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Membrane traffic between single membrane-bounded organelles plays integral roles in various cell activities in eukaryotic cells. Recent comparative genomics has indicated that membrane trafficking pathways are diversified among eukaryotic lineages, which is associated with lineage-specific acquisition of new trafficking pathways and secondary loss of preexisting trafficking routes. Our long-term goal is unraveling 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 model of liverworts, *Marchantia polymorpha*. We also aim to elucidate detailed molecular mechanisms and physiological functions of membrane trafficking in higher-ordered plant functions.

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

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

1-1 Characterization of RAB and SNARE proteins in the liverwort, *Marchantia polymorpha*

For information on the diversification of membrane trafficking pathways during land plant evolution, we systematically identified RAB GTPases and SNARE proteins in *Marchantia polymorpha*. Comparison of organization of these protein families with other plant lineages, followed by their 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) relocating conserved machinery components to distinct trafficking events, and 3) secondary loss of conserved machinery components, during evolution.

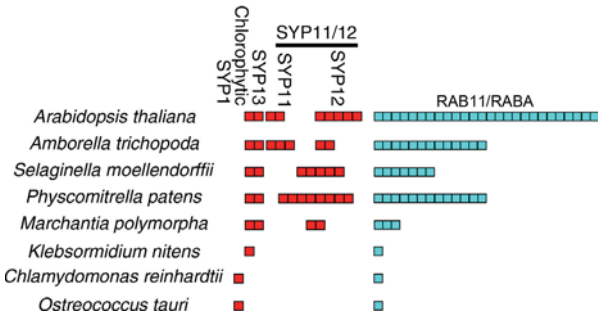

Figure 1. *SYP1* and *RAB11* genes in the genomes of green plants. Genes are indicated as individual red (for *SYP1*) or cyan (for *RAB11*) boxes. Numbers of these genes increased during land plant evolution, suggesting secretion-related functions have been diversified in land plants.

1-2 Analysis of the liverwort-specific organelle, 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 (Figure 2), whose origin and mechanisms of biogenesis remain unclear. We are currently analyzing the molecular function of the SYP1 member, as well as characterizing membrane trafficking pathways responsible for oil body biogenesis. We have succeeded in isolating several mutants defective in the function and morphogenesis of the oil body through a forward-genetic approach. Analyses of a mutant with an increased number of oil bodies identified a transcription factor regulating oil body biogenesis, which activates transcription of genes responsible for secondary metabolite biosynthesis and putative transporters, as well as the SYP1 member.

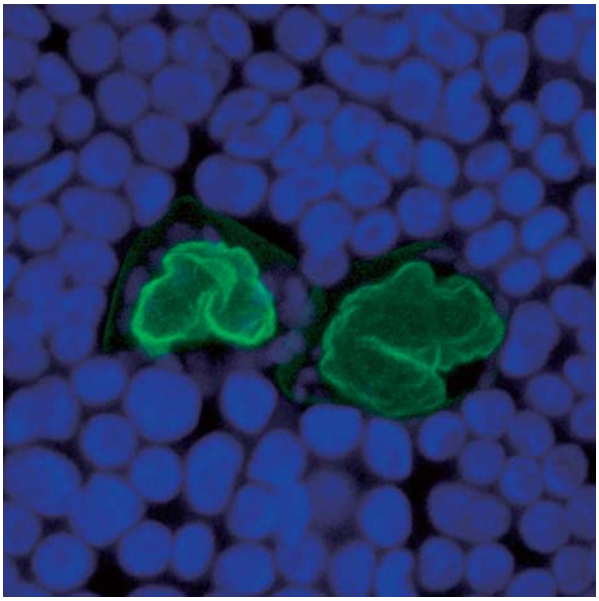

Figure 2. Oil bodies in a thallus of *M.* polymorpha, which are visualized by the YFP-tagged SYP1 member (green). Autofluorescence from chlorophyll is also shown (blue).

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2017. The former title is indicated by an asterisk (*).

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, which include protein degradation, protein storage, and regulation of turgor pressure. To fulfill these vacuolar functions, a wide variety of vacuolar proteins and other components must be correctly transported to the vacuole, which is mediated by membrane trafficking. To understand molecular mechanisms of vacuolar transport in plants, we analyzed molecular functions of RAB5 and RAB7, and found that these proteins act sequentially in the vacuolar transport pathway in Arabidopsis cells. Furthermore, we also found that two additional vacuolar transport pathways, the RAB5-dependent but RAB7-independent pathway and the AP-3-dependent pathway operate in vacuolar transport in Arabidopsis. We are now exploring details of molecular mechanisms of these vacuolar transport pathways, especially focusing our interest on the RAB5-dependent but RAB7-independent pathway, because this trafficking pathway has not been described in non-plant systems. A tethering complex, CORVET, is known to act as an effector of Vps21/RAB5 to regulate endosomal transport in yeast and animal cells. CORVET subunits are also conserved in Arabidopsis, but molecular function of CORVET remained unclear. We found that VPS3, one of the CORVET subunits, acts in the RAB5-dependent and RAB7-independent vacuolar transport pathway in Arabidopsis (Figure 3). 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. These findings demonstrated that the plant vacuolar transport system has been diverged from vacuolar/lysosomal transport systems in non-plant systems.

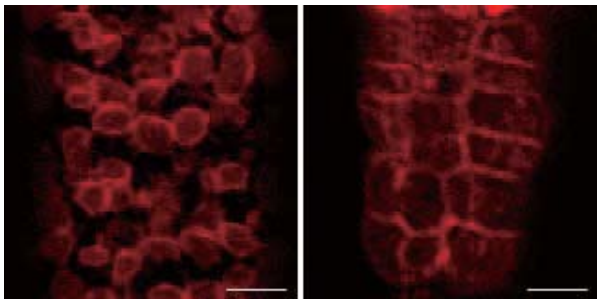

Figure 3. Subcellular localization of mRFP-SYP22 in WT-like and *vps3* embryos. SYP22 is one of the cargo molecules of the RAB5-dependent and RAB7-independent vacuolar transport pathway. mRFP-tagged SYP22 localized to the vacuolar membrane in WT-like embryo cells (left), whereas it mislocalized to the plasma membrane in the *vps3* mutant embryo cells (right). Bars = 10 μm.

We also conducted detailed analyses of vacuolar SNARE proteins. Defective functions in vacuolar SNAREs affect both vacuolar transport and vacuolar morphology. The *sgr3-1* (*shoot gravitropism3*) mutant was isolated as a mutant defective in shoot gravitropism, which resulted from a point 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* should be a good tool for dissecting functions of the vacuolar SNARE. We are exploring vacuolar dynamics regulated by SYP22 by analyzing the effect of the *sgr3-1* mutation in a detailed manner. Co-immunoprecipitation and MS analyses indicated that the *sgr3-1* mutation specifically stabilizes a SNARE complex comprising VAMP71, SYP22, and αSNAP. We also found that these molecules and HOPS components accumulate on the vacuolar membrane in *sgr3-1*. These results highlighted a unique characteristic of the *sgr3-1* mutation, which will provide valuable information to better understand mechanisms of SNARE-mediated membrane fusion.

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

2-1 Analyses of functions of the plant-specific RAB GTPase ARA6 in stress responses in *Arabidopsis thaliana*

ARA6 is a plant-unique RAB GTPase, whose close homologs are only found in green plant lineages. To elucidate why only plants harbor the ARA6 members, we analyzed functional significance of ARA6 in biotic and abiotic stress responses. We found that ARA6 is recruited to the extrahaustorial membranes formed by the fungal pathogen causing powdery mildew and the oomycete causing downy mildew, suggesting modulation of host membrane trafficking by pathogenic microbes (Inada *et al*., 2016). We also found that overexpression of constitutive active ARA6 repressed the full proliferation of powdery mildew fungi (Inada et al., 2017).

2-2 Membrane trafficking in plant gametogenesis

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

Cytokinesis in land plants is achieved by re-direction of the secretory pathway, and KNOLLE/SYP111 and KEULE/SEC11 play important roles in membrane fusion at the forming cell plate in somatic cells of Arabidopsis. Conversely, any deleterious effects on gametogenesis have been reported for mutations in these genes thus far. We found that other SYP1 and SEC1 members are highly expressed during male gametogenesis. The analyses of these proteins during male gametogenesis are currently underway.

Distinct from seed plants, basal land plants including *M. polymorpha* utilize sperm as the male gamete in sexual reproduction. We visualized the process of sperm formation, especially spermiogenesis, using fluorescently-tagged organelle markers (Figure 4). The majority of the endomembranous organelles such as the Golgi apparatus was removed from the maturing cells, and the plasma membrane was also reorganized during spermiogenesis. A TEM analysis also indicated that the number of degradative organelles such as the multivesicular endosomes, vacuoles, and autopha-

gosomes, was transiently increased during this process. To reveal the molecular mechanisms of organelle degradation and cytoplasm removal, we are now analyzing the contribution of autophagy. The autophagy-defective mutations resulted in defective morphogenesis and motility of sperm, indicating crucial functions of autophagy during spermiogenesis in *M. polymorpha*.

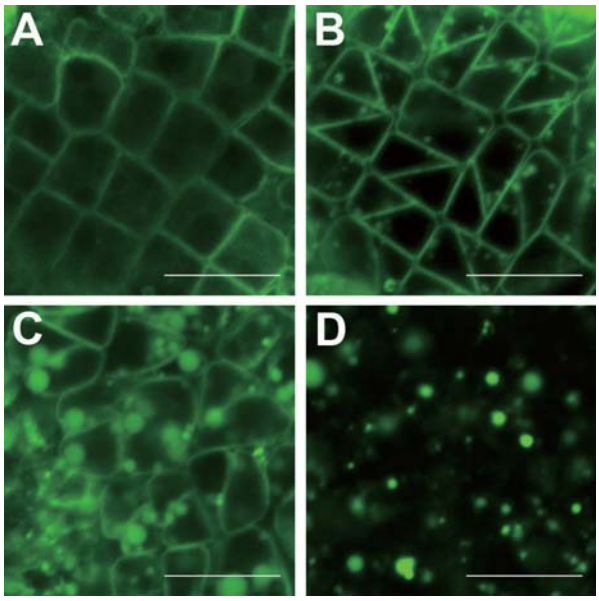

Figure 4. Reorganization of the plasma membrane during spermatogenesis in *M. polymorpha*. Spermatogenesis proceeds from A to D. A plasma membrane-resident protein visualized with Citrine is targeted to intracellular spherical structures, and completely removed from the plasma membrane of mature spermatids. Bars = 10 μm. (Adopted from Minamino et al., 2017)

Publication List:

〔Original papers〕

- Akita, K., Kobayashi, M., Sato, M., Kutsuna, N., Ueda, T., Toyooka, K., Nagata, N., Hasezawa, S., and Higaki, T. (2017). Accumulation of fluorescent proteins derived from a trans-Golgi cisternal membrane marker and paramural bodies in interdigitated apoplastic space in Arabidopsis leaf epidermis. Protoplasma *254*, 367-377.
- Bowman, J.L., Kohchi, T., Yamato, K.T., Jenkins, J., Shu, S., Ishizaki, K., Yamaoka, S., Nishihama, R., Nakamura, Y., Berger, F., et al. (2017). Insights into land plant evolution garnered from the Marchantia polymorpha genome. Cell *171*, 287-304.
- Cui, Y., Zhao, Q., Xie, H.T.,Wong, W.S., Gao, C., Ding, Y., Tan, Y., Ueda, T., Zhang, Y., and Jiang, L. (2017). MON1/CCZ1-mediated Rab7 activation regulates tapetal programmed cell death and pollen development in Arabidopsis. Plant Physiol. *173*, 206-218.
- Inada, N., Ebine, K., Ito, E., Nakano, A., and Ueda, T. (2017). Constitutive activation of plant-specific RAB5 GTPase confers increased resistance against adapted powdery mildew fungus. Plant Biotech. *34*, 89-95.
- Ito, Y., Toyooka, K., Fujimoto, M., Ueda, T., Uemura, T., and Nakano A. (2017). The trans-Golgi network and the Golgi stacks behave independently during regeneration after Brefeldin A treatment in tobacco BY-2 cells. Plant Cell Physiol. *58*, 811-821.
- Matsui, H., Nomura, Y., Egusa, M., Hamada, T., Hyon, G.S., Kaminaka, H., Watanabe, Y., Ueda,, T., Trujillo, M., Shirasu, K., and Nakagami, H. (2017). The GYF domain protein PSIG1 dampens the induction of cell death during plant-pathogen interactions. PLoS Genet. *13*, e1007037.
- Minamino, N., Kanazawa, T., Nishihama, R., Yamato, T.K., Ishizaki, K., Kohchi, T., Nakano, A., and Ueda, T. (2017). Dynamic reorganization of the endomembrane system during spermatogenesis in Marchantia polymorpha. J. Plant Res. *130*, 433-441.
- Ung, H., Karia, P., Ebine, K., Ueda, T., Yoshioka, K., and Moeder, W. (2017). Triphosphate tunnel metalloenzyme function in senescence highlights a biological diversification of this protein superfamily. Plant Physiol. *175*, 473-485.

〔Original paper (E-publication ahead of print)〕

- Sánchez-Rodríguez, C., Shi, Y., Kesten, C., Zhang, D., Sancho-Andrés, G., Ivakov, A., Lampugnani, E.R., Sklodowski, K., Fujimoto, M., Nakano, A., Bacic, A., Wallace, I.S., Ueda, T., van Damme, D., Zhou, Y., and Persson, S. The cellulose synthases are cargo of the TPLATE adaptor complex. Mol. Plant 2017 Dec 5.

〔Review article〕

- Kanazawa, T., and Ueda, T. (2017). Exocytic trafficking pathways in plants: why and how they are redirected. New Phytol. *215*, 952-957.