaliana* (hereafter referred to as Arabidopsis) are classified into the RAB11 group and are involved in various cellular functions (i.e., cytokinesis, endocytosis, exocytosis, tip growth of root hairs and pollen tubes, and defense responses) (de Graaf et al 2005; ChoIt al 2008; Szumlanski and Nielsen 2009; Feraru et al 2012; Asaoka et al 2013; Choi et al 2013).

RAB5 is a widely conserved RAB GTPase among eukaryotic lineages (Field et al 2007;

Pereira-Leal 2008) that localizes on early endosomes and the plasma membrane to regulate various endocytic events, including homotypic early endosomal fusion, endosomal motility, subcompartmentalization of the endosomal membrane, and signaling from early endosomes in the animal system (Grosshans et al 2006). RAB5 is also uniquely diversified in plants. Plants possess an additional type of RAB5 group (the ARA6 group) in addition to the conventional RAB5 groups conserved among eukaryotes. The Arabidopsis genome encodes two conventional RAB5s (RHA1/RABF2a and ARA7/RABF2b) and a plant-specific RAB5 (ARA6/RABF1) (Ueda et al 2001; Ueda et al 2004). ARA6 exhibits unique structural features, although it shares considerably high similarity with conventional RAB5. ARA6 is N-myristoylated and palmitoylated at its N-terminus instead of isoprenylated at its C-terminus, which is a conserved modification in the conventional RAB GTPases and known to be essential for their functions. ARA6-like RAB5 members are conserved in land plants and some algal species, but clear homologs have not been identified in eukaryotic lineages other than plants (Ebine et al 2011; Hoepflinger et al 2013). Conventional and plant-unique RAB5s are localized on partially overlapping but different endosomal populations in Arabidopsis and act in different trafficking pathways; for example, ARA7 and RHA1 regulate the vacuolar trafficking pathway (Sohn et al 2003; Bolte et al 2004; Kotzer et al 2004; Bottanelli et al 2011; Ebine et al 2011), while ARA6 acts in the pathway from endosomes to the plasma membrane (Ebine et al 2011).

Conventional and plant-unique RAB5s also seem to have distinct functions in basal plants; for example, the ARA6 homolog (CaARA6) and conventional RAB5 (CaARA7) are differently localized in the characean alga *Chara australi*s (Hoepflinger et al 2013; Hoepflinger et al 2015).

In animal cells, the activation of RAB5 is mediated by several distinct GEFs, such as RABEX-5, RIN family members, RME-6, and ALS2 (Carney et al. 2006). These RAB5 GEFs commonly harbor the distinctive functional Vps9 domain originally identified in the yeast Vps9p (Burd et al 1996; Hama et al 1999). In addition to the Vps9 domain, animal Rab5 GEFs contain several known protein domains (Carney *et al.*, 2006; Mattera *et al.*, 2006; Penengo *et al.*, 2006). For example, RIN family members possess the Ras association (RA) and SH2 (Src-homology) domains. ALS2 contains a regulator of chromosome condensation 1 (RCC1)-like domain, a Dbl homology (DH) domain, a pleckstrin homology (PH) domain, and eight consecutive membrane occupation and recognition nexus (MORN) motifs. RME-6 contains a Ras GAP-related domain, and RABEX-5 contains ubiquitin-binding regions and a zinc finger domain.

Distinct from the animal system, I previously demonstrated that Arabidopsis VPS9a (VACUOLAR PROTEIN SORTING 9a) with the Vps9 domain is practically the sole RAB5 GEF in the vegetative tissues of Arabidopsis (Goh et al 2007). VPS9a consists of helical

bundles followed by the Vps9a domain in the N-terminal half and C-terminal region (CTR), where no known protein domains and motifs have been identified. Interestingly, VPS9a activates both conventional and plant-unique RAB5, although these molecules are distinctly involved in endosomal trafficking (Goh *et al.*, 2007; Ebine et al., 2011). Because conventional RAB5 seems to fulfill counteracting functions with ARA6 (Ebine et al 2011), there should be a mechanism to distinctly regulate the two types of RAB5 in plants. In this study, I examined the function of the CTR of VPS9a in Arabidopsis. Biochemical analyses of the truncated version of VPS9a indicated that this region was specifically responsible for the modulation of GEF activity toward ARA6. I also found that this domain was required for the proper localization and oligomerization of VPS9a. Our results indicate that the CTR of VPS9a plays important roles in the regulation and coordination of the two RAB5 groups in plants.

Material and methods

Plant materials and growth conditions

Transgenic plants were generated using fluorescence-tagging of full-length proteins (Tian et al 2004). The cDNA encoding GFP or mRFP was inserted in front of the stop codon of the genomic sequence of *VPS9a*, which includes 2.5 kb 5'-flanking and 0.9 kb 3'-flanking sequences. VPS9aS304*-GFP and VPS9aS304*-mRFP were generated by replacing the

genomic sequence in *VPS9a* corresponding to the deleted region (from S304 to the stop codon) with the cDNA for GFP or mRFP. All chimeric genes were subcloned into the binary vector pGWB1 (Nakagawa et al 2007), which was a kind gift from Dr. T. Nakagawa (Shimane University). VPS9a-mRFP, VPS9a265-Venus and VPS9aY225A-GFP was expressed in *vps9a-2* (GABI_557C02), and VPS9aS304*-GFP and VPS9aS304*-mRFP were expressed in *vps9a-1* (SALK_018174). CTR-GFP was controlled by an estradiol-inducible promoter (Zuo et al 2000). The cDNA fragments of CTR was cloned into pMDC7 for estradiol inducible expression (Curtis and Grossniklaus 2003). Transformation of *A. thaliana* plants was performed via floral dipping using *Agrobacterium tumefaciens* (strain GV3101::pMP90) (Clough and Bent 1998). Transgenic plants expressing combinations of the GFP and mRFP fusion proteins were generated by cross-pollination; F1 and F2 generations were used for microscopic observation. All plants were grown on 1× Murashige Skoog (MS; Murashige and Skoog, 1962) medium plates supplemented with 30 mM sucrose, vitamins, and 0.3% phytagel (pH 5.7) in a climate-controlled chamber at 23 °C under continuous light.

Yeast two-hybrid assay

The cDNAs for the wild-type and mutant versions of RAB5 were subcloned into pAD-GAL4-2.1 (Stratagene). The cDNA for VPS9a and its mutant versions (S304*, S395*,

CTR, and ΔPRS) were subcloned into pAD-GAL4-GWRFC, which was kindly provided by Dr. T. Demura (NAIST). ΔPRS was generated by deleting the corresponding region of 36 amino acids (354-386aa) from the *VPS9a* cDNA. Plasmids containing each RAB5 and VPS9a were introduced into the AH109 strain (Clontech). Empty vectors were used for negative controls. Transformation was performed at least twice independently and at least three colonies were checked for the interaction for each transformation.

Expression and purification of GST fusion proteins

Full-length and truncated versions (S304*, S395*, and CTR) of VPS9a and RAB5 proteins (ARA7, ARA6, ARA6Nd1 and ARA6Nd2) fused to GST were expressed in *Escherichia coli* BL21 (DE3) using the pGEX 4T-1 vector (GE Healthcare). The cells expressing fusion proteins were collected and resuspended in lysis buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1 % β-mercaptoethanol, 1% Triton and protease inhibitor cocktail (GE Healthcare)], sonicated, and centrifuged at 10,000x*g* for 30 min. Supernatants were loaded onto glutathione-Sepharose 4B columns (GE Healthcare) and washed with 10 column volumes of washing buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1 % β-mercaptoethanol). Then, the fusion proteins were eluted with elution buffer (20 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl).

In vitro pull-down assay

RAB5 members were expressed in the yeast strain YPH414 (*MATa Δpep4:TRP1 ura3 lys2 ase2 trp1 his3 leu2*) under the control of the constitutive TDH3 promoter. HA-tagged full-length and truncated versions (CTR) of VPS9a were expressed in YPH414 under the regulation of the galactose-inducible *GAL1* promoter in galactose-containing medium. Collected cells were collapsed by vortexing with glass beads in PBS plus protease inhibitor cocktail (GE Healthcare) and 0.05% TIen20 (Wako); collected lysates were co-incubated with GST-VPS9a, GST-VPS9aS304*, GST-VPS9aS395*, or GST-CTR prebound to the glutathione-Sepharose 4B resin (GE Healthcare) for 60 min at room temperature. The protein complexes bound to resins were washed three times and subjected to immunoblot analyses. I confirmed that the results presented here were reproducible by independent assays repeated at least three times.

Nucleotide-exchange assay

Nucleotide exchange in the purified GST-tagged RAB5 was measured as reported previously (Goh et al 2007) using a fluorescence spectrophotometer (model F-2500; Hitachi High Technologies) (Figure 7). The assay was repeated at least three times for each RAB5 GTPase.

Antibodies

Antibodies against VPS9a, ARA7, and RHA1 were prepared as described previously (Goh et al 2007). Anti-HA monoclonal antibody was purchased from Zymed. The dilution of each antibody used in immunoblotting was as follows: anti-VPS9a, 1:1000; anti-ARA7, 1:1000; anti-RHA1, 1:800; anti-GFP, 1:1000; and anti-HA, 1:1000.

Microscopy

Roots of transgenic plants expressing GFP- and/or mRFP-tagged proteins were placed on glass slides (76×26 mm; Matsunami) and covered with a 0.12-0.17-mm-thick cover slip (24×60 mm; Matsunami). The root epidermal cells were observed under the LSM780 confocal microscope with an oil immersion lens (×63, numerical aperture = 1.40). For the Wm or BFA treatment, transgenic plants grown on 1× MS medium plates (0.3 % phytagel) for 5 days at 23 °C under continuous light were incubated with Wm (33 μM; Sigma-Aldrich) or BFA (50 μM; Sigma-Aldrich) in liquid MS medium at 23 °C for 1 hr and observed under the LSM780 confocal microscope. Quantification of colocalization between GFP- and mRFP-tagged proteins was carried out as previously described (Ito *et al.*, 2012).

Results

VPS9a localizes on the endosomes and partially the TGN

For detailed comparison of VPS9a with ARA7 and ARA6 in the same cell, I generated transgenic plants expressing VPS9a-GFP, ARA6-Venus and mRFP-ARA7. As observed in Figure 9, VPS9a exhibited the same localization with ARA6 and ARA7.

I have investigated into the subcellular localization of VPS9a in relation to that of several established organelle markers. I established transgenic plants with co-expression of VPS9a-GFP and organelle markers. These included mRFP- or Venus- tagged mRFP-SYP43 which is a marker for the TGN (Uemura et al 2004; Ebine et al 2011) and mRFP-VAMP722 for TGN(Zhang et al 2011; Asaoka et al 2013), the ARA6-Venus and mRFP-ARA7(Lee et al 2004; Ueda et al 2004), for the MVE, ST-mRFP, for the *trans*-Golgi(Boevink et al 1998; Wee et al 1998), mRFP-VAM3, for the Vacuole(Uemura et al 2002) (Figure 9A).

For the more quantitative comparison for subcellular localization of VPS9a and other organelle markers, I used a macro in Metamorph software (Ito et al 2012). I classified the resulting distances into three groups; (1) colocalized: a distance between two centers that was below the resolution limit of the objective lens (0.24 μm in this study), (2) associated; a distance less than the sum of the radii of two punctate signals (<1 μm in this study), and (3) independent; a distance larger than the sum of the radii of two punctate signals (>1 μm) (Figure 9B).

VPS9a-GFP colocalized most closely with MVE markers, ARA7 and ARA6, and partially with TGN markers, VAMP722 and SYP43.

The VPS9a CTR is required for interaction with ARA6

I have previously demonstrated that VPS9a interacted with two types of RAB5 in Arabidopsis, although the two groups of RAB5 have distinct (and seemingly counteracting) functions (Goh et al 2007; Uejima et al 2010; Ebine et al 2011). I hypothesized that the CTR of the VPS9a protein (Figure 10A) could have a specific role in its GEF activity for the plant RAB5, because none of the Vps9 domain-containing proteins identified in non-plant systems harbored a similar domain. To verify this possibility, I generated a truncated mutant of VPS9a in which the Ser located after the conserved Vps9 domain was replaced with a stop signal, yielding a truncated VPS9a protein consisting of 303 amino acids (VPS9aS304*). The Vps9 domain of VPS9a consists of 265 amino acids, and VPS9a is predicted to have a coiled coil motif behind the Vps9 domain. Given this coiled coil motif in VPS9a function, I decided to generate a truncate version of VPS9a containing the coiled coil motif. I tested the interaction between VPS9a and RAB5 members using the yeast two-hybrid method. I confirmed the interaction between full-length VPS9a and the GDP-fixed mutants (SN) of RAB5 members that were reported previously (Goh et al 2007) (Fig. 1b). Intriguingly, the deletion of the CTR specifically

abolished the interaction with ARA6SN in this assay (Figure 10B), whereas the nucleotide binding-deficient mutant of ARA6 (NI) retained the ability to interact with VPS9aS304* (Figure 10C). I also confirmed the interaction between conventional RAB5 and VPS9aS304* using the pull-down assay, which further supported the hypothesis that the CTR deletion did not affect the interaction between VPS9a and conventional RAB5. Thus, these results suggested that the CTR was required for the interaction between VPS9a and ARA6 but not the interaction with conventional RAB5.

Regulatory effect of the CTR on the GEF activity on ARA6

To examine the effect of the CTR deletion on the GEF activity against RAB5 members, I compared nucleotide exchange activities between full-length and truncated versions of VPS9a. The activity was measured by monitoring changes in fluorescence from intrinsic tryptophan residues upon exchange of the binding nucleotide from GDP to GMP-PNP (a non-hydrolysable analog of GTP). VPS9a exerted nucleotide exchange activity toward ARA7 and ARA6 as previously reported (Goh et al 2007) (Figure 19A and B). VPS9aS304* exhibited GEF activity similar to full-length VPS9a toward ARA7, suggesting that the CTR did not have a notable regulatory role in the activation of conventional RAB5 (Figure 11A . In contrast, VPS9aS304* exhibited increased GEF activity toward ARA6 compared with full-length VPS9a (Figure 11B).

Taken together the VPS9a CTR specifically exerted a negative effect on VPS9a GEF activity toward ARA6.

CTR-mediated oligomerization of VPS9a

Yeast Vps9p harbors the ubiquitin-binding CUE domain in addition to the Vps9 domain, and dimerization of the CUE domains leads to the binding of another ubiquitin to enable lattice contact between the CUE domains and ubiquitin (Prag et al 2003). Although the CTR of VPS9a does not contain the CUE domain, it is still possible that VPS9a forms oligomers mediated by the CTR. I tested this possibility using the yeast two-hybrid and *in vitro* pull-down assays. Full-length VPS9a interacted with full-length VPS9a and the CTR but not VPS9aS304* in the yeast two-hybrid experiment (Figure 12A). The *in vitro* pull-down assay consistently demonstrated that HA-tagged full-length VPS9a pulled down GST-tagged full-length VPS9a and the CTR but not VPS9aS304* (Figure 12B). The HA-tagged CTR also pulled down GST-tagged full-length VPS9a and the CTR (Figure 12B). These results indicated that VPS9a formed a homo-oligomer in a manner that was mediated by the CTR.

To obtain further information on the region required for oligomerization, I constructed additional deletion mutants of VPS9a. The VPS9aS395* mutant lacked the latter half of the CTR (Figure 10A); this mutant was used to test the interaction between RAB5 members and

VPS9a using the yeast two-hybrid method. VPS9aS395* interacted with ARA7 and full-length VPS9a but not ARA6 (Figure 13A). Thus, the C-terminal half of the CTR is required for the interaction with ARA6, but this region is not required for the homo-oligomerization of VPS9a. This interaction was also confirmed by the pull-down assay using purified GST-tagged full-length or truncated versions of VPS9a and the yeast lysate prepared from yeast cells expressing HA-tagged VSP9a (Figure 13B). Furthermore, I found that plant RAB5 GEFs shared a distinctively conserved region consisting of 36 amino acids in the CTR that is referred to as the plant-specific high similarity region (PSR). I generated a mutant lacking the PSR (VPS9aΔPSR) and tested whether this mutant formed homo-oligomers. VPS9aΔPSR did not interact with full-length VPS9a in the yeast two-hybrid assay (Figure 13C). Collectively, the results strongly indicated that VPS9a oligomerization was mediated by the PSR and that homo-oligomerization was a common feature of plant RAB5 GEEs that harbor the PSR sequence.

Function and subcellular localization of mutant VPS9a

I examined the functionality and subcellular localization of VP9aS304* by introducing this mutant tagged with the green fluorescent protein (GFP) under regulation of the *VPS9a* promoter into the *vps9a-1* mutant that exhibited embryonic lethality (Goh et al 2007).

VPS9aS304*-GFP rescued the lethal phenotype of *vps9a-1*, exhibiting only residual growth abnormalities, if any (Figure 14A). Thus, the CTR is not essential for plant growth under laboratory conditions.

Next, I examined the subcellular localization of VPS9aS304*-GFP. This chimeric protein was localized to the punctate compartment, which was similar to the pattern observed for full-length VPS9a tagged with GFP and expressed in the *vps9a-1* mutant (Figure 14B). To examine the effect of the CTR truncation on the subcellular localization of VPS9a, I compared the subcellular localization of VPS9aS304*-mRFP and VPS9a-GFP. To examine the possible effect of the distinct fluorescent tags on subcellular localization, I also coexpressed VPS9a-GFP and VPS9a-mRFP in the Arabidopsis plant; in this plant, VPS9a tagged with GFP or mRFP overlapped almost completely in the punctate compartment, which was also confirmed by the quantification analysis (Figure 14C and D). In contrast, VPS9aS304*-mRFP and VPS9a-GFP localized in distinct populations of punctate compartments with overlap (Figure 14C and D); punctate compartments with only VPS9a-GFP were observed (for example, arrowhead in Figure 14C). Then, I tested the effect of wortmannin (Wm; an inhibitor of phosphatidylinositol 3- and 4- kinases that induces the dilation of multivesicular endosomes in Arabidopsis root cells) (Jaillais et al 2008) and brefeldin A (BFA; an inhibitor of activating factors for ARF GTPases that induces agglomeration of organelles in Arabidopsis root cells)

(Grebe et al 2003) on punctate compartments bearing fluorescently tagged VPS9a and/or VPS9aS304*. I did not observe difference in the responses of VSP9a-GFP and VPS9a-mRFP to this drug; a subpopulation of the fluorescent compartments was dilated upon Wm treatment. Conversely, Wm differentially affected the punctate compartments bearing VPS9aS304*-mRFP and VPS9a-GFP; a substantial population of VPS9a-GFP-positive compartments retained punctate morphology after Wm treatment, whereas VPS9aS304*-mRFP-positive compartments were more sensitive to Wm treatment (Figure 14C). BFA also conferred distinct effects; a considerable population of VPS9aS304*-mRFP and VPS9a-GFP aggregated into the same compartments, but compartments bearing only VPS9a-GFP were more resistant to BFA treatment (Figure 14C). I then quantified the effect of Wm in the root epidermal cells from transgenic plants expressing VPS9a-GFP or VPS9aS304*-GFP to confirm the distinct effects of Wm on punctate compartments bearing VPS9a-GFP and VPS9aS304*-GFP. VPS9aS304*-GFP-positive compartments were more sensitive to Wm treatment (Figure 14E). Thus, these results suggested that the CTR was responsible for targeting VPS9a to the Wm-resistant compartment. Alternatively, the CTR could be required to maintain the endosomal compartment resistant to Wm.

VPS9a265* has increased GEF activity,even higher than VPS9aS304*, toward ARA6.

The truncated VPS9a mutant 265*, which is positioned at end of the Vps9 domain, contains a helical bundle and the Vps9 domain (Figure 15A). The GST-VPS9a265* recombinant protein, demonstrated increased GEF activity towards ARA6, even higher than S304*, but not toward ARA7 (Figure 15B). To observe the subcellular localization of VPS9a265*, I fused a Venus fused protein that was driven by its own promoter to the truncated mutant. 265-Venus is expressed in plants (Figure 15D), and localizes to the cytosol, not to the dot-like structures (Figure 15C). It may not be necessary for VPS9a RAB5 activation on the endosome membrane. *The vps9a-2* mutant, a weak mutant allele that exhibits leaky phenotypes in multiple developmental stages, revealed abnormality in root elongation and cell division. The *vps9a-2* mutant phenotypes were complemented by VPS9a265-Venus (Figures 15E and F).

CTR-GFP localized to the cytosol.

To observe the subcellular localization of CTR, I generated transgenic plants expressing a GFP-tagged CTR. GFP-CTR was not expressed with its own promoter or the 35S promoter; thus, it was transiently expressed in wild-type *A. thaliana* using an estradiol-inducible promoter (Zuo et al., 2000). Plants were grown on MS-0 plates for 5 days and then transferred to MS plates with estradiol (10 μM) for 2 days. CTR-GFP localized only to the cytosol *in planta* (Figure 16).

ARA6 specific N-terminal region is not involved in GEF activity.

I demonstrated that the CTR is involved in ARA6 regulation. Conversely, I examined whether the ARA6 N-terminal region is involved in GEF activity by generating two deletion mutants of ARA6, Nd1 and Nd2 (Figure 17A), and measured their GEF activities. Nd1 and Nd2 showed GEF activities to the same extent as full-length ARA6 (Figure 17B). Thus, the ARA6-specific N-terminal region is not involved in GEF activity.

GEF activity does not affect VPS9a subcellular localization.

During my master's research, I found that VPS9a D185 and Y225 are essential role for GEF activity and interactions with RAB5s *in vitro*. However, VPS9aD185A and Y225A rescued the lethal *vps9a-1*phenotype. These plants had no phenotypes under laboratory conditions. To investigate in detail, I observed subcellular localization of VPS9aY225A-GFP in *vps9a-2*. Y225 is located in the Vps9 domain (Figure 18A). The GFP-fused protein was driven by its own promoter. The *vps9a-2* phenotypes were also complemented by VPS9aY225A-GFP (Figures 18C and D). VPS9aY225A-GFP localized predominantly to punctate organelles and to the cytosol. The organelles labeled by VPS9aY225A-GFP were sensitive to both wortmannin and BFA (Figure 18B). These results indicate that VPS9a GEF activity does not affect its

subcellular localization.

Discussion

ARA6-specific regulatory region in VPS9a

Arabidopsis has two types of RAB5 GTPases, conventional RAB5s and plant-specific ARA6. Although these two types of RAB5 reside on multivesicular endosomes (Haas et al 2007) and share the common activating factor VPS9a, their roles in endosomal trafficking are different. Indeed, ARA6 and conventional RAB5 appear to play counteracting roles in endosomal trafficking (Ebine et al 2011). Thus, I postulated that the upstream activating factor of RAB5 members (VPS9a) could distinctly regulate RAB5 members. In this study, I demonstrated that deletion of the CTR resulted in a specific increase in GEF activity towards ARA6 in a biochemical GEF assay, suggesting that the CTR is specifically responsible for the regulation of ARA6 activity. Although VPS9aS304* exhibited elevated GEF activity towards ARA6 compared with full-length VPS9a, I did not detect an interaction between VPS9aS304* and ARA6 in our yeast two-hybrid experiment. This is most likely due to the fact that the high GEF activity of VPS9aS304* towards ARA6 resulted in the rapid dissociation of ARA6 from VPS9aS304*, because the interaction with the GEF was generally detected only for GDP-bound RAB GTPases (Hama et al 1999; Tall et al 2001; Sato et al 2005; Goh et al 2007;

Zhu et al 2009).

My results revealed that VPS9a contains two distinctive functional regions, the Vps9 domain and the CTR. I did not detect a significant sequence similarity between the CTR and any other RAB5 GEFs identified from non-plant systems. Thus, it can be said that the CTR has been uniquely acquired during plant evolution and recruited to regulate of the plant-specific RAB5 (ARA6).

CTR mediates VPS9a homo-oligomerization

Some RAB GEFs have been reported to act as dimers, which is exemplified by the yeast Vps9p and animal ALS2 (Prag et al 2003; Kunita et al 2004). A rice RAB5 GEF (GLUP6) has also been shown to form an oligomer (Fukuda et al 2013). My results indicated that VPS9a formed an oligomer via a process that was mediated by the CTR. In yeast, Vps9p dimerization is responsible for binding multiple ubiquitin molecules (Prag et al 2003). However, the VPS9a CTR does not contain any known ubiquitin binding motifs; thus, how VPS9a dimerization affects the function of VPS9a remains unclear. As mentioned above, the CTR was specifically required for the regulation of ARA6 activation. Thus, VPS9a dimerization may affect the regulatory functions of the CTR *in vivo*, which in turn may enable the selective activation of either of the two RAB5 groups in plants (Figure 19). I also found that RAB5 GEFs in plants

shared a distinctively conserved region (PSR) in the CTR that seemed to be responsible for VPS9a dimerization. The PSR is also conserved in putative RAB5 GEFs in green algae, including *Chara australis*, *Volvox carteri*, and *Chlamydomonas reinhardtii*. Among these species, an orthologous product of ARA6 is encoded in the *C. australis* genome, whereas *V. carteri* and *C. reinhardtii* do not possess *ARA6* orthologs. Thus, it was possible that the CTR and dimerization of RAB5 GEFs could had functions other than the regulation of ARA6 in algal species without *ARA6* homologs. This hypothesis should be examined in future comparative studies between the Arabidopsis VPS9a and algal RAB5 GEF proteins.

The CTR is required for the subcellular localization of VPS9a

The results of the complementation analysis using the *vps9a-1* mutant indicated that the truncated mutant of VPS9a (VPS9aS304*) restored the embryonic lethality observed in the mutant without the marked abnormalities in the complemented mutant plants. Thus, deletion of the CTR exerted little effect on plant growth under laboratory conditions. This result could be consistent with our previous finding that the loss of function of *ARA6* and expression of constitutively active ARA6 do not result in visible phenotypic abnormalities (Ebine et al 2011), whereas the nucleotide status of ARA6 in the transgenic plants and physiological significance of oligomerization of VPS9a *in planta* should be verified in future studies. The CTR was also

required for the subcellular localization of VPS9a and/or insensitivity of endosomes to Wm treatment. ARA7 has been demonstrated to localize on the TGN in addition to multivesicular endosomes (Stierhof and El Kasmi 2010; Choi et al 2013); thus, some population of conventional RAB5 may be activated on the TGN, which should require the presence of VPS9a. The Wm-insensitive population of VPS9a-positive compartments may represent the TGN, because the treatment with Wm under experimental conditions employed in this study does not affect SYNTAXIN OF PLANTS 43 (SYP43)-labeled TGN morphology (Ebine et al 2011; Ito et al 2012). Alternatively, VPS9a could play a role in recruiting an unknown factor that conferred sensitivity to Wm to multivesicular endosomes in a CTR-dependent manner.

In this study, I identified a specific role for the CTR of VPS9a, which did not exhibit similarity with known protein domains. Future detailed analyses of the mode of interaction between VPS9a and the two types of RAB5 GTPases are expected to provide more information on the function of the CTR. In animal systems, an effector molecule of RAB5 (Rabaptin-5) has been shown to form a tripartite complex with GTP-bound RAB5 and its GEF Rabex-5 (Horiuchi et al 1997); the formation of this complex results in enhanced nucleotide exchange activity of Rabex5, leading to positive feedback for RAB5 activation (Horiuchi et al 1997; Lippé et al 2001). Thus, it is reasonably presumed that an effector protein of ARA6 and/or canonical RAB5 also has a similar function. The identification and characterization of such an

effector protein would also be an interesting future project that would potentially unravel how the two types of RAB5 in plants are regulated by the common activating factor VPS9a.

Mechanisms of localization and activation of VPS9a.

Details regarding VPS9a localization still remain unclear. Full-length VPS9a and VPS9aS304* were localized to the endosomes, while VPS9a265* and CTR were not. One should consider the possibility that amino acids 266 to 304 constitute a membrane-binding motif. It is also likely that other factors could support to recruitment of VPS9a to the endosome membrane.

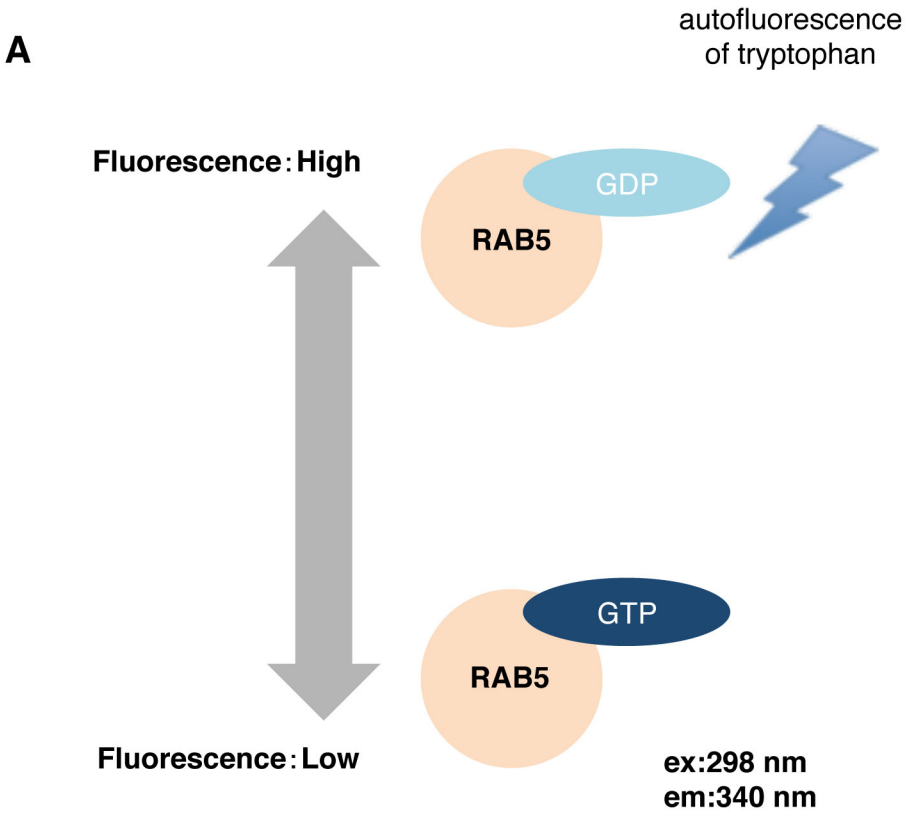
VPS9a265-Venus appeared to partially localize to the nucleus. In mammals, a truncated mutant lacking the endosomal targeting domain localizes to some extent (Chen et al 2008; Blümer et al 2013).

Y225A-GFP localized to the endosomes, consistent with the mammalian RAB5 GEF RABEX-5(Xu et al 2010; Blümer et al 2013). Mutations that reduce tRAB5 GEF activity did not influence its subcellular localization. Although VPS9aY225A exhibited much lower GEF activity in vitro, it rescued the embryonic lethality of *vps9a-1*. One copy of ARA7 or RHA1 in *A. thaliana* is sufficient for survival under laboratory conditions (Ebine et al 2011), suggesting that RAB5 exists in excess in wild-type *A. thaliana*. The effect of the Y225A mutation could be visualized when the copy numbers of conventional RAB5s were reduced.

It is unclear where VPS9a activates RAB5 groups in plant cells. Other factors may also be involved in the GEF activity of VPS9a *in vivo*. Identification of interacting proteins or potential regulators will be important for better understand mechanism of localization and activation mechanisms of VPS9a.

Accession numbers

The Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are At3g19770 (VPS9a), At3g54840 (ARA6/RABF1), At5g45130 (RHA1/RABF2a), At4g19640 (ARA7/RABF2b), At3g05710 (SYP43), At2g33120 (VAMP722) and At5g46860 (VAM3).



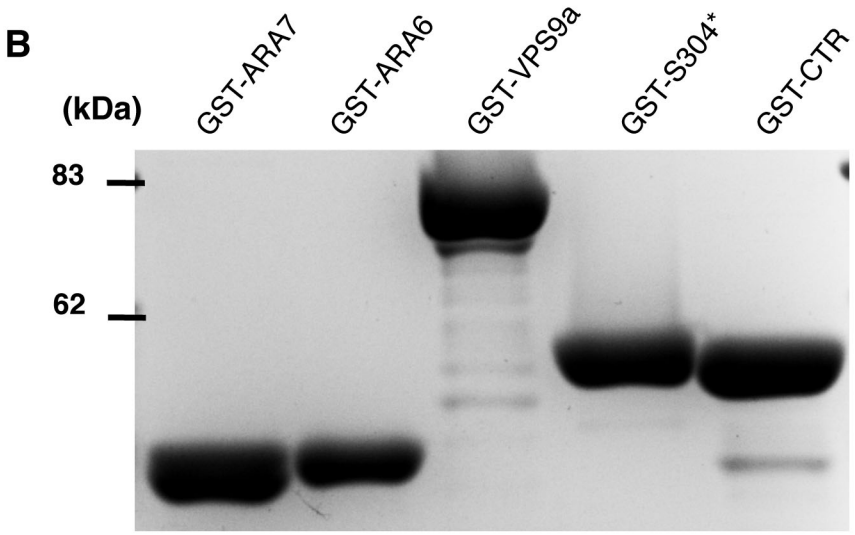


Figure 7. Measurement of GEF activity

(A) Schematic diagram of measurement of tryptophan autofluorescence. The reaction initially contained ARA7 and ARA6 (1 μM) with/without full-length or deletion mutant of VPS9a. GMP-PNP (GTP-nonhydrolyzable analog) was added at 100 sec, and the GDP form to GTP form transition was monitored by tryptophan autofluorescence of GTPase at 340 nm.

(B) CBB staining image of purified proteins.

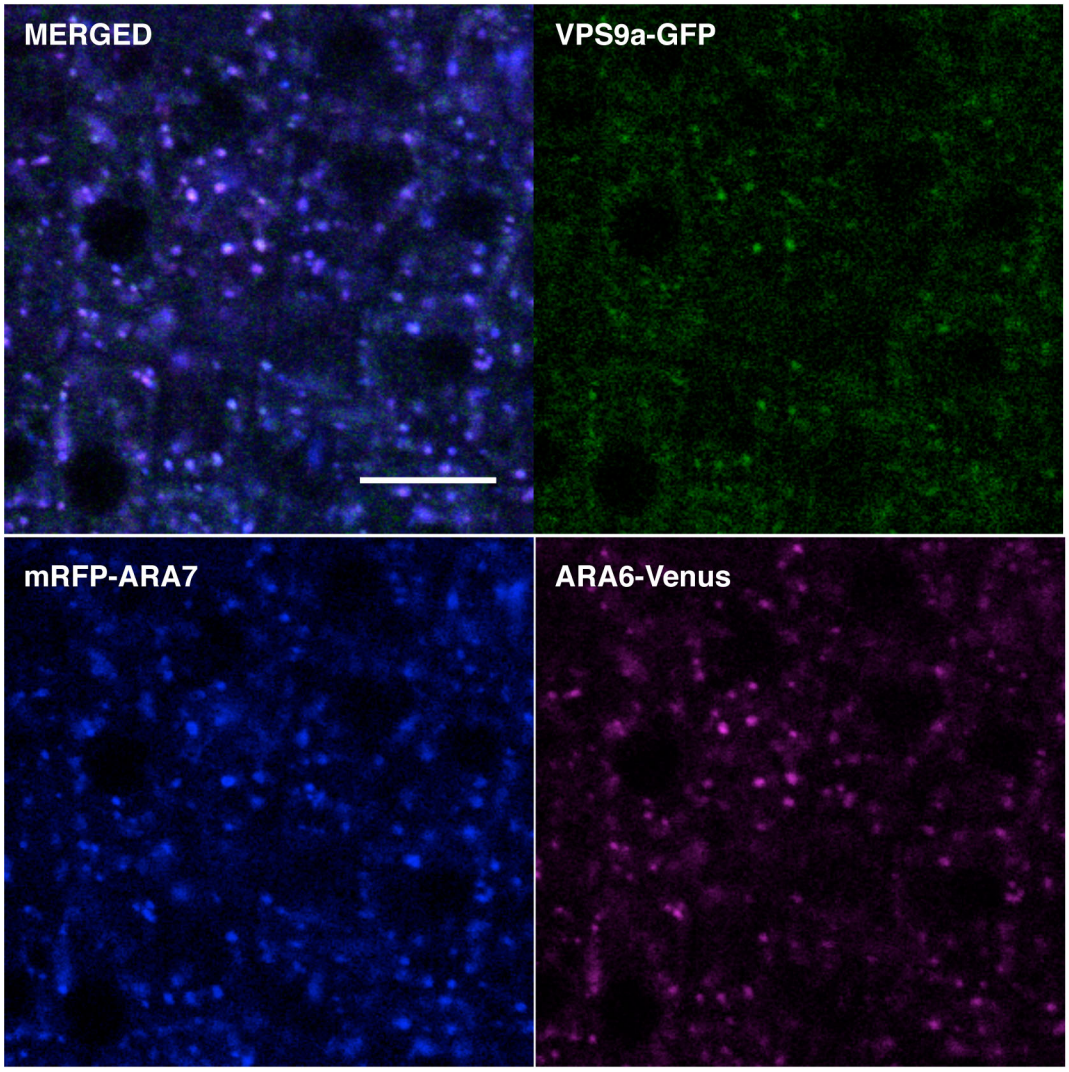


Figure 8. VPS9a colocalizes with ARA6 and ARA7 on endosomes.
Subcellular localization of VPS9a-GFP (green), mRFP-ARA7 (magenta) and ARA6-Venus (blue) with BFA treatment. Bar =10 μm.

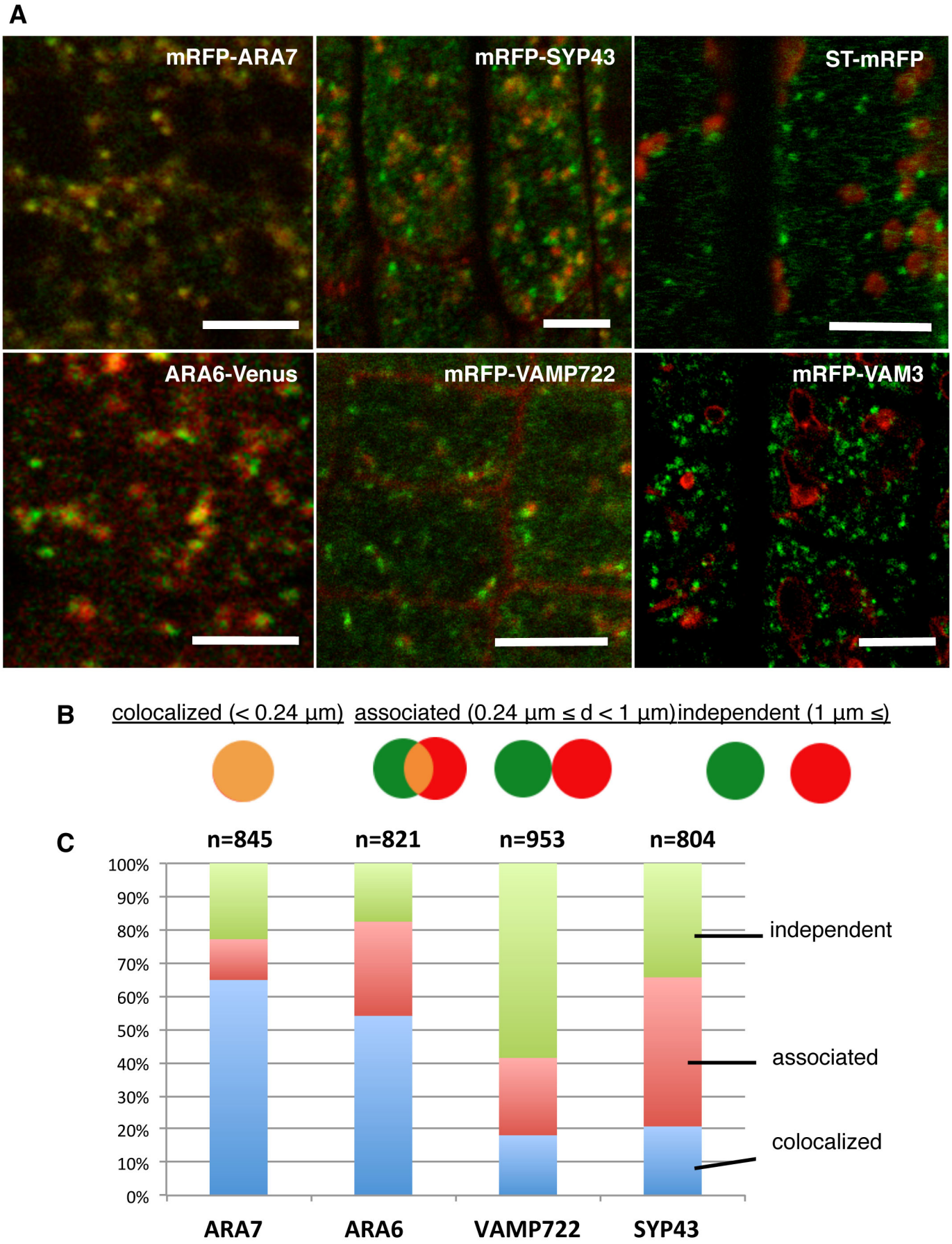


Figure 9. Subcellular localization of VPS9a-GFP and organelle markers.

(A) VPS9a-GFP (green) and Organelle maerkers (red); mRFP-ARA7, ARA6-Venus, mRFP-SYP43, mRFP-VAMP722, ST-mRFP and mRFP-VAM3. 5-day root epidermal cells. Bars = 5 μm

(B) Schematic representation of the criteria for classfying the relationship between VPS9a-GFP and amarker for a subcellular organelle.

(C) Quantitative analysis of subcellular localization with VPS9a-GFP and organelle markers. N= the total number of fluorescent points analyzed.

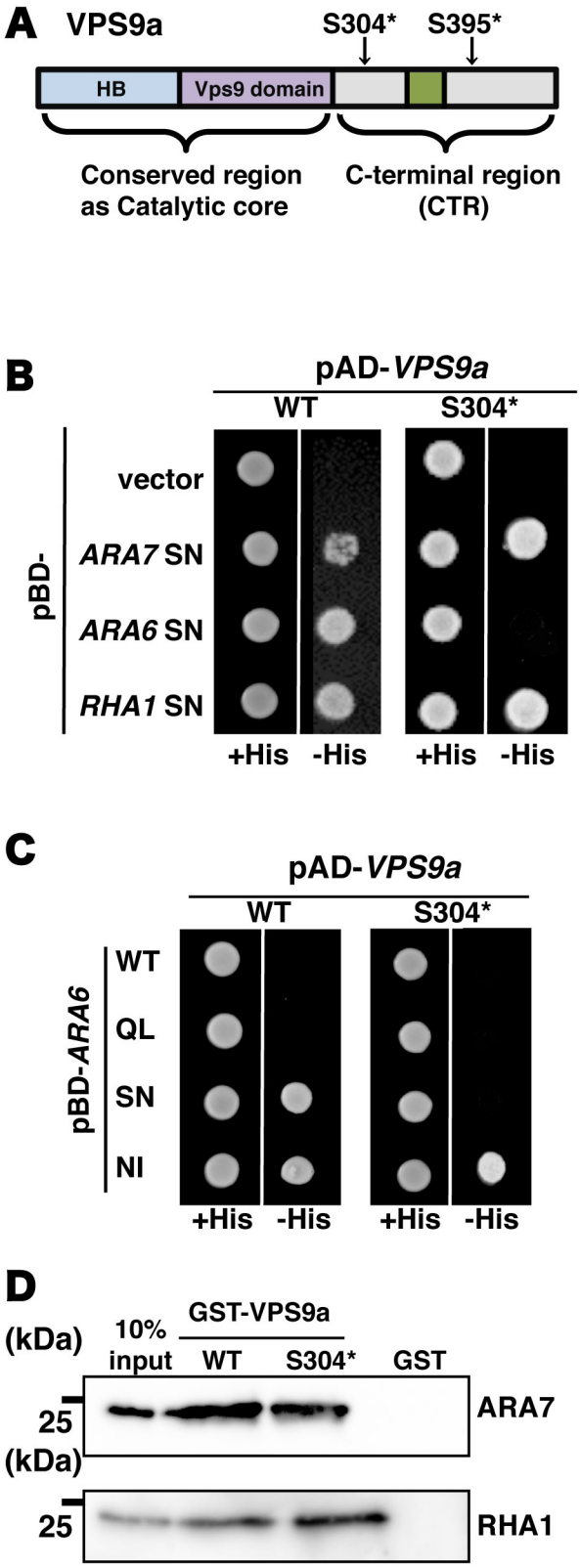


Figure 10. The CTR of VPS9a mediates the interaction with ARA6.

(A) The schematic structure of VPS9a. VPS9a consists of the N-terminal helical bundle (HB), Vps9 domain, and C-terminal region (CTR) comprising the plant-specific region (PSR, green box). Mutations used in this study (S304* and S395*) are also indicated.

(B) Interactions between RAB5 members and VPS9a detected by the yeast two-hybrid assay. The transformants were spotted onto selection plates and incubated at 30 °C for 5 days. Interactions between two proteins were tested using the *HIS3* gene.

(C) Interaction between VPS9a and wild-type and mutant versions of ARA6. QL; GTP-freeze, SN; GDP-freeze, and NI; nucleotide-free mutants.

(D) Invitro pull-down assay between full-length (WT) or truncated (S304*) VPS9a and ARA7 or RHA1.

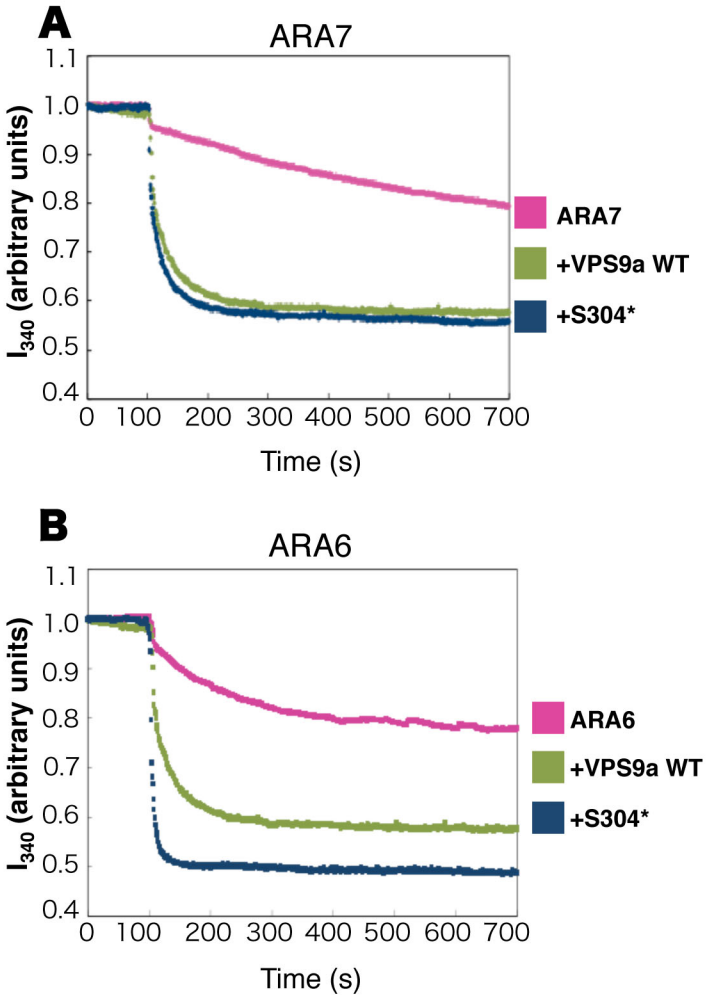


Figure 11. The CTR has a regulatory role in the activation of ARA6.
The conformational changes of ARA7 (upper) and ARA6 (lower) were detected by measuring autofluorescence from Trp in the absence (pink) or presence of 1 μM GST-VPS9a (green) or 1 μM GST-VPS9aS304* (blue).

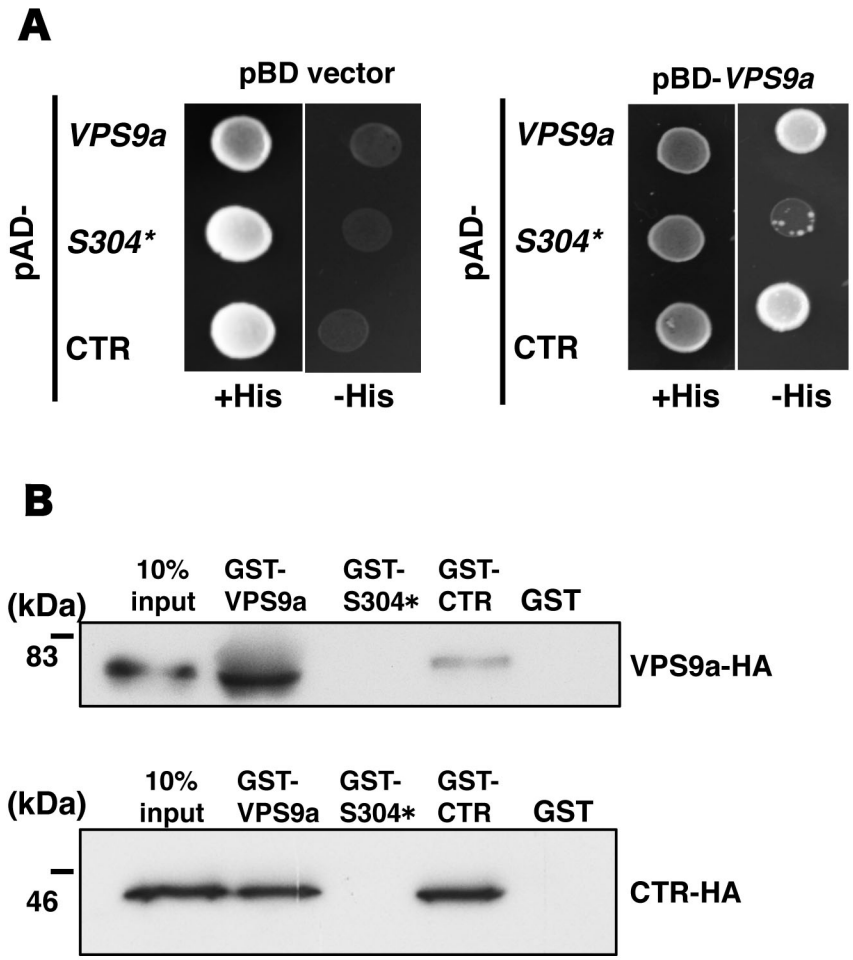


Figure 12. VPS9a forms oligomers via a process mediated by the CTR.

(A) Homo-oligomer formation of VPS9a detected by the yeast two-hybrid assay.

(B) Homo-oligomer formation of VPS9a detected by the pull-down assay. Samples were analyzed by immunoblotting with the anti-HA antibody.

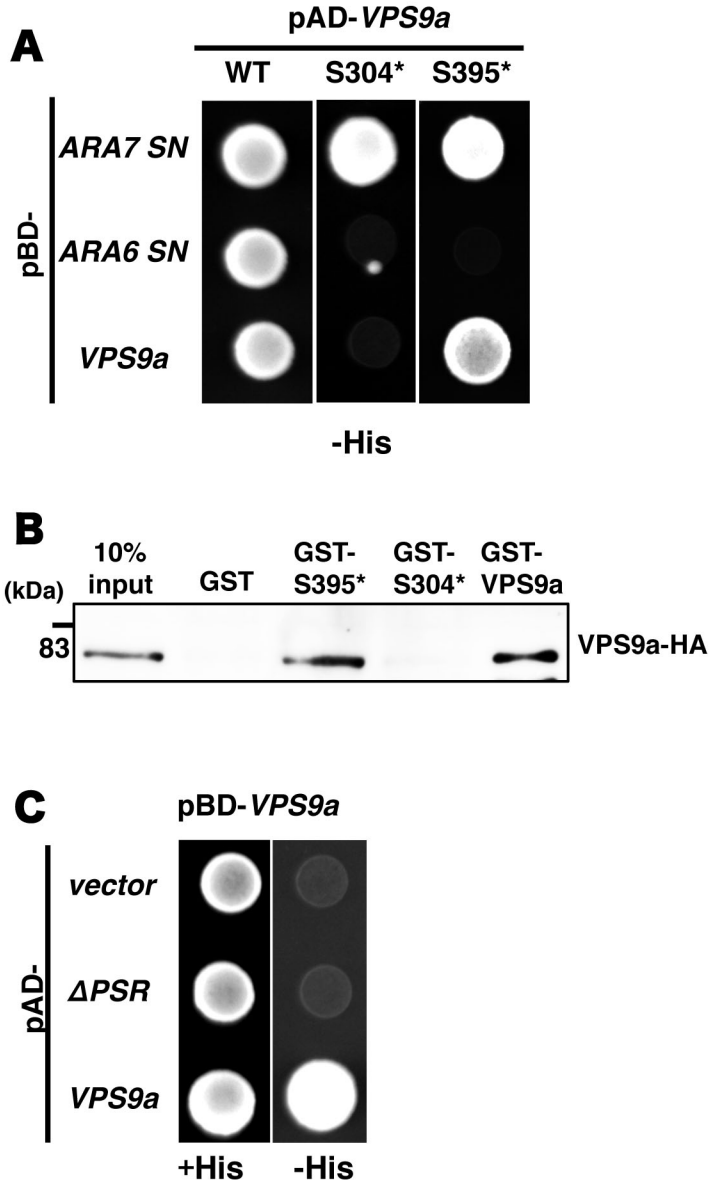
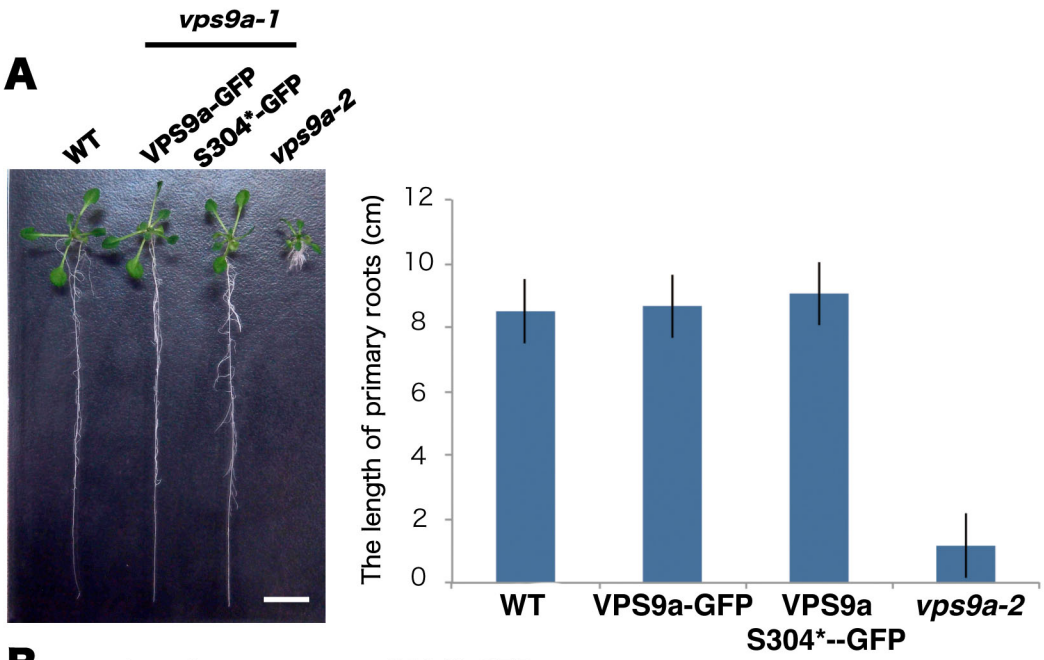


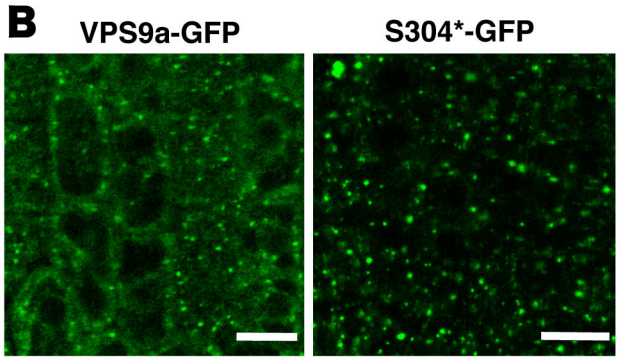
Figure 13. The plant-specific region (PSR) is required for oligomer formation of VPS9a.

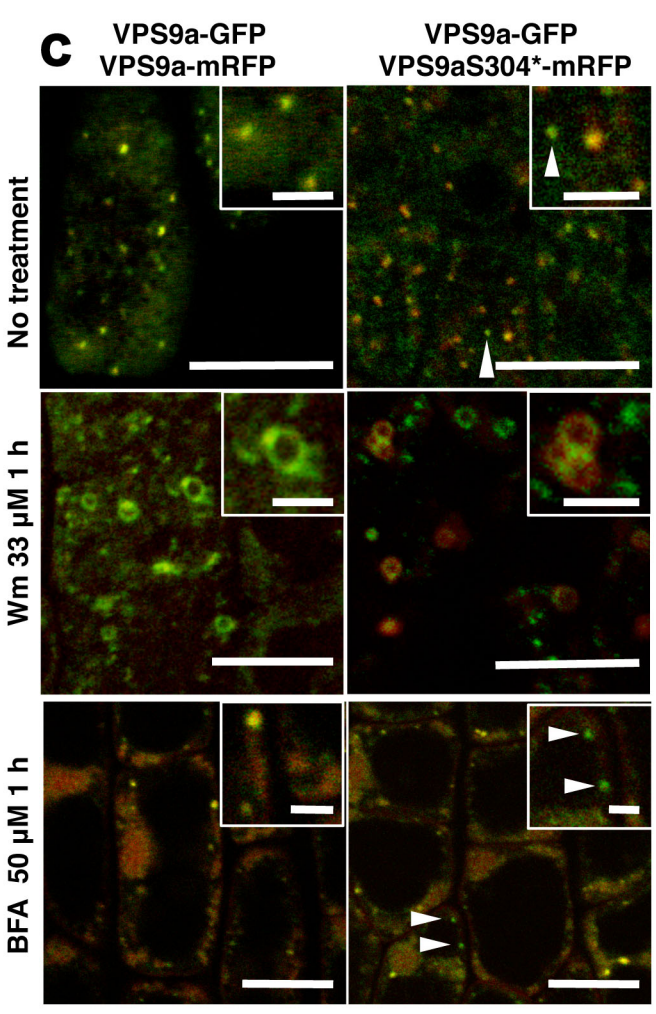
(A) Interaction between full-length (WT) or truncated versions (S304* and S395*) of VPS9a and ARA7SN, ARA6SN, or VPS9a detected by the yeast two-hybrid assay. SN; GDP-freeze mutant.

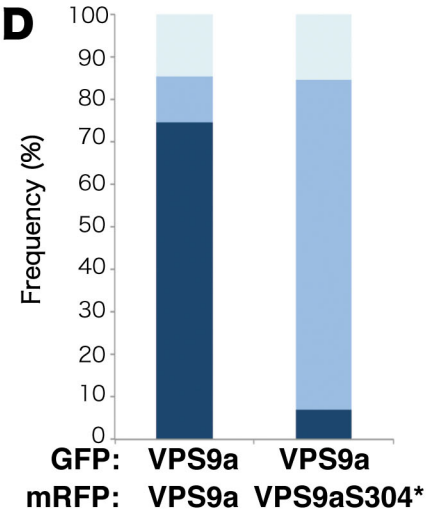
(B) Interaction between full-length (WT) or truncated versions (S304* and S395*) of VPS9a and HA-tagged full-length of VPS9a detected by the pull-down assay.

(C) VPS9a without the PSR (ΔPSR) does not interact with full-length VPS9a in the yeast two-hybrid assay.


A
vps9a-1
WT
VPS9a-GFP
S304*-GFP
vps9a-2
The length of primary roots (cm)
0
2
4
6
8
10
12
WT
VPS9a-GFP
VPS9a S304*--GFP
vps9a-2


B
VPS9a-GFP
S304*-GFP


C
VPS9a-GFP
VPS9a-mRFP
VPS9a-GFP
VPS9aS304*-mRFP
No treatment
Wm 33 μM 1 h
BFA 50 μM 1 h


D
Frequency (%)
0
10
20
30
40
50
60
70
80
90
100
GFP:
mRFP:
VPS9a
VPS9a
VPS9a
VPS9aS304*

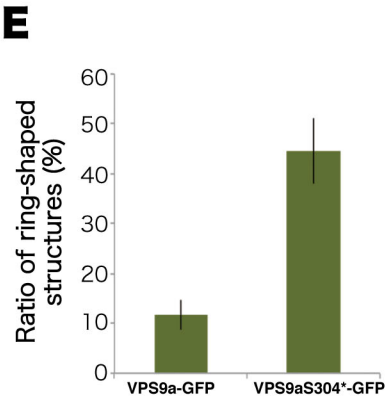

E
Ratio of ring-shaped structures (%)
0
10
20
30
40
50
60
VPS9a-GFP
VPS9aS304*-GFP

Figure 14. Deletion of the CTR results in alteration of the subcellular localization of VPS9a.

(A) Fourteen-day-old wild-type and *vps9a-1* mutant plants expressing VPS9a-GFP or VPS9aS304*-GFP or the *vps9a-2* mutant (left). Scale bar = 1 cm. The *vps9a-1* phenotype was rescued by VPS9aS304*-GFP to a similar extent as VPS9a-GFP. This finding was supported by the quantification of the lengths of the primary roots (right). Results are presented as mean ± standard deviation.

(B) Subcellular localization of VPS9a-GFP and VPS9aS304*-GFP in root tip cells from 5-day-old plants. Bars = 10 μm.

(C) Root tip cells from 5-day-old plants expressing VPS9a-GFP (green) and VPS9a-mRFP or VPS9aS304*-mRFP (red) treated without (upper panels) or with (lower panels) Wm. Bars = 10 μm.

(D) Quantitative analysis of colocalization between VPS9a-GFP and VPS9a-mRFP or VPS9aS304*-mRFP using the method described in (Ito *et al.*, 2012). The distance from the center of each GFP signal to the center of the nearest mRFP signal was measured. n = 580 GFP signals for the transgenic plants expressing VPS9a-GFP and VPS9a-mRFP, and 507 GFP signals for the plant expressing VPS9a-GFP and VPS9aS304*-mRFP.

(E) Ratios of ring-shaped structures in total numbers of fluorescent compartments in root tip cells from 5-day-old plants expressing VPS9a-GFP or VPS9aS304*-GFP after Wm treatment. Four images from at least three independent roots were examined. Results are presented as mean ± standard deviation.

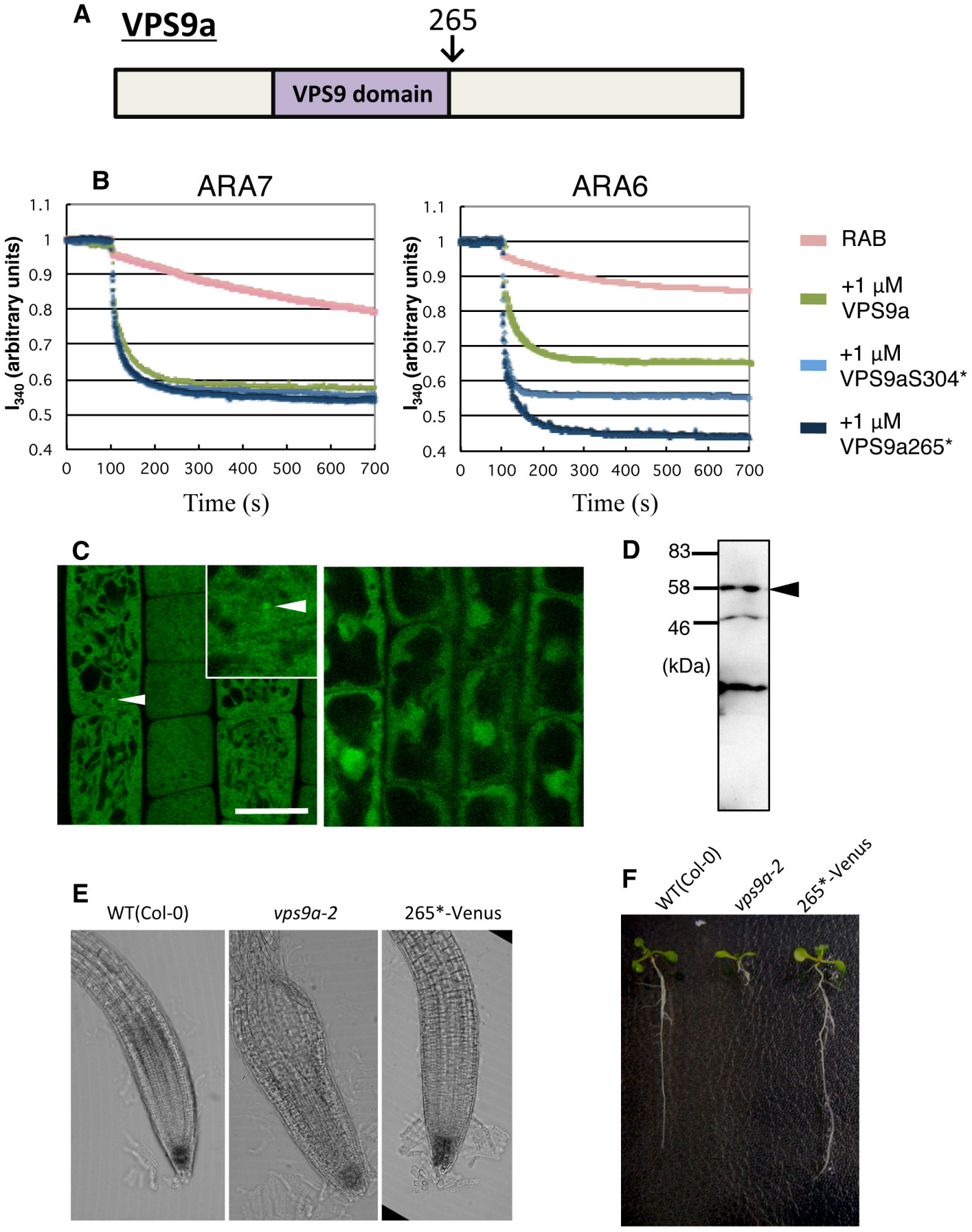


Figure 15. VPS9a265* showed specific increased in the activity toward ARA6

(A) Position of 265 in VPS9a.

(B) In vitro GEF assay of GST-VPS9a265*

(C) Subcellular localization of VPS9a265*-Venus in *A.thaliana*. Bar = 10 μM

(D) Immunoblot analysis of expression of VPS9a265-Venus. Arrow head indicates VPS9a265*-Venus.

(E) Images of root tip of VPS9a265*-Venus plants.

(F) Root elongation of VPS9a265*-Venus in vps9a-2.

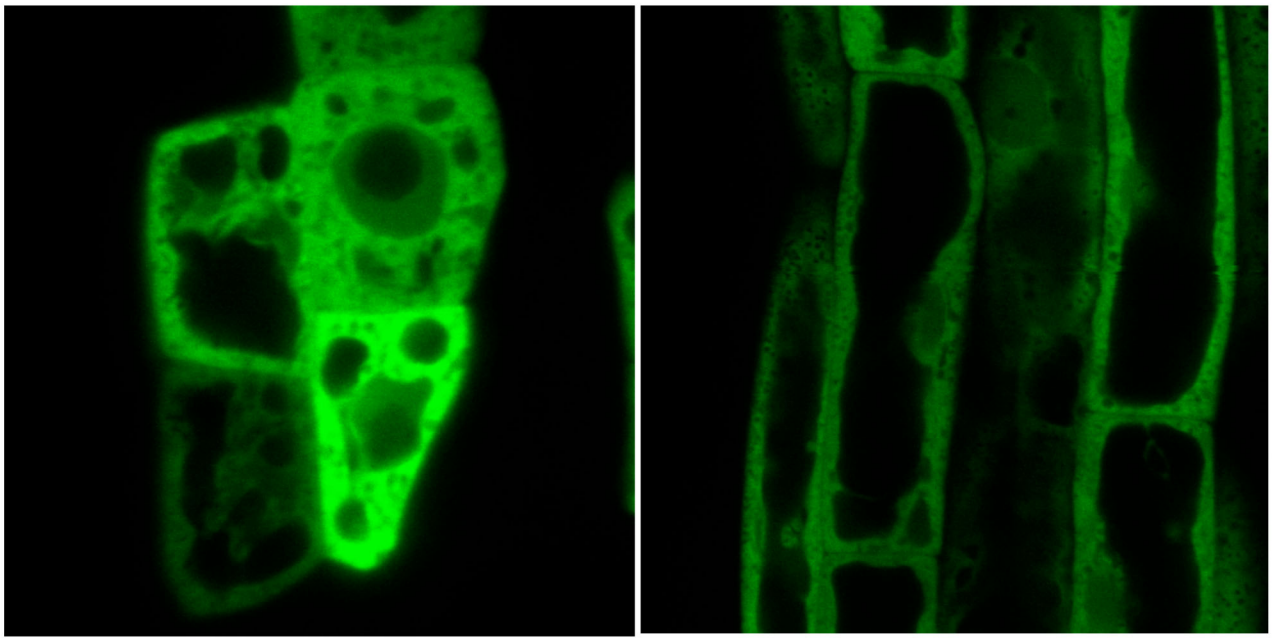


Figure 16. Subcellular localization of CTR (305-520)-GFP in *A.thaliana*.

CTR-GFP was controlled by an estradiol inducible promoter in Root tip cells of *A.thaliana*. Root tip cells (left) and elongation zone (right) . Plants were grown on MS-0 plate for 5 days, transferred onto MS plate with β-estradiol (10 μM) and cultured for 2 more days.

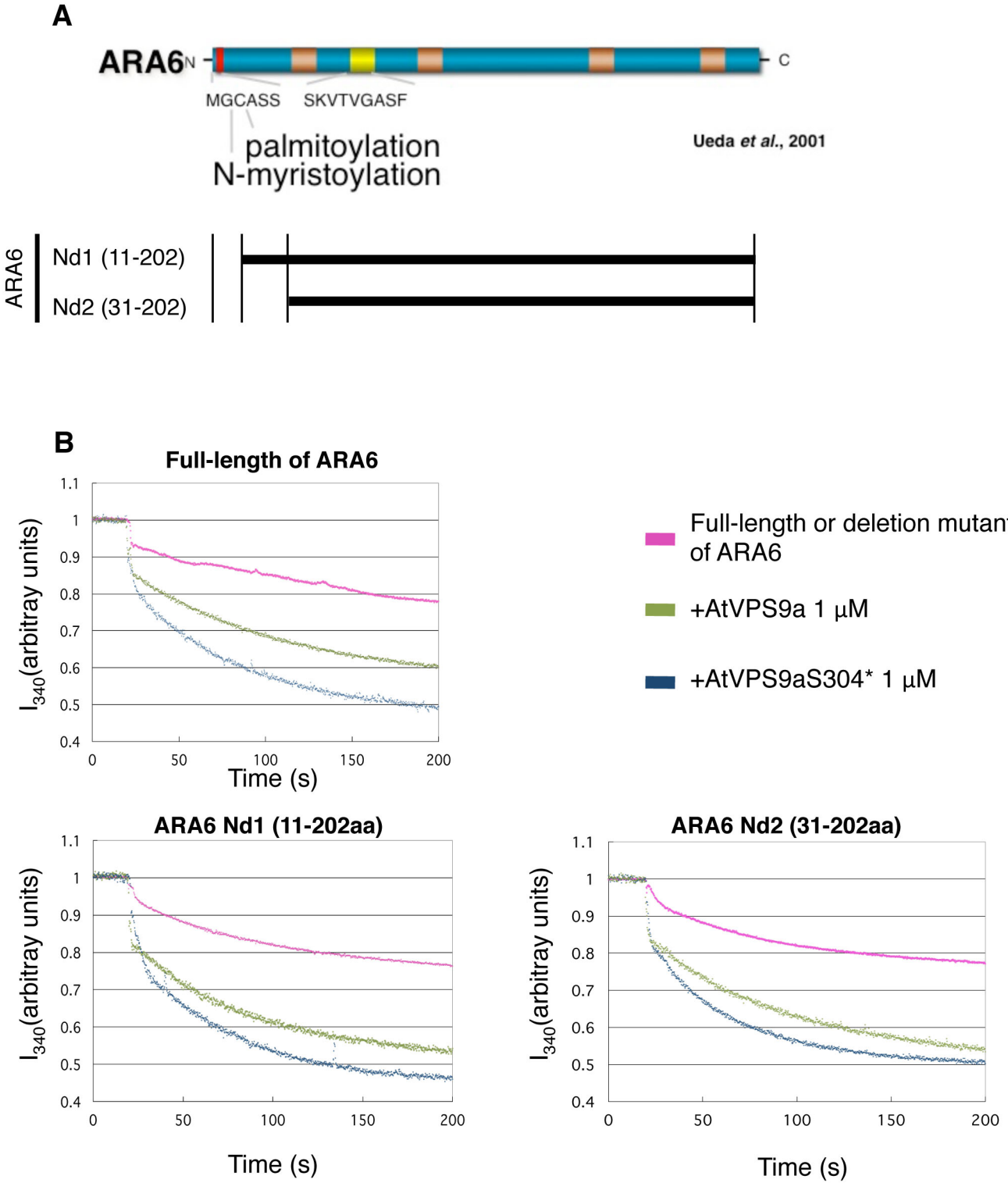


Figure 17. GEF activity to ARA6 N-terminal deletion mutants.

(A) Structure of ARA6 deletion mutants.

(B) GEF activity toward deletion mutants of ARA6. The conformational changes of Nd1 (left bottom) and Nd2 (right bottom) were detected by measuring autofluorescence from Trp in the absence (pink) or presence of 1 μM GST-VPS9a (green) or1 μM GST-VPS9aS304* (blue).

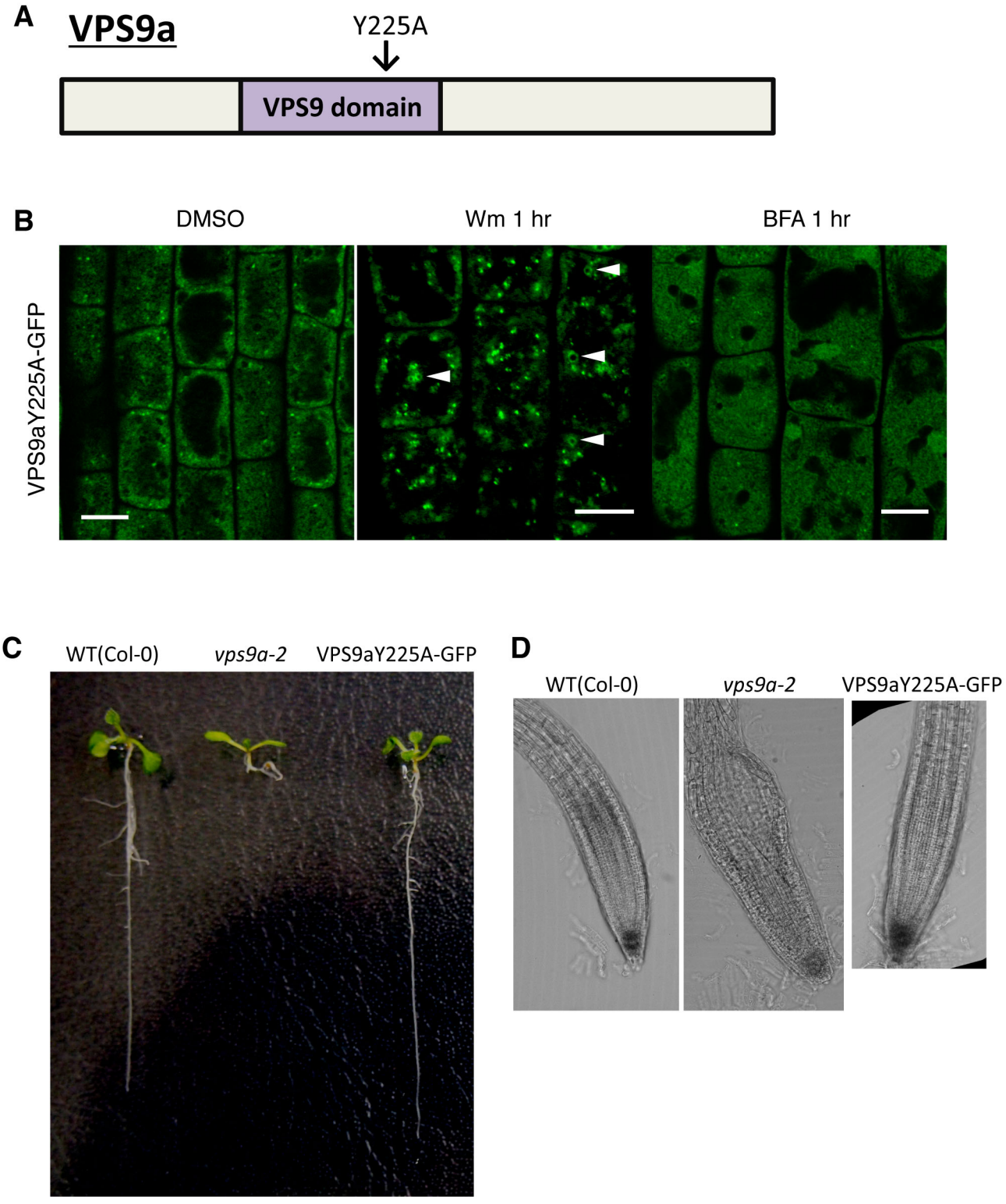


Figure 18. Expression of Y225A-GFP complemented the phenotypes of *vps9-2*
(A) Position of Y225A in VPS9a.
(B) Subcellular localization of VPS9aY225A-GFP with drag treatment. Bars=
(C) Root elongation of VPS9a-Y225A-GFP in *vps9a-2*.
(D) Images of root tip of VPS9aY225A-Venus plants.

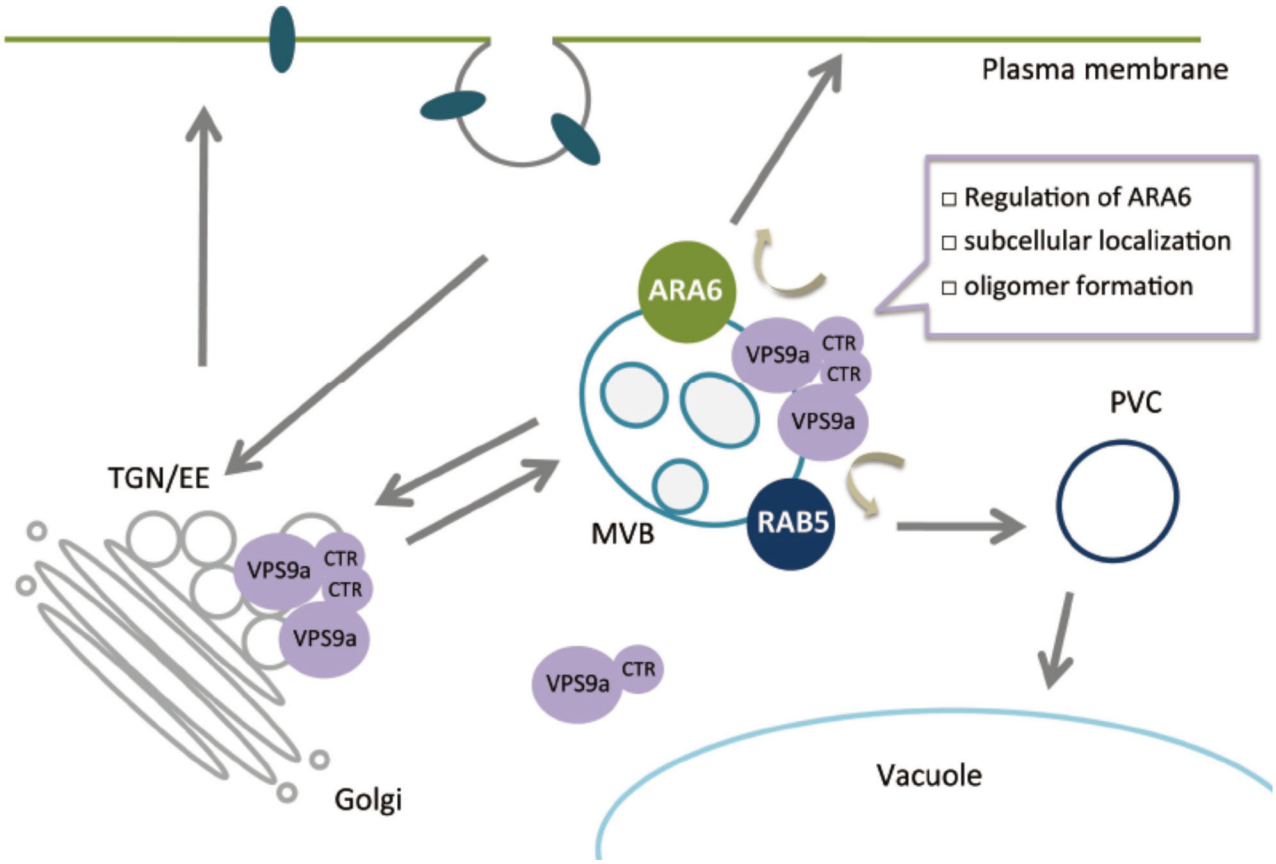


Figure 19. Schematic model of the function of VPS9a on endocytic pathway.

VPS9a localizes mainly on endsomes and partially TGNs, and activates two RAB5 groups, ARA6 and ARA7. CTR of VPS9a is involved in ARA6 regulation, subcellular localization and homo-oligomer formation. The oligomerization of of VPS9a may affect the regulatory function of the CTR in vivo, which in turn may enable the selective activation of either of the two RAB5 groups.

Chapter 2

Analysis of theVPS9 homolog in *Marchantia polymorpha*

Abstract

The membrane trafficking pathways have been diversified in a specific manner for each eukaryotic lineage, likely to fulfill specific functions in organelles. *Marchantia polymorpha* is a key model of basal plants, because of its important location in evolutionary studies.

M. polymorpha has two RAB5 members, MpRAB5 and MpARA6, which were localized on endosomes in vivo (A. Era, unpublished). *M. polymorpha* has one gene containing the Vps9 domain in the genome, named as MpVPS9. MpVPS9 encodes a protein consisting of 606 amino acids residues. I did not find any known motif or domain for the C-terminal half of MpVPS9.

I demonstrated that MpVPS9 activates and interacts with MpRAB5 and MpARA6. MpARA6 is sensitive to MpVPS9 like AtARA6. MpVPS9 also forms homo-oligomer mediated by CTR, suggesting that MpVPS9 possesses similar properties to AtVPS9a

Introduction

Two RAB5 groups in *Marchantia polymorpha*

The membrane trafficking pathway has been diversified in a specific way for each eukaryotic lineage, likely to fulfill specific functions in the organelles. In plants, a recent investigation has reported that some of the SNAREs exhibited distinct subcellular localization in *A. thaliana* and *M. polymorpha* (Kanazawa et al 2015). Characean algae ARA6-like protein was localized to the plasma membrane, charasome and TGN, though not restricted to the MVE (Hoepflinger et al 2013). It is possible that ARA6 different localization might reflect diversification of ARA6 functions from algae to land plants.

Conventional RAB5s are evolutionarily conserved in eukaryotes, while ARA6 homologues are conserved in the genomes from green algae to land plants (Ebine et al 2011; Hoepflinger et al 2013).

M. polymorpha is a key model of basal plant, and it occupies an important position in evolutionary studies. Components of membrane trafficking machinery, such as RAB GTPases and SNARE molecules, are also well conserved in *M. polymorpha*.

M. polymorpha has one ARA6-type RAB5 and one conventional-typB5 in its genome. MpRAB5 and MpARA6 localize to dot-like structures in the endocytic pathway (A. Era, unpublished). Moreover MpRAB5-positive endosomes exhibited sensitivity to Wm treatment,

whereas MpARA6-positive endosomes did not (A. Era, unpublished). Surprisingly, MpARA6^{Q91L}-Citrine, which is constitutively active, localized to the plastid (A.Era, unpublished). These findings suggest that ARA6 in *A. thaliana* regulates the pathway from endosomes to the plasma membrane (Ebine et al 2011), while ARA6 in *M. polymorpha* regulates the pathway from endosomes to chloroplasts (A. Era, unpublished). To examine whether Vps9 homologs have common activation systems for the two RAB5 groups in plants, I identified and characterized the VPS9 homolog in *M. polymorpha.*

Material and methods

Yeast two-hybrid assay

Wild-type and mutant versions RAB5 cDNA were subcloned into the pAD-GAL4-2.1 vector (Stratagene). MpVPS9 cDNA was subcloned into the pAD-GAL4-GWRFC vector, which was kindly provided by Dr. T. Demura (NAIST). The plasmids containing each RAB5 and MpVPS9 were introduced into the AH109 strain (Clontech). Empty vectors were used for negative controls. The representative colonies were cultured in selective medium without leucine and tryptophan (designated as "+H"), and medium without leucine, tryptophan and histidine (designated as "-H"). Transformation was performed at least twice independently and at least three colonies were checked for the interactions for each transformation.

Expression and purification of GS- fusion proteins

GST-tagged MpRAB5, MpARA5, MpVPS9 (full-length and R311*), AtARA7, AtARA6 and AtVPS9a were expressed in *Escherichia coli* (Rossetta (DE3)) using the pGEX 4T-1 vector (GE Healthcare). The cells expressing fusion proteins were collected, resuspended in lysis buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% β-mercaptoethanol, 1% Triton, and protease inhibitor cocktail (GE Healthcare)], sonicated, and centrifuged at 10,000x*g* for 30 min. The supernatants were loaded onto glutathione-Sepharose 4B columns (GE Healthcare) and washed with 10 column volumes of washing buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1 % β-mercaptoethanol). Then, the fusion proteins were eluted with elution buffer (20 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl).

In vitro pull-down assay

HA-tagged MpVPS9 was expressed in the yeast strain YPH414 (*MATa Δpep4:TRP1 ura3 lys2 ase2 trp1 his3 leu2*) under the regulation of a galactose-inducible *GAL1* promoter in galactose-containing medium. The collected cells were collapsed by vortexing with glass beads in PBS plus protease inhibitor cocktail (GE Healthcare) and 0.05% Tween20 (Wako). The collected lysates were co-incubated with GST-MpRAB5, GST-MpARA6 and GST-MpVPS9,

which were prebound to the glutathione-Sepharose 4B resin (GE Healthcare) for 60 min at room temperature. The protein complexes bound to the resins were washed three times and subjected to immunoblot analyses. We confirmed that the results presented here were reproducible by independent assays were that repeated at least three times.

Nucleotide exchange assay

Nucleotide exchange on the purified GST-tagged RABs was measured by monitoring the change of autofluorescence from intrinsic tryptophan accompanying structural conversion from inactive to active state (Pan et al., 1995; Antonny et al., 2001). Each purified protein was preloaded with GDP and incubated with or without GEF in reaction buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.5 mM $MgCl_2$] for 100 sec at 25 ℃. Then, GMP-PNP was added to a final concentration of 0.1 mM to start the nucleotide exchange reaction. The fluorescence shift was detected with a fluorescence spectrophotometer (model F-2500, Hitachi High Technologies) at an excitation wavelength of 298 nm and an emission wavelength of 340 nm. The assay was repeated at least three times for each reaction.

Antibodies

Antibodies against MpVPS9 and MpARA6 were generated against purified protein of MpVPS9.

Anti-HA monoclonal antibody was purchased from Zymed. The dilution of each antibody used in immunoblotting was as follows: anti-MpVPS9, 1:200; anti-MpARA6 1:200 and anti-HA, 1:1000.

Results

Identification and characterization of MpVPS9

M. polymorpha has one gene that encodes a protein containing the Vps9 domain in its genome, and I named this protein MpVPS9. MpVPS9 encodes 606 amino acid residues. Similarity analysis revealed that RAB5 GEFs share similarity with only the Vps9 domain at the N-terminal half of the protein. I did not find any known motifs or domains in the C-terminal half of MpVPS9. From alignment analysis, the PSR is conserved widely in plants (Figures 20A and B), but not with the yeast RAB5 GEF Vps9p.

MpVPS9 shows high amino acid sequence similarity to AtVPSS9a, especially in the Vps9 domain and the PSR. The Vps9 domain and more, specifically the residues essential for GEF activity of VPS9a, such as D185 and Y225 in AtVPS9a, are also conserved in MpVPS9.

An anti-MpVPS9 antibody was generated against purified protein of MpVPS9, and I utilized this antibody toanalyzed endogenous MpVPS9 and yeast lysate expressing MVPS9-HA. MpVPS9 was detected in a larger form than the predicted molecular weight (Figure 21A). The recombinant proteins, GST-MpVPS9 and GST-MpVPS9 R311*, were detected using the anti-MpVPS9 antibody (Figure 21B)

MpVPS9 interacts with RAB5 members

I examined whether MpVPS9 interacts with conventional RAB5 by yeast two-hybrid assay. MpVPS9 interacted with MpRAB5 SN and MpARA6SN, but not with MpRAB5 QL (Figures 22A and B). These interactions were not detected by an in vitro pull-down assay (Figure 22D).

MpVPS9 forms homo-oligomerization mediated by CTR

To examine the functions of MpVPS9 in more detail, I focused on the CTR of MpVPS9. From alignment analysis, MpVPS9 R311 corresponds to AtVPS9a S304. MpVPS9 R311* interacts with conventional RAB5 SN, but not with ARA6 SN (Figure 22B). I also examined whether MpVPS9 forms a homo-oligomer detected by in vitro assay. MpVPS9 interacted with MpVPS9 and the CTR of MpVPS9 in the yeast two-hybrid assay (Figure 22C) and pull-down assay (Figure 22D). These results indicate that MpVPS9 forms homo-oligomer mediated by the CTR.

MpVPS9 activates MpRAB5 and MpARA6 in vitro

To investigate whether MpVPS9 acts as a RAB5 GEF, I measured its GEF activity. As I predicted, MpVPS9 stimulated the nucleotide exchange of MpRAB5 (Figure 23). MpVPS9 also activated the plant-specific MpARA6, but not MpRAB7. Interestingly MpVPS9 could activate MpARA6 even at low concentrations (Figure 24). MpARA6 appeared to be more sensitive to MpVPS9 than AtARA6.

AtVPS9a activates MpARA6 and MpRAB5 to a similar extent to MpVPS9

To examine whether the activation of the proteins are different from one other, I measured GEF activity of AtVPS9a toward MpARA6. AtVPS9a exhibited GEF activity toward MpRAB5, and activated MpARA6 to the same extent as MpVPS9 (Figures 25A and B).

Chimeric ARA6 (At-Mp-At) revealed similarity to MpARA6.

What causes differential subcellular localization and GEF activity between AtARA6 and MpARA6? ARA6-like proteins have high similarity from algae to land plants. In *A. thaliana*, deletion mutants of AtARA6 were activated by VPS9a to a similar extent as full-length ARA6. This finding suggests that the N-terminal region of ARA6 is not involved in the GEF activity. Thus, I generated chimeric ARA6 proteins, the 48 amino acids that were swapped between AtARA6 and MpARA6 indicated in the red box (Figure 26A). The 48 amino acids contained the effector domain of ARA6. Chimeric ARA6 (At-Mp-At) was used in the in vitro GEF assay. Chimeric ARA6 (At-Mp-At) was activated by AtVPS9a, though it was not sensitive to the GEF activity of AtVPS9a (Figure 26B). Thus other regions of MpARA6 could be involved in sensitivity to the GEF activity.

Discussion

MpVPS9 also acts as a RAB5 GEF in *M. polymorpha*.

There are two RAB5 subgroups in plants; conventional RAB5s are conserved in eukaryotes, and plant-unique ARA6 is conserved in land plants and some green algae. In contrast, the Vps9 homolog guanine exchange factors are also evolutionarily conserved.

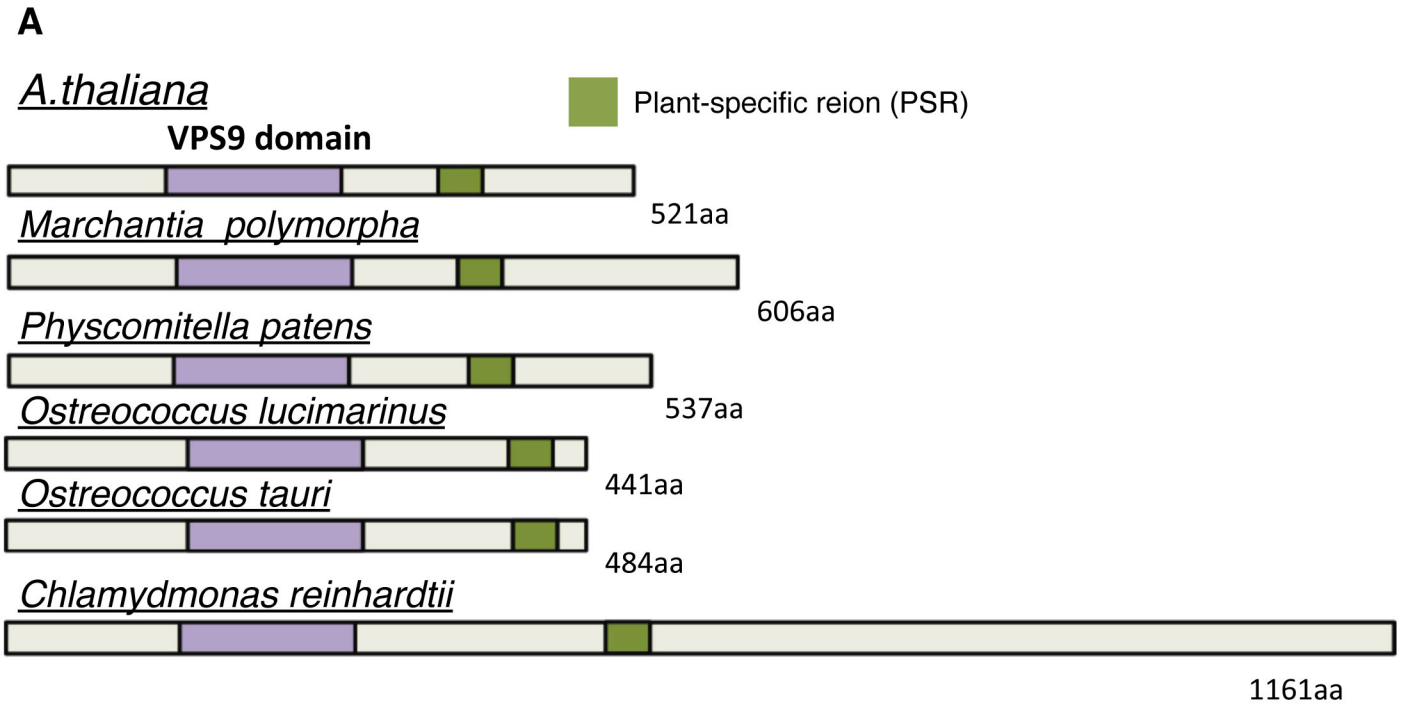
In this study, I attempted to characterize the Vps9 homolog in *M. polymorpha*, MpVPS9. As expected, MpVPS9 activated two RAB5 members, MpRAB5 and MpARA6.

MpVPS9 also exhibited similarity to AtVPS9a. It appears that MpVPS9 did not interact with MpARA6. MpVPS9 also forms homo-oligomer mediated by its CTR. In vitro GEF assay between MpARA6 and AtVPS9a indicates that MpVPS9 and AtVPS9a possess similar GEF activities toward the two RAB5 groups. A common RAB5 activation mechanism must be conserved in bryophytes and flowering plants.

ARA6 diversification and RAB5 GEFs

MpARA6 was shown to be more sensitive to the GEF activities of MpVPS9 and AtVPS9a than AtARA6, even though ARA6 exhibited high similarity. Chimeric ARA6 showed GEF activity susceptibility similar to AtARA6. It remains unclear why ARA6-like proteins are differently localized in spite of the high similarity between characean algae and land plants. AtVPS9a

exhibited GEF activity toward MpRAB5, and activated MpARA6 to a similar extent to MpVPS9, suggesting that the diversification of ARA6 functions cause a different reaction of the GEF activity.



B

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AtVPS9a              -SETPMKKAESISDLENKG-ATLLKD-TEPSKVFQ-EYPYIFASAGDLRIGDVEGLLNSY 376
AtVPS9b              -SETPIKKADPITDLENKGAATLLNDRSEATKIFQ-EYPYMFASVGDLKIGDVEDLLNNY 362
OsGLUP6              ASSNPVERVQSISDLEKKGAAELLKD-DDLNKKIQ-EYPFLFARSGDLTVADVENLLNSY 384
S.moellendorffii     AKESSE---LSVAKLEAGGASGVVEA-DRSGQLAK-EYPFLYASAGDLRVEDVESLLTQY 354
P.patens             ASGSSDKDTMTVAKLEALGLPDVLEA-DKTGQLAR-DYPYLYASAGDLKVMDVEGLLADY 389
O.lucimarinus        RAAAPYVPWRTTEDVEAEGATQLTALDVAGNLTLSSDYKFLYAKVEDLTVGDVARLLHDY 415
O.tauri              SAAPAYIPWRTTEDVEAEGATALTALDVAGELALS-DYKFLYAKVDDLTVGDVSRLLNDY 458
C.reinhardtii        TSQLVPLPPKSVSQLEADGVRLVLRAEAAGELRAR--YRYLYASPEGLTLRDVSQLLAAY 515
V.carteri            QKQFIPPRPKSVSELERDGIRLVIKAEAAGELRAK--YPYLYCIKESLTLHDVSQLLVSY 430
Vps9p                KFNELFSPIGEPTQEEALKSEQSNKEEDVSSLIKK-----IEENERKDTLNTLQNMFPDM 423
                                . *                          :        :  :  ::

AtVPS9a              KQLVFKYVCLTKG---LGDGTSLAPSSSPLQASSGFNT--------SKESEDHRRSSSDV 425
AtVPS9b              KQLVFKYVCLSKG---LGDATSLTPCISPLQAS--------------KVSENHTTLSSDF 405
OsGLUP6              KQLVLKYVALSQG---MGINLENPPVQSMQTVS-------------------------D 415
S.moellendorffii     KELVLRYVALRKGLESIG------RKPAIVEEPT--------------SPAPALAAPVPA 394
P.patens             KEIVLKYAALYKAVQGMGSATGNRRTSSPLLDIS--------------ESSGILQTAAEF 435
O.lucimarinus        KGLALQYESLSRGVAKVLTTLDARLZ---------------------------------- 441
O.tauri              KGLALQYESLSRGVAKVLASLHRAPZ---------------------------------- 484
C.reinhardtii        KELAIKYETLAQAVENNVLKYFSDLEVYQAGVAPALPAGPTHAAQPHQQPQPHQPRHPPP 575
V.carteri            KELVLRYETLAQAVENNVVKYFTELEAFQAG-LPTEPLGTTAAAAWSAAPPPPPP--PPP 487
Vps9p                DPSLIEDVCIAKK-SRIGPCVDALLSLSEZ------------------------------ 452
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Figure 20. VPS9 homologs in plants.
(A) Schematic structure of RAB5 GEF of plants.
(B) Alingment anlysis using ClustalX revealed that PSR conserved in plant Vps9 domain containing proteins. Yeast Vps9p does not have PSR. Green line indicates PSR.

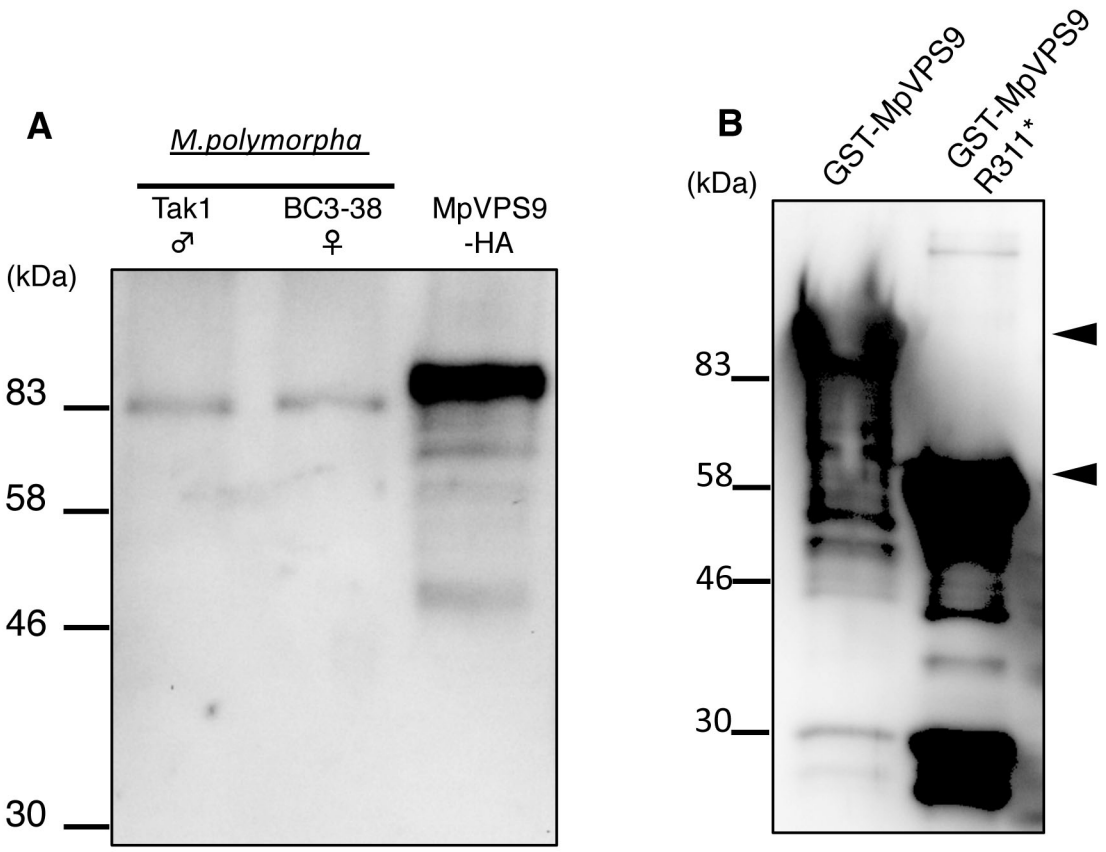

Figure 21. Immunoblot analysis, using with an anti-MpVPS9 antibody.

(A) Immunoblot analysis of endogenous MpVPS9 and yeast lysate expressingMpVPS9-HA.

(B) Immunoblot analysis of recombinant MpVPS9. Arrowheads indicate GST-MpVPS9 and GST-MpVPS9 R311*

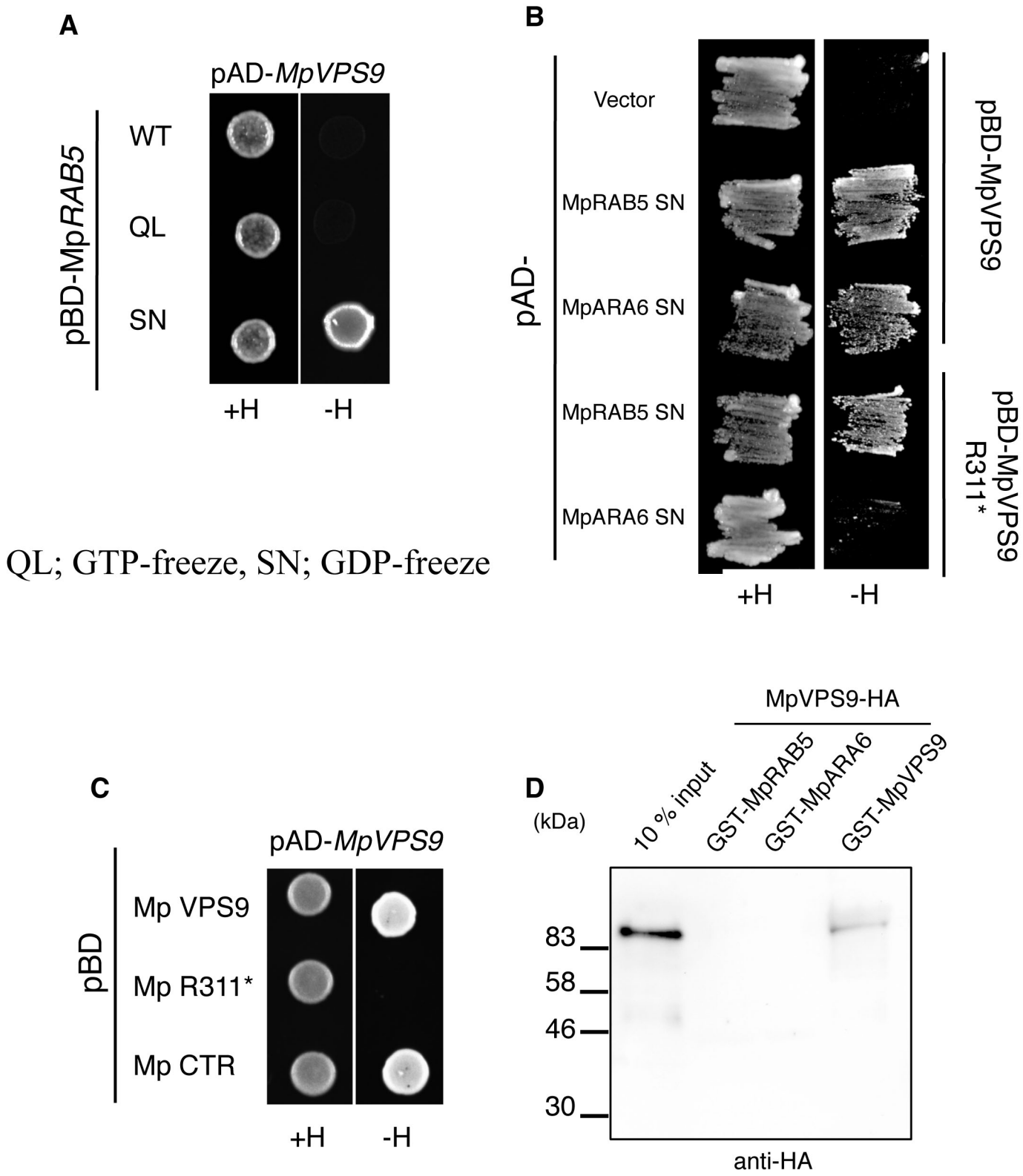

Figure 22. MpVPS9 showed similar to AtVPS9a function.

(A) Interaction between MpVPS9 and MpRAB5 detected by the yeast two-hybrid assay. QL; GTP-freeze, SN; GDP-freeze mutants.

(B) Interaction between MpVPS9 R311* and RAB5 members in *M.polymorpha* detected by the yeast two-hybrid assay.

(C) Homo-oligomer formation of MpVPS9a detected by the yeast two-hybrid assay.

(D) Interaction between MpVPS9 and RAB5 members, and homo-oligomer formation of MpVPS9 detected by the pull-down assay

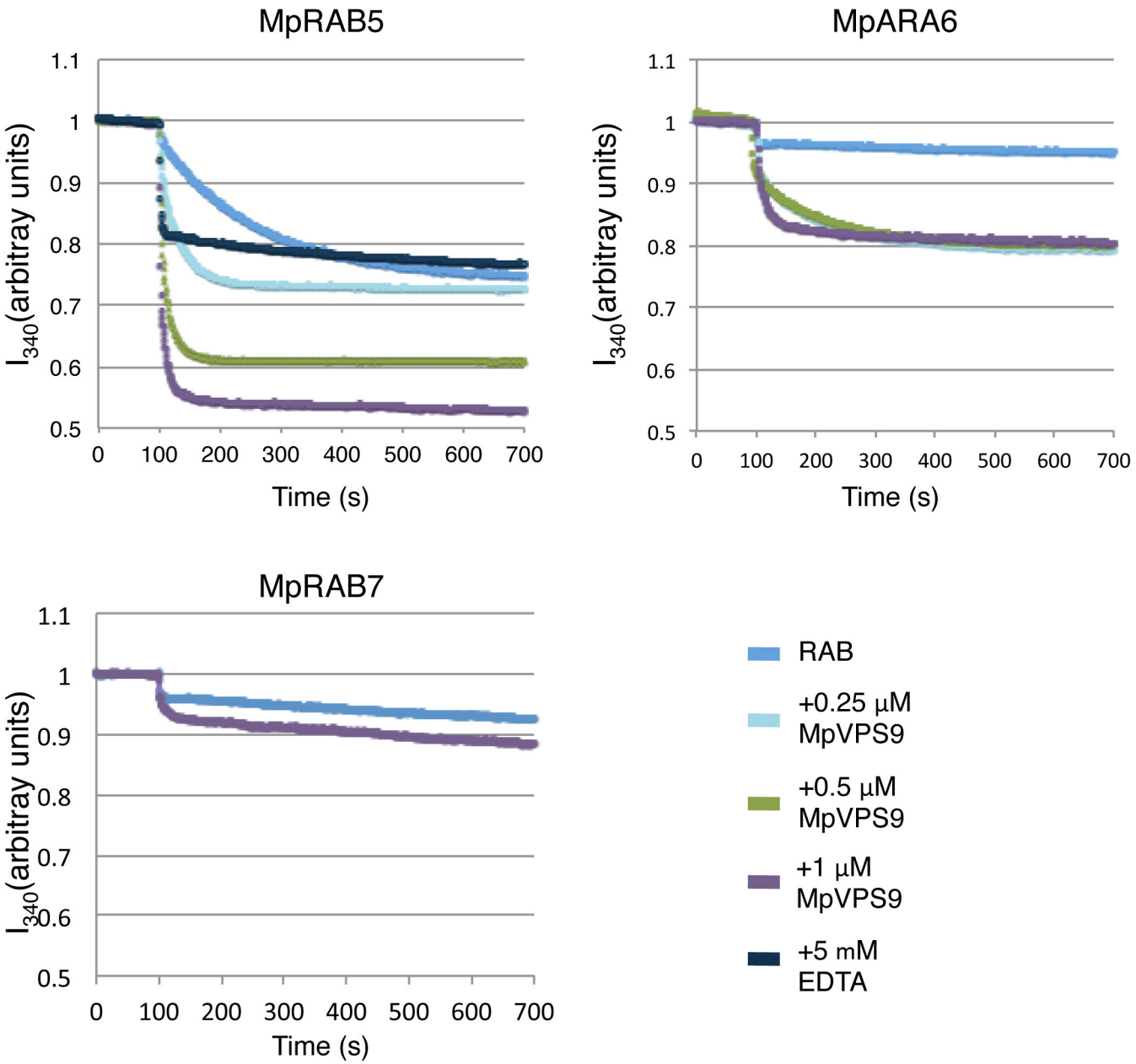

Figure 23. MpVPS9 activates MpRAB5 and MpARA6.

The conformational change of MpARA6 was detected by measuring autofluprescence from Trp in the absence (blue) or presence of 0.25 µM(light blue), 0.5 µM (green), 1 µM (purple) MpVPS9 and 5 µM EDTA (black).

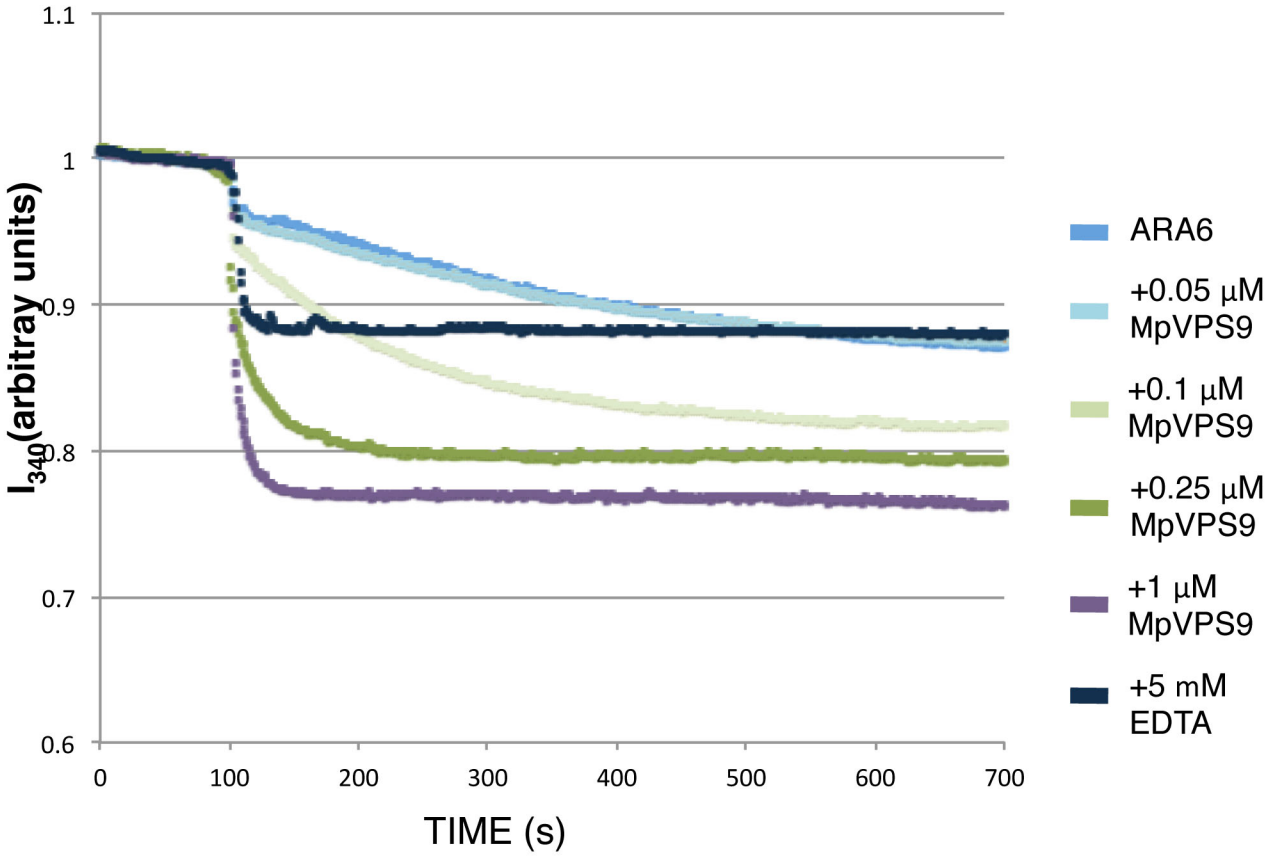

Figure 24. MpARA6 is sensitive to MpVPS9 compared to AtARA66.

The conformational changes MpARA6 was detected by measuring autofluorescence from Trp in the absence (blue) or presence of 0.05 µM(light blue), 0.1 µM (light green), 0.25 µM (green), 1 µM (purple) MpVPS9 and 5 µM EDTA (black).

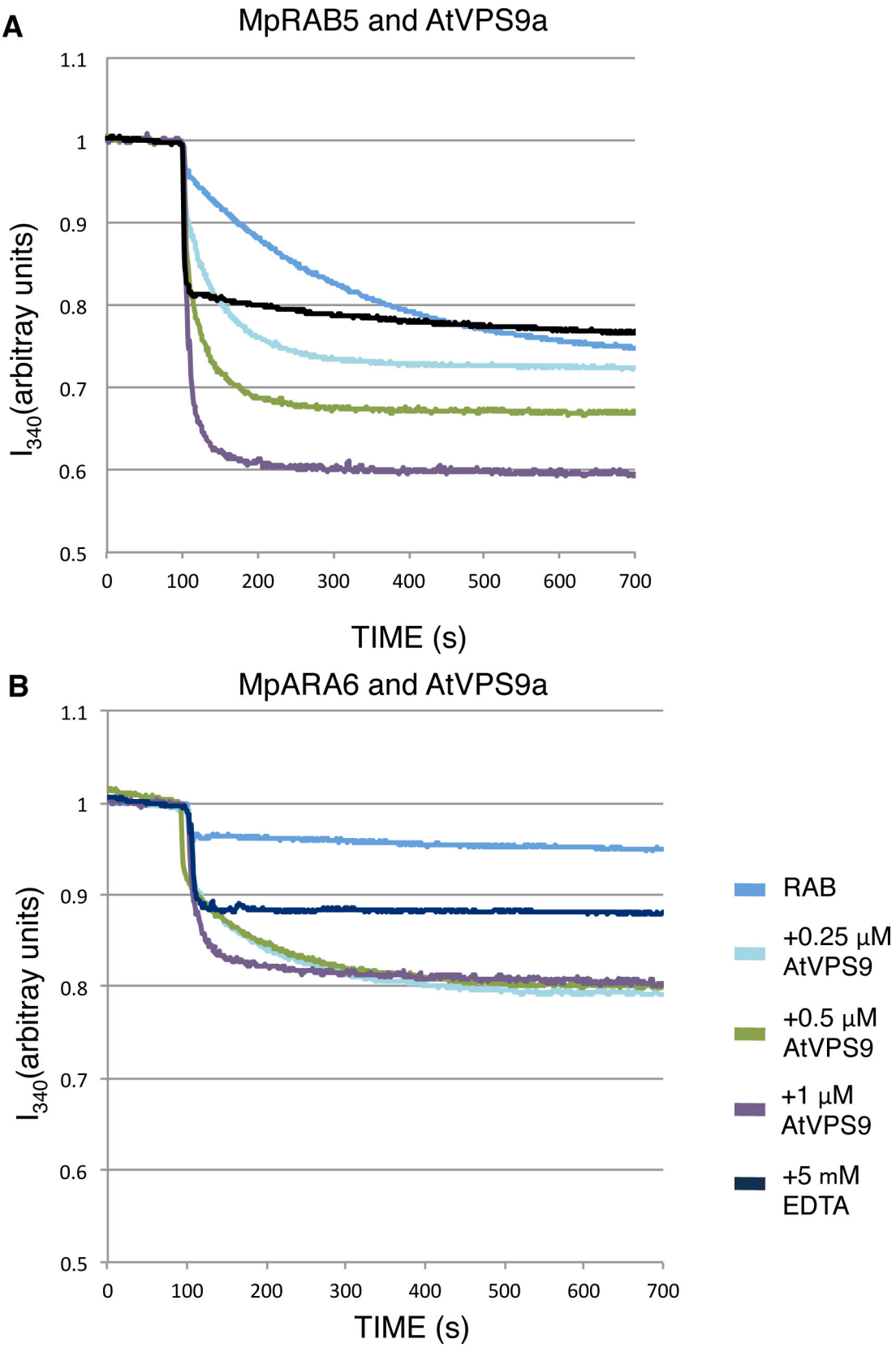

Figure 25. AtVPS9a activates MpRAB5 and MpARA6 to the same extent as MpVPS9.

The conformational changes MpRAB5 and MpARA6 were detected by measuring autofluprescence from Trp in the absence (blue) or presence of 0.25 µM(light blue), 0.5 µM (green), 1 µM (purple) MpVPS9 and 5 µM EDTA (black).

(A) In vitro GEF assay of MpRAB5 and AtVPS9a.

(B) In vitro GEF assay of MpARA6 and AtVPS9a.

A

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A_thaliana      MGCASSLPDRNSGTLSGLSNSENAVPADAKNLRVKLVLLGDSGVGKSCIVLRFVRGQFDA TSKVTVGASFLSQTIALQDSTTVKFEIWDTAGQERYSALAPLYYRGAGVAVIVYDITSPE
A_lyrata        MGCATSLPDRNSGALSGLSNSENAVPADAKNLRVKLVLLGDSGVGKSCIVLRFVRGQFDA TSKVTVGASFLSQTIALQDSTTVKFEIWDTAGQERYSALAPLYYRGAGVAVIVYDITSPE
Oryza           MGCSSSVPARSTGGLNNISNDNS--ATDSKDLRAKLVLLGDSGVGKSCIVLRFVRGQFDP TSKVTVGASFLSQTLALEDSTIVKFEIWDTAGQERYAALAPLYYRGAAAAVVVYDITSPE
Zea             MGCSSSVPARNTGGLNTISNDSSP-DASSKDLRAKLVLLGDSGVGKSCIVLRFVRGQFDP TSKVTVGASFLSQTLALEDSTIVKFEIWDTAGQERYAALAPLYYRGAAAAIVVYDITSPE
Vitis           MGCSSSLPDRNSGRLGG-LNAENGGAPDAKNLRVKLVLLGDSGVGKSCIVLRFVRGQFDP TSKVTVGASFLSQTIALQDSTTVKFEIWDTAGQERYAALAPLYYRGAAVAVVVYDITSPE
Lotus           MGCGSSLPDRDSRPFGR-DNPENGGGQDAKNLRVKLVLLGDSGVGKSCIVLRFVRGQFDP TSKVTVGASFLSQTIALQDSTTVKFEIWDTAGQERYAALAPLYYRGAAVAVIVYDITSPD
Populus         MGCSSSLPDRSTGRLGGLNNSESGGVADAKNLRVKLVLLGDSGVGKSCIVLRFVRGQFDP TSKVTIGASFLSQTIALQDSTTIKFEIWDTAGQERYAALAPLYYRGAAVAVIVYDITSPE
Ricinus         MGCASSLPDRNSGRLGGLNNSDNNGVSDAKNLRVKLVLLGDSGVGKSCIVLRFVRGQFDP TSKVTIGASFLSQTIALQDSTTVKFEIWDTAGQERYAALAPLYYRGAAVAVIVYDITSPD
Mesenbryanthemum MGCASSAPDRTVSRGSSSLNSANEGASDAKNLKVKLVLLGDSGVGKSCIVLRFVRGQFDP TSKVTVGASFLSQTIALQDSSTVKFEIWDTAGQERYAALAPLYYRGAAVAVIVYDITSPE
Marchantia      MGCAASAPARQ--GRLAGMNQDAVETFDSKNLRIKLVLLGDSGVGKSCIVLQFVRGQFDP SSKVTVGASFLSQTVALKDSTTVKFEIWDTAGQERYASLAPLYYRGASAAVVVYDITSPD
                ***.:* * *        *      .:*:*: ****************:*******. :****:********:**:**: :**************:*********..*::*******:

A_thaliana      SFKKAQYWVKELQKHGSPDIVMALVGNKADLHEKREVPTEDGMELAEKNGMFFIETSAKT ADNINQLFEEIGKRLPRPAPSSZ----------------------
A_lyrata        SFKKAQYWVKELQKHGSPDIVMALVGNKADLHEKREVPTEDGIGLAEKNGMFFIETSAKT ADNINQLFEEIGKRLPRPAPSS----------------------
Oryza           SFSKAQYWVKELQKHGSPDIIMVLVGNKADLHENRHVSSQEAQEYAEKNNMVFIETSAKT ADNINQVFEEIAKRLPRPTAS----------------------
Zea             SFSKAQYWVKELQKHGSPGIVMVLVGNKADLHDNRSVSSQDAQDYAEKNNMFFIETSAKT ADNINQLFEEIAKRLPRPTAS----------------------
Vitis           SFNKAQYWVKELQKHGSPDIVMALVGNKADLHENREVPVQDGIDYAEKNGMFFIETSAKT ADNINQLFEEIAKRLPRPSSS----------------------
Lotus           SFSKAQYWVKELQKHGNPDIVMALVGNKADLLEKREVAVQDGIDYAEKNGMFFIETSAKT ADNINELFEEIAKRLPRPPTT----------------------
Populus         TFNKAQYWVKELQKHGSPDIVMALVGNKADLHEKREVPTQDGIEYAEKNGMFFIETSAKT ADNINQLFEEIAKRLPRPSST----------------------
Ricinus         SFNKAQYWVKELQKHGSPDIVMALVGNKADLHDNREVPVQDGKEYAEKNGMFFIETSAKT ADNINQLFEPGFGQGGPCSDSGNGKQLVGILQVGEGLSLTSLHF
Mesenbryanthemum SFTKAQYWVKELQKHGSPDIIMALVGNKADLQERREVPAQDGIEYAEKNGMFFIETSAKT ADNINQLFEEIAKRLPRLLPS----------------------
Marchantia      SFQKAQFWVKELQKHGSPGIVMALVGNKADLEESREVASEDSQAYAESNGMFFIETSAKL ATNVNLLFEEIGQRLPRGQPTQKPZ----------------------
                :* ***:*********.*.*:*.******** : * *. ::.   **.*.*.*******  * *:* :**    :      :
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B

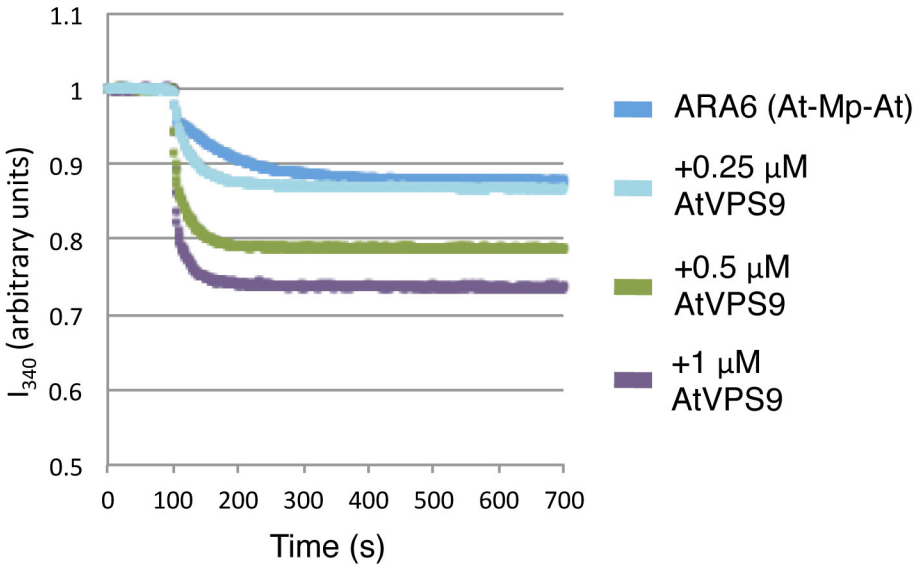

Figure 26. In vitro GEF assay of chimeric ARA6 (At-Mp-At)

(A) ARA6 in land plants and structure of chimeric ARA6. Red box indicates the area which was swapped in the chimeric proteins.

(B) Measurement of ApVPS9 GEF activity toward chimeric ARA6 (At-Mp-At). The baseline of chimera got higher than AtARA6 but sensitivity was almost equal to AtARA6.

General discussion

Studies of membrane trafficking in plants have been progressing rapidly. As described in the general introduction, plant membrane trafficking is involved in a wide variety of plant functions. Recent studies indicate that plants have developed a unique membrane traffic system. Plant TGNs act as early endosomes and RAB5-localizing organelles are the multivesicular endosomes, which are known as the late endosomal compartment in animals. Ebine and colleagues reported that plants have developed a complicated vacuolar transport system distinct from that of non-plant systems (Ebine et al 2014). The acquisition of ARA6 allowed development of unique post-Golgi trafficking systems.

Plant-unique RAB5, ARA6, acts in the pathway from endosomes to the plasma membrane, unlike conventional RAB5s. In spite of its high degree of similarity, ARA6 has diversified functions within land plants (Ebine et al 2011; A. Era unpublished).

Despite of the varying functions between conventional RAB5s and ARA6, VPS9 homologs activate both of the two RAB5 groups in *A. thaliana*. In addition to the Vps9 domain, animal RAB5 GEFs, but not plant RAB5 GEFs, contain several known protein domains and motifs.

In this study, I report that VPS9a consists of a conserved region which acts as the catalytic core and the plant specific C-terminal region (CTR). VPS9a activates conventional RAB5s which

regulate the pathway from endosomes to the vacuole. VPS9a also activates ARA6 through its CTR which negatively regulates the pathway from endosomes to the plasma membrane in *A. thaliana* (Figure 27). MpARA6 might regulate the pathway from endosomes to chloroplasts in *M. porymorpha*. The CTR of VPS9a is involved in the negative regulation of ARA6, homo-oligomerization, and subcellular localization. Specifically the PSR which is involved in homo-oligomer formation, is widely conserved in plant RAB5 GEFs, but not in animal RAB5 GEFs and other counterparts.

The CTR might be involved in the regulation of two RAB5 group members. The PSR is conserved widely in Vps9 homologs in plants and involved in homo-oligomerization. Homo-oligomer formation might be required for forming microdomains on the endosomes where VPS9a activates RAB5s. The PSR is conserved in green algae which have no ARA6 types of RAB5, suggesting that the PSR could not be directly involved in regulation of ARA6.

Animal RAB5 GEFs are localized to early endocytic compartments, while VPS9a-GFP localizes on the MVE and partially on TGNs. Consistent with the mammalian RAB5 GEF, RABEX-5, Y225A mutation that reduces the GEF activity in RAB5 GEF did not affect its subcellular localization. Although RAB GEFs are major determinants for specific RAB membrane targeting, VPS9a265-Venus, which could activate RAB5 members, was localized to the cytosol. It will be needed to further investigate where RAB5 members are activated in plant

cells. Homo-oligomerization of VPS9a might increase VPS9a recruitment to endosomes and aid forming a special microdomain on endosomes.

MpVPS9 analysis has provided important insights into the significance of the activation mechanism of two RAB5 group members. MpVPS9 shows high amino acid sequence similarity to AtVPS9a, especially in the Vps9 domain and the PSR. D185 and Y225 of Vps9 domain in AtVPS9a are essential residues for the GEF activity of VPS9a, and are also conserved in MpVPS9. The PSR of MpVPS9 could also be involved in homo-oligomer formation. These results suggest that activation mechanism is conserved in land plants. However, in addition to conventional RAB5s and ARA6, there is only one close homolog, RAB21 in *M. polymorpha* and *O. sativa*. GLUP6 and OsGEF2, Vps9 homologs of *O. sativa*, act as RAB5 GEF (Fukuda et al 2013; Wen et al 2015). MpVPS9 and GLUP6 activated MpRAB21 and OsRAB21, respectively (N. Minamino, unpublished; L. Wen, unpublished). VPS9a and MON1-CCZ-1, which act as RAB5 GEF and RAB7 GEF, respectively, are only plant RAB GEFs that have identified thus far (Goh et al 2007; Cui et al 2014; Ebine et al 2014). Our knowledge on RAB GEFs in plants is still limited. However, my findings on RAB5 and RAB21 in plants indicate that different RAB5 activation systems could have been developed in land plants.

My results demonstrated that the CTR of VPS9a regulates the GEF activity toward ARA6. I used only purified proteins in this study, though it is known that REI-1 and MON1-CCZ1 act as

a novel RAB11 GEF and Ypt7 GEF, respectively, exhibited increased GEF activity in the presence of liposomes (Cabrera et al 2014; Sakaguchi et al 2015). The functions of VPS9a and MpVPS9 would be clarified in more detail by measuring the GEF activity under the optimized conditions mimicking the *in vivo* conditions, .

Vps9p and RABEX-5, which act as yeast and animal RAB5 GEFs, respectively, contain ubiquitin binding domains or motifs. This may allow interaction between RAB5 GEFs and ubiquitinated transmembrane cargo proteins /other ubiquitinated proteins, which recruit VPS9a onto endosome membranes (Mattera and Bonifacino 2008; Shideler et al 2015). However, Vps9 homologs in plants are predicted to not contain ubiquitin binding domains or motifs. VPS9a interacting proteins have not yet been reported, except for RAB5s. It remains unclear how VPS9a has been cooperates with other proteins. In our laboratory, plant unique RAB5 effectors2 (PUF2) was identified as a novel ARA6 effector (E. Ito unpublished). PUF2 also interacts with inactive ARA7 and VPS9a, and is involved in VPS9a recruitment. PUF2 is probably involved in the regulation of the differential activation of the two RAB5 groups via recruitment of VPS9a onto endosomes.

Vps9 homologs are conserved in some green algae, including *O. tauri*, *C. reinhardii* and *V. carteri*, which have no ARA6-type of RAB5. The investigation of Vps9 homologs in other plant species and identification of their interacting proteins would provide important insights

into future studies.

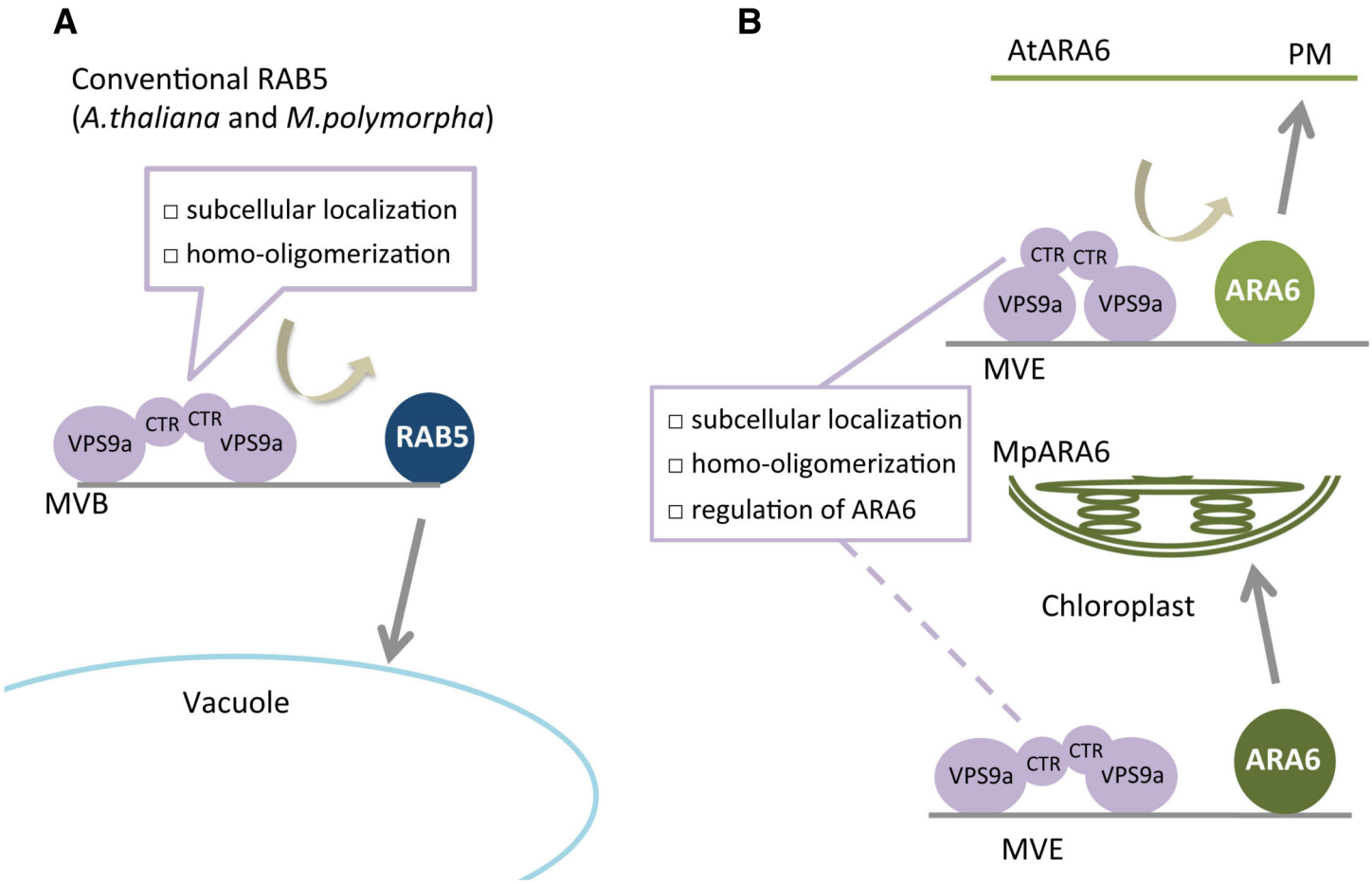

Figure 27. Model of activation mechanism by VPS9a

(A) VPS9a activates conventional RAB5s similar to animal RAB5 GEFs. Conventional RAB5s regulate the pathway from the endosome to the vacuole.

(B) Vps9 domain-containing proteins activate ARA6s. AtARA6 regulates the pathway from endosomes to the plasma membrane in *A.thaliana*. While MpARA6 might regulate the pathway from endosomes to chloroplasts in *M.polymorpha*. CTR is involved in regulation of ARA6, homo-oligomerization and subcellular localization.

References

Asaoka R, Uemura T, Ito J, Fujimoto M, Ito E, Ueda T, Nakano A (2013) Arabidopsis RABA1 GTPases are involved in transport between the trans-Golgi network and the plasma membrane, and are required for salinity stress tolerance. Plant J 73:240–249.

Blümer J, Rey J, Dehmelt L, Maze T, Wu YW, Bastiaens P, Goody RS, Itzen A (2013) RabGEFs are a major determinant for specific Rab membrane targeting. J Cell Biol 200:287–300.

Boevink P, Oparka K, Cruz SS, Martin B, Betteridge A, Hawes C (1998) Stacks on tracks: The plant Golgi apparatus traffics on an actin/ER network. Plant J 15:441–447.

Bolte S, Brown S, Satiat-Jeunemaitre B (2004) The N-myristoylated Rab-GTPase m-Rabmc is involved in post-Golgi trafficking events to the lytic vacuole in plant cells. J Cell Sci 117:943–954.

Bottanelli F, Foresti O, Hanton S, Denecke J (2011) Vacuolar Transport in Tobacco Leaf Epidermis Cells Involves a Single Route for Soluble Cargo and Multiple Routes for Membrane Cargo. Plant Cell 23:3007–3025.

Boutté Y, Frescatada-Rosa M, Men S, Chow C-M, Ebine K, Gustavsson A, Johansson L, Ueda T, Moore I, Jürgens G, Grebe M (2010) Endocytosis restricts Arabidopsis KNOLLE syntaxin to the cell division plane during late cytokinesis. EMBO J 29:546–558.

Bravo-Sagua R, Torrealba N, Paredes F, Morales PE, Pennanen C, López-Crisosto C, Troncoso R, Criollo A, Chiong M, Hill JA, Simmen T, Quest AF, Lavandero S (2014) Organelle communication: Signaling crossroads between homeostasis and disease. Int J Biochem Cell Biol 50:55–59.

Burd CG, Mustol PA, Schu P V, Emr SD (1996) A yeast protein related to a mammalian Ras-binding protein, Vps9p, is required for localization of vacuolar proteins. Mol Cell

Biol 16:2369–2377.

Cabrera M, Nordmann M, Perz A, Schmedt D, Gerondopoulos A, Ungermann C, Barr F, Piehler J, Engelbrecht-vandre S (2014) The Mon1 – Ccz1 GEF activates the Rab7 GTPase Ypt7 via a longin-fold – Rab interface and association with PI3P- positive membranes. J Cell Sci 127:1043–1051.

Camacho L, Smertenko AP, Pérez-Gómez J, Hussey PJ, Moore I (2009) Arabidopsis Rab-E GTPases exhibit a novel interaction with a plasma-membrane phosphatidylinositol-4-phosphate 5-kinase. J Cell Sci 122:4383–4392.

Carney DS, Davies B a., Horazdovsky BF (2006) Vps9 domain-containing proteins: Activators of Rab5 GTPases from yeast to neurons. Trends Cell Biol 16:27–35.

Chen D, Wilkinson CRM, Watt S, Penkett CJ, Toone WM, Jones N, Bähler J (2008) Multiple pathways differentially regulate global oxidative stress responses in fission yeast. Mol Biol Cell 19:308–317.

Choi S, Tamaki T, Ebine K, Uemura T, Ueda T, Nakano A (2013) RABA members act in distinct steps of subcellular trafficking of the FLAGELLIN SENSING2 receptor. Plant Cell 25:1174–87.

Chow C-M, Neto H, Foucart C, Moore I (2008) Rab-A2 and Rab-A3 GTPases define a trans-golgi endosomal membrane domain in Arabidopsis that contributes substantially to the cell plate. Plant Cell 20:101–123.

Clough SJ, Bent AF (1998) Floral dip: A simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16:735–743.

Cui Y, Zhao Q, Gao C, Ding Y, Zeng Y, Ueda T, Nakano a., Jiang L (2014) Activation of the Rab7 GTPase by the MON1-CCZ1 Complex Is Essential for PVC-to-Vacuole Trafficking and Plant Growth in Arabidopsis. Plant Cell 26:2080–2097.

Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol 133:462–469.

Dacks JB, Field MC (2007) Evolution of the eukaryotic membrane-trafficking system: origin, tempo and mode. J Cell Sci 120:2977–2985.

Dacks JB, Poon PP, Field MC (2008) Phylogeny of endocytic components yields insight into the process of nonendosymbiotic organelle evolution. Proc Natl Acad Sci U S A 105:588–593.

de Graaf BHJ, Cheung AY, Andreyeva T, Levasseur K, Kieliszewski M, Wu H (2005) Rab11 GTPase-regulated membrane trafficking is crucial for tip-focused pollen tube growth in tobacco. Plant Cell 17:2564–2579.

Dettmer J, Hong-Hermesdorf A, Stierhof Y-D, Schumacher K (2006) Vacuolar H+-ATPase activity is required for endocytic and secretory trafficking in Arabidopsis. Plant Cell 18:715–730.

Dhonukshe P, Baluška F, Schlicht M, Hlavacka A, Šamaj J, Friml J, Gadella TWJ (2006) Endocytosis of cell surface material mediates cell plate formation during plant cytokinesis. Dev Cell 10:137–150.

Dhonukshe P, Tanaka H, Goh T, Ebine K, Mähönen AP, Prasad K, Blilou I, Geldner N, Xu J, Uemura T, Chory J, Ueda T, Nakano A, Scheres B, Friml J (2008) Generation of cell polarity in plants links endocytosis, auxin distribution and cell fate decisions. Nature 456:962–966.

Ebine K, Fujimoto M, Okatani Y, Nishiyama T, Goh T, Ito E, Dainobu T, Nishitani A, Uemura T, Sato MH, Thordal-Christensen H, Tsutsumi N, Nakano A, Ueda T (2011) A membrane trafficking pathway regulated by the plant-specific RAB GTPase ARA6. Nat Cell Biol 13:853–859.

Ebine K, Inoue T, Ito J, Ito E (2014) Plant Vacuolar Trafficking Occurs through Distinctly Regulated Pathways. Curr Biol 24:1375–1382.

Ellinger D, Glockner a., Koch J, Naumann M, Sturtz V, Schutt K, Manisseri C, Somerville SC, Voigt C a. (2014) Interaction of the Arabidopsis GTPase RabA4c with Its Effector PMR4 Results in Complete Penetration Resistance to Powdery Mildew. Plant Cell

tpc.114.127779–.

Feraru E, Feraru MI, Asaoka R, Paciorek T, De Rycke R, Tanaka H, Nakano A, Friml J (2012) BEX5/RabA1b Regulates trans-Golgi Network-to-Plasma Membrane Protein Trafficking in Arabidopsis. Plant Cell 24:3074–3086.

Field MC, Natesan SKA, Gabernet-Castello C, Lila Koumandou V (2007) Intracellular trafficking in the trypanosomatids. Traffic 8:629–639.

Fukuda M, Wen L, Satoh-Cruz M, Kawagoe Y, Nagamura Y, Okita TW, Washida H, Sugino A, Ishino Y, Ishino S, Ogawa M, Sunada M, Ueda T, Kumamaru T (2013) A guanine nucleotide exchange factor for Rab5 proteins is essential for intracellular transport of the proglutelin from the Golgi apparatus to the protein storage vacuole in rice endosperm. Plant Physiol 162:663–74.

Geldner N, Friml J, Stierhof YD, Jürgens G, Palme K (2001) Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. Nature 413:425–428.

Goh T, Uchida W, Arakawa S, Ito E, Dainobu T, Ebine K, Takeuchi M, Sato K, Ueda T, Nakano A (2007) VPS9a, the common activator for two distinct types of Rab5 GTPases, is essential for the development of Arabidopsis thaliana. Plant Cell 19:3504–3515.

Grebe M, Xu J, Möbius W, Ueda T, Nakano A, Geuze HJ, Rook MB, Scheres B (2003) Arabidopsis sterol endocytosis involves actin-mediated trafficking via ARA6-positive early endosomes. Curr Biol 13:1378–1387.

Green EG, Ramm E, Riley NM, Spiro DJ, Goldenring JR, Wessling-Resnick M (1997) Rab11 is associated with transferrin-containing recycling compartments in K562 cells. Biochem Biophys Res Commun 239:612–616.

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

Haas TJ, Sliwinski MK, Martínez DE, Preuss M, Ebine K, Ueda T, Nielsen E, Odorizzi G, Otegui MS (2007) The Arabidopsis AAA ATPase SKD1 is involved in multivesicular endosome function and interacts with its positive regulator LYST-INTERACTING

PROTEIN5. Plant Cell 19:1295–1312.

Hama H, Tall GG, Horazdovsky BF (1999) Vps9p is a guanine nucleotide exchange factor involved in vesicle-mediated vacuolar protein transport. J Biol Chem 274:15284–15291.

Hoepflinger MC, Geretschlaeger A, Sommer A, Hoeftberger M, Hametner C, Ueda T, Foissner I (2015) Molecular Analysis and Localization of CaARA7 a Conventional RAB5 GTPase from Characean algae. Traffic 16:534–554.

Hoepflinger MC, Geretschlaeger A, Sommer A, Hoeftberger M, Nishiyama T, Sakayama H, Hammerl P, Tenhaken R, Ueda T, Foissner I (2013) Molecular and biochemical analysis of the first ARA6 homologue, a RAB5 GTPase, from green algae. J Exp Bot 64:5553–5568.

Horiuchi H, Lippé R, McBride HM, Rubino M, Woodman P, Stenmark H, Rybin V, Wilm M, Ashman K, Mann M, Zerial M (1997) A novel Rab5 GDP/GTP exchange factor complexed to Rabaptin-5 links nucleotide exchange to effector recruitment and function. Cell 90:1149–1159.

Ito E, Fujimoto M, Ebine K, Uemura T, Ueda T, Nakano A (2012) Dynamic behavior of clathrin in Arabidopsis thaliana unveiled by live imaging. Plant J 69:204–216.

Ivanov R, Gaude T (2009) Endocytosis and endosomal regulation of the S-receptor kinase during the self-incompatibility response in Brassica oleracea. Plant Cell 21:2107–2117.

Jaillais Y, Fobis-Loisy I, Miège C, Gaude T (2008) Evidence for a sorting endosome in Arabidopsis root cells. Plant J 53:237–247.

Kanazawa T, Era A, Minamino N, Shikano Y, Fujimoto M, Uemura T, Nishihama R, Yamato KT, Ishizaki K, Nishiyama T, Takayuki Kohchi, Akihiko Nakano, Ueda T (2015) SNARE Molecules in Marchantia polymorpha: Unique and Conserved Features of the Membrane Fusion Machinery. Plant Cell Phsiology. doi: doi: 10.1093/pcp/pcv076

Kotzer AM, Brandizzi F, Neumann U, Paris N, Moore I, Hawes C (2004) AtRabF2b (Ara7) acts on the vacuolar trafficking pathway in tobacco leaf epidermal cells. J Cell Sci 117:6377–6389.

Kunita R, Otomo A, Mizumura H, Suzuki K, Showguchi-Miyata J, Yanagisawa Y, Hadano S, Ikeda JE (2004) Homo-oligomerization of ALS2 through its unique carboxyl-terminal regions is essential for the ALS2-associated Rab5 guanine nucleotide exchange activity and its regulatory function on endosome trafficking. J Biol Chem 279:38626–38635.

Lam SK, Siu CL, Hillmer S, Jang S, An G, Robinson DG, Jiang L (2007) Rice SCAMP1 defines clathrin-coated, trans-golgi-located tubular-vesicular structures as an early endosome in tobacco BY-2 cells. Plant Cell 19:296–319.

Lee G-J, 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.

Lippé R, Miaczynska M, Rybin V, Runge A, Zerial M (2001) Functional synergy between Rab5 effector Rabaptin-5 and exchange factor Rabex-5 when physically associated in a complex. Mol Biol Cell 12:2219–2228.

Mackiewicz P, Wyroba E (2009) Phylogeny and evolution of Rab7 and Rab9 proteins. BMC Evol Biol 9:101.

Mattera R, Bonifacino JS (2008) Ubiquitin binding and conjugation regulate the recruitment of Rabex-5 to early endosomes. EMBO J 27:2484–2494.

Mattera R, Yien CT, Weissman AM, Bonifacino JS (2006) The Rab5 guanine nucleotide exchange factor Rabex-5 binds ubiquitin (Ub) and functions as a Ub ligase through an atypical Ub-interacting motif and a zinc finger domain. J Biol Chem 281:6874–6883.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tabacco tissue cultures. Physiol Plant 15:473–497.

Nakagawa T, Kurose T, Hino T, Tanaka K, Kawamukai M, Niwa Y, Toyooka K, Matsuoka K, Jinbo T, Kimura T (2007) Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. J Biosci Bioeng 104:34–41.

Paciorek T, Zazímalová E, Ruthardt N, Petrásek J, Stierhof Y-D, Kleine-Vehn J, Morris DA, Emans N, Jürgens G, Geldner N, Friml J (2005) Auxin inhibits endocytosis and promotes

its own efflux from cells. Nature 435:1251–1256.

Penengo L, Mapelli M, Murachelli AG, Confalonieri S, Magri L, Musacchio A, Di Fiore PP, Polo S, Schneider TR (2006) Crystal Structure of the Ubiquitin Binding Domains of Rabex-5 Reveals Two Modes of Interaction with Ubiquitin. Cell 124:1183–1195.

Pereira-Leal JB (2008) The Ypt/Rab family and the evolution of trafficking in fungi. Traffic 9:27–38.

Platta HW, Stenmark H (2011) Endocytosis and signaling. Curr Opin Cell Biol 23:393–403.

Prag G, Misra S, Jones EA, Ghirlando R, Davies BA, Horazdovsky BF, Hurley JH (2003) Mechanism of ubiquitin recognition by the CUE domain of Vps9p. Cell 113:609–620.

Preuss ML, Schmitz AJ, Thole JM, Bonner HKS, Otegui MS, Nielsen E (2006) A role for the RabA4b effector protein PI-4K beta 1 in polarized expansion of root hair cells in Arabidopsis thaliana. J Cell Biol 172:991–998.

Ren M, Xu G, Zeng J, De Lemos-Chiarandini C, Adesnik M, Sabatini DD (1998) Hydrolysis of GTP on rab11 is required for the direct delivery of transferrin from the pericentriolar recycling compartment to the cell surface but not from sorting endosomes. Proc Natl Acad Sci U S A 95:6187–6192.

Robatzek S, Chinchilla D, Boller T (2006) Ligand-induced endocytosis of the pattern recognition receptor FLS2 in Arabidopsis. Genes Dev 20:537–542.

Rutherford S, Moore I (2002) The Arabidopsis Rab GTPase family: Another enigma variation. Curr Opin Plant Biol 5:518–528.

Sakaguchi A, Sato M, Sato K, Gengyo-Ando K, Yorimitsu T, Nakai J, Hara T, Sato K, Sato K (2015) REI-1 Is a Guanine Nucleotide Exchange Factor Regulating RAB-11 Localization and Function in C. elegans Embryos. Dev Cell 35:211–221.

Sato M, Sato K, Fonarev P, Huang C-J, Liou W, Grant BD (2005) Caenorhabditis elegans RME-6 is a novel regulator of RAB-5 at the clathrin-coated pit. Nat Cell Biol 7:559–569.

Schwartz SL, Cao C, Pylypenko O, Rak A, Wandinger-Ness A (2007) Rab GTPases at a glance.

J Cell Sci 120:3905–3910.

Shideler T, Nickerson DP, Merz a. J, Odorizzi G (2015) Ubiquitin binding by the CUE domain promotes endosomal localization of the Rab5 GEF Vps9. Mol Biol Cell 26:1345–1356.

Sohn EJ, Kim ES, Zhao M, Kim SJ, Kim H, Kim Y-W, 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.

Sousa E, Kost B, Malhó R (2008) Arabidopsis phosphatidylinositol-4-monophosphate 5-kinase 4 regulates pollen tube growth and polarity by modulating membrane recycling. Plant Cell 20:3050–3064.

Stenmark H (2009) Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol 10:513–525.

Stierhof YD, El Kasmi F (2010) Strategies to improve the antigenicity, ultrastructure preservation and visibility of trafficking compartments in Arabidopsis tissue. Eur J Cell Biol 89:285–297.

Szumlanski AL, Nielsen E (2009) The Rab GTPase RabA4d regulates pollen tube tip growth in Arabidopsis thaliana. Plant Cell 21:526–544.

Tall GG, Barbieri MA, Stahl PD, Horazdovsky BF (2001) Ras-Activated Endocytosis Is Mediated by the Rab5 Guanine Nucleotide Exchange Activity of RIN1. Dev Cell 1:73–82.

Tian G-W, Mohanty A, Chary SN, Li S, Paap B, Drakakaki G, Kopec CD, Li J, Ehrhardt D, Jackson D, Rhee SY, Raikhel N V, Citovsky V (2004) High-throughput fluorescent tagging of full-length Arabidopsis gene products in planta. Plant Physiol 135:25–38.

Ueda T, Uemura T, Sato MH, Nakano A (2004) Functional differentiation of endosomes in Arabidopsis cells. Plant J 40:783–789.

Ueda T, Yamaguchi M, Uchimiya H, Nakano A (2001) Ara6, a plant-unique novel type Rab GTPase, functions in the endocytic pathway of Arabidopsis thaliana. EMBO J

20:4730–4741.

Uejima T, Ihara K, Goh T, Ito E, Sunada M, Ueda T, Nakano A, Wakatsuki S (2010) GDP-bound and nucleotide-free intermediates of the guanine nucleotide exchange in the Rab5??Vps9 system. J Biol Chem 285:36689–36697.

Uemura T, Ueda T, Ohniwa RL, Nakano A, Takeyasu K, Sato MH (2004) Systematic analysis of SNARE molecules in Arabidopsis: dissection of the post-Golgi network in plant cells. Cell Struct Funct 29:49–65.

Uemura T, Yoshimura SH, Takeyasu K, Sato MH (2002) Vacuolar membrane dynamics revealed by GFP-AtVam3 fusion protein. Genes to Cells 7:743–753.

Ullrich O, Reinsch S, Urbé S, Zerial M, Parton RG (1996) Rab11 regulates recycling through the pericentriolar recycling endosome. J Cell Biol 135:913–924.

Viotti C, Bubeck J, Stierhof Y-D, Krebs M, Langhans M, van den Berg W, van Dongen W, Richter S, Geldner N, Takano J, Jürgens G, de Vries SC, Robinson DG, Schumacher K (2010) Endocytic and secretory traffic in Arabidopsis merge in the trans-Golgi network/early endosome, an independent and highly dynamic organelle. Plant Cell 22:1344–1357.

Wee EG, Sherrier DJ, Prime TA, Dupree P (1998) Targeting of active sialyltransferase to the plant Golgi apparatus. Plant Cell 10:1759–1768.

Wen L, Fukuda M, Sunada M, Ishimoto S, Ishino Y, Okita TW, Ogawa M, Ueda T, Kumamaru T (2015) Guanine nucleotide exchange factor 2 for Rab5 proteins coordinated with GLUP6/GEF regulates the intracellular transport of the proglutelin from the Golgi apparatus to the protein storage vacuole in rice endosperm Liuying. J Exp Bot. doi: 10.1104/pp.113.217869

Xu L, Lubkov V, Taylor LJ, Bar-Sagi D (2010) Feedback regulation of ras signaling by rabex-5-mediated ubiquitination. Curr Biol 20:1372–1377.

Zhang L, Zhang H, Liu P, Hao H, Jin JB, Lin J (2011) Arabidopsis R-SNARE proteins VAMP721 and VAMP722 are required for cell plate formation. PLoS One. doi:

10.1371/journal.pone.0026129

Zhang X, He X, Fu X-Y, Chang Z (2006) Varp is a Rab21 guanine nucleotide exchange factor and regulates endosome dynamics. J Cell Sci 119:1053–1062.

Zhao Y, Yan A, Feijó JA, Furutani M, Takenawa T, Hwang I, Fu Y, Yang Z (2010) Phosphoinositides regulate clathrin-dependent endocytosis at the tip of pollen tubes in Arabidopsis and tobacco. Plant Cell 22:4031–4044.

Zhu H, Liang Z, Li and G (2009) Rabex-5 Is a Rab22 Effector and Mediates a Rab22-Rab5 Signaling Cascade in Endocytosis. Mol Biol Cell 20:4072–4729.

Zuo J, Niu Q-W, Chua N-H (2000) An estrogen-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J 24:265–273.

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