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Membrane traffic between single membrane-bounded organelles plays an integral role in various cell activities in eukaryotic cells. Recent comparative genomics have indicated that membrane trafficking pathways are diversified among eukaryotic lineages, which is associated with the lineage-specific acquisition of new trafficking pathways and the secondary loss of preexisting trafficking routes. Our long-term goal is to reveal how plants have acquired their unique membrane trafficking systems during evolution, which will be achieved by comparative analyses using the model plant *Arabidopsis thaliana* and a liverwort model, *Marchantia polymorpha*. We also aim to elucidate the detailed molecular mechanisms and physiological functions of membrane trafficking in higher-ordered plant functions.

I. Diversification of membrane trafficking pathways associated with the acquisition of novel machinery components

Although the basic framework of membrane trafficking is well conserved among eukaryotic lineages, recent comparative genomics have suggested that each lineage has acquired unique membrane trafficking pathways during evolution. RAB GTPases and SNARE proteins are evolutionarily conserved key regulators active in the tethering and/or fusion of membrane vesicles with target membranes. It has been proposed that lineage-specific diversification of these key factors is closely associated with the acquisition of lineage-specific membrane trafficking pathways, whose molecular basis remains unknown.

1-1 Characterization of RAB GTPases in the liverwort, *Marchantia polymorpha*

To gain information on the diversification of membrane trafficking pathways during land plant evolution, we systematically analyzed RAB GTPases in *Marchantia polymorpha*. Comparisons of the organization of this protein family with other plant lineages, followed by functional analyses in *M. polymorpha*, indicated that diversification of membrane trafficking pathways in land plants has been achieved by 1) acquisition of novel machinery components, 2) relocation of conserved machinery components to distinct trafficking events, and 3) secondary loss of conserved machinery components during evolution (Minamino *et al*., 2018).

1-2 Analysis of the liverwort-specific organelle: the oil body

Through analyses of SNARE members in *M. polymorpha*, we found that a member of the SYP1 group is localized to the membrane of an organelle specific to liverworts (the oil body) whose origin and biogenesis remain unclear. We are currently characterizing membrane trafficking pathways responsible for oil body biogenesis, as well as analyzing the function of a master regulator of oil body biogenesis.

1-3 Mechanisms and dynamics of vacuolar transport

The vacuole is the largest organelle in plant cells, and occupies over 90% of mature plant cells. The vacuole fulfills various functions in plant physiology and development, such as protein degradation, protein storage, and regulation of turgor pressure. To perform these vacuolar functions, a wide variety of vacuolar proteins and other components must be properly transported to the vacuole, the entirety of which is mediated by membrane trafficking. To understand the molecular mechanisms of vacuolar transport in plants, we analyzed the molecular functions of RAB5 and RAB7 in *A. thaliana* cells. A tethering complex, CORVET, is known to act as an effector of Vps21/RAB5 in regulating endosomal transport in yeast and animal cells. CORVET subunits are also conserved in *A. thaliana*, but the molecular function of CORVET remains unclear. We found that VPS3, one of the CORVET subunits, acts in the RAB5-dependent and RAB7-independent vacuolar transport pathway in *A. thaliana*. Furthermore, we also found that another tethering complex sharing the core complex with CORVET, the HOPS complex, regulates a different trafficking event from CORVET. Our results further indicated that different fusion machineries comprising distinct R-SNARE proteins are involved in CORVET- and HOPS-mediated trafficking pathways (Figure 1). These findings demonstrated that the plant vacuolar transport system has diverged from vacuolar/lysosomal transport systems in non-plant systems (Takemoto *et al*., 2018).

We also conducted detailed analyses of vacuolar SNARE proteins. Defective functions of vacuolar SNAREs affect both vacuolar transport and morphology. The *sgr3-1* (*shoot gravitropism3*) mutant was isolated as a mutant that is defective in shoot gravitropism, which resulted from a point

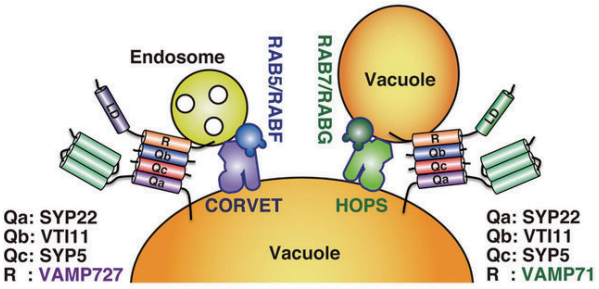

Figure 1. Schematic illustration of tethering-fusion modules acting in vacuolar transport in *A. thaliana* cells (Takemoto *et al*., 2018).

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2018. The former title is indicated by an asterisk (*).

mutation in *SYP22/VAM3*, one of the SNARE proteins residing on the vacuole and acting in vacuolar transport. Intriguingly, *sgr3-1* exhibits abnormal vacuolar morphology, although vacuolar transport is not markedly affected in this mutant. Therefore *sgr3-1* may be a useful means of examining the functions of the vacuolar SNARE. We are exploring vacuolar dynamics regulated by SYP22 by analyzing the effect of the *sgr3-1* mutation in a more detailed manner.

1-4 Integration of two RAB5 groups in plants

RAB5 is a member of RAB GTPase acting in endosomal transport in eukaryotic cells that has existed within almost all eukaryotic organisms, as well as their common ancestor: LECA. Plants possess two different RAB5 groups, canonical and plant-unique types, which act via unknown counteracting mechanisms. We identified an effector molecule of the plant-unique RAB5 in *A. thaliana*, ARA6, which we designated as PLANT-UNIQUE RAB5 EFFECTOR 2 (PUF2). Preferential co-localization with canonical RAB5 on endosomes and genetic interaction analysis (Figure 2) indicated that PUF2 coordinates vacuolar transport with canonical RAB5, although PUF2 was identified as an effector of ARA6. Competitive binding of PUF2 with GTP-bound ARA6 and GDP-bound canonical RAB5, interacting together with the shared activating factor VPS9a, showed that ARA6 negatively regulates canonical RAB5-mediated vacuolar transport by titrating PUF2 and VPS9a. These results suggest a unique and unprecedented function for RAB effectors involving the integration of two RAB groups to orchestrate endosomal trafficking in plant cells.

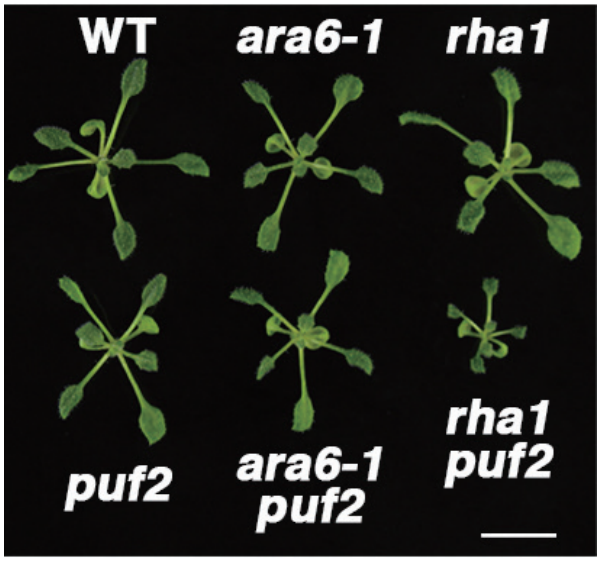

Figure 2. Genetic interactions among mutations in canonical (*RHA1*) and plant-unique (*ARA6*) *RAB5* and *PUF2* (Ito *et al*., 2018).

II. Significance of membrane trafficking in higher-ordered plant functions

2-1 Membrane trafficking in plant gametogenesis

Gametogenesis in plants involves membrane trafficking-mediated processes. We are now analyzing the molecular mechanisms of gametogenesis in Arabidopsis and *M. polymorpha*, especially focusing our attention on secretory and degradative trafficking pathways during male gamete formation.

Cytokinesis in land plants is achieved by the re-direction of the secretory pathway. As such, KNOLLE/SYP111 and KEULE/SEC11 play important roles in membrane fusion at the forming cell plate in somatic cells of Arabidopsis. Conversely, no deleterious effects on gametogenesis have been reported regarding mutations in genes for these proteins thus far. We found that other SYP1 and SEC1 members are highly expressed during male gametogenesis. Analyses of the functions of these proteins during male gametogenesis are currently underway.

Distinct from seed plants, basal land plants including *M. polymorpha* utilize the spermatozoid with two (or more) motile flagella as the male gamete in sexual reproduction. We visualized the process of spermatozoid formation, especially spermiogenesis, using fluorescently-tagged organelle markers. The majority of the endomembranous organelles such as the Golgi apparatus were removed from maturing spermatozoid cells, and the plasma membrane was also reorganized during spermiogenesis. Inspection by transmission electron microscope and live-cell imaging analyses also indicated that the number of degradative organelles such as the multivesicular endosomes, vacuoles, and autophagosomes, was transiently increased during this process. To reveal the molecular mechanisms of cytoplasm removal and organelle remodeling, we are now analyzing the contribution of autophagy in these processes. Autophagy-defective mutants exhibited defects in the areas of morphogenesis, cytoplasm removal, and motility of spermatozoids, indicating the numerous crucial roles played by autophagy during spermiogenesis in *M. polymorpha*. We are also analyzing the role of RAB GTPases in flagella formation. Through a comprehensive analysis of RAB GTPases in *M. polymorpha*, we found that a RAB GTPase plays an essential role in generating fully functional flagella.

2-2 Membrane trafficking in sexual reproduction

During plant reproduction, sperm cells are delivered to ovules through growing pollen tubes. This process involves tip-localized receptor kinases regulating the integrity and/or guidance of pollen tubes, whose localizations must be strictly regulated. We found that a pair of AP180 N-terminal homology domain-containing proteins (ANTH proteins), PICALM5a and PICALM5b, are responsible for the tip-localization of ANXUR receptor kinases acting in an autocrine signaling pathway required for pollen tube integrity in *A. thaliana*. The *picalm5a picalm5b* double mutant exhibits reduced fertility, and the double mutant pollen is defective in pollen tube integrity with premature bursts. The tip localizations of ANXUR proteins are severely impaired in *picalm5a picalm5b* pollen tubes (Figure 3), whereas another receptor kinase PRK6 acting in pollen tube guidance is not affected. Based on these results, we propose that PICALM5 proteins serve as specific loading adaptors to recycle ANXUR proteins (Muro *et al*., 2018).

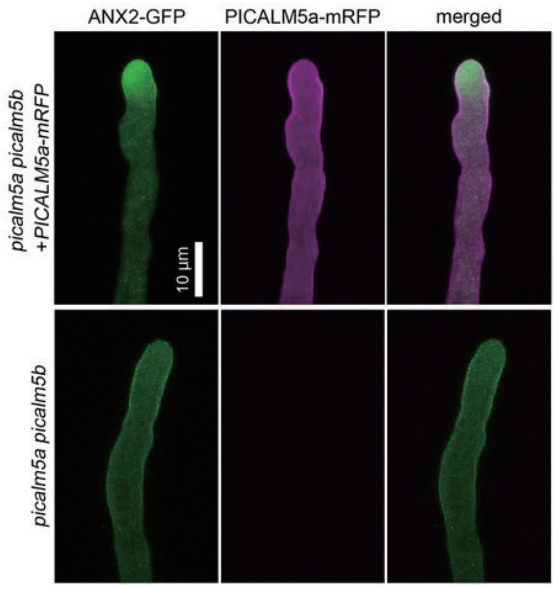

Figure 3. PICALM5s are required for tip-localization of ANX2 (Muro *et al*., 2018).

Publication List:

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