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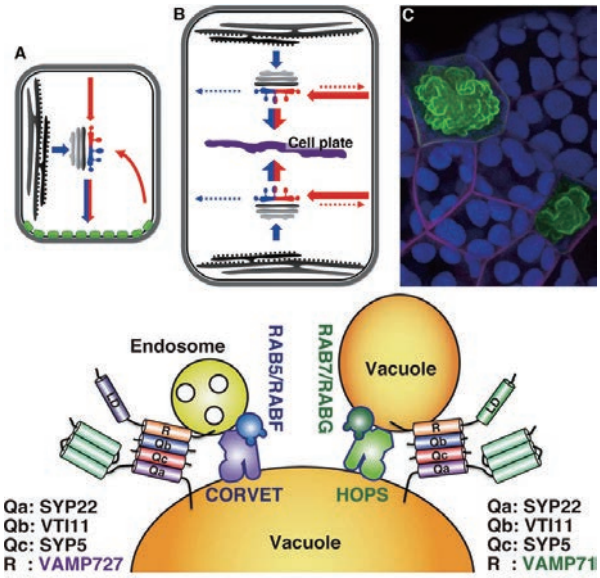

Visual overview of this lab's work.

Membrane traffic between single membrane-bounded organelles plays an integral role in various activities in eukaryotic cells. Recent comparative genomics has indicated that membrane trafficking pathways are diversified among eukaryotic lineages, which are 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. This 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 has 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. Comparisons of these protein families' organizations among plant lineages, followed by functional analyses of each gene product in *A. thaliana* and *M. polymorpha*, indicated that diversification and specialization of membrane trafficking pathways in land plants have 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.

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

The oil body is an organelle specific to liverworts, whose origin and biogenesis mechanism remain unclear. We are studying the oil body in *M. polymorpha*, as a model of newly-acquired organelles in specific lineages during evolution. Through comprehensive analyses of SNARE members and organelle markers in *M. polymorpha*, we identified a member of the SYP1 group. Secretory cargos were targeted to the oil body (Figure 1), which indicated that the oil body is formed by the redirection of the secretory pathway. We additionally proposed the oil body cycle hypothesis; the periodic redirection of the secretory pathway mediates oil body development and growth of the oil body cell. Furthermore, we revealed that the oil body acts as a defense against potential arthropod herbivory by using mutants of the master regulator of oil body formation that we identified, MpERF13.

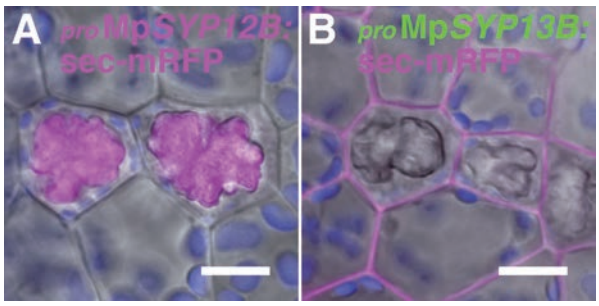

Figure 1. The redirection of the secretory pathway in oil body cells. The secretory cargo, sec-mRFP, is targeted to oil bodies (A) and the extracellular space (B), when expressed in hypothetical "oil body phase" and "plasma membrane phase," respectively. Bars = 10 μm. (Kanazawa *et al.*, 2020)

The morphology and distribution pattern of the oil body, *e.g.* its shape, color, number, and a density of oil body cells in tissues diverge among liverwort species, which, therefore, are regarded as important features for taxonomical classification in liverworts. However, molecular mechanisms of oil body morphogenesis remain to be determined. We have successfully isolated some mutants defective in oil body morphogenesis in *M. polymorpha*, which would lead to identification of unknown factors involved in oil body biogenesis.

1-2 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 the 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, which is a process distinctly regulated from non-plant systems (for example, Takemoto *et al*., 2018).

Defective vacuolar SNARE functions affect both vacuolar transport and morphology. The *sgr3-1* (*shoot gravitropism3*) mutant was isolated as a mutant deemed defective in shoot gravitropism. This resulted from a point mutation in *SYP22/VAM3*, which is one of the SNARE proteins residing on the vacuole and active in vacuolar transport. The *sgr3-1* mutant exhibits abnormal vacuolar morphology, although vacuolar transport is not markedly affected in this mutant. We also found that machinery components for homotypic vacuolar membrane fusion including VAMP71, SYP22, and the tethering HOPS complex were accumulated at specific domains in the vacuolar membrane in *sgr3-1*. These results suggested that vacuolar membrane homotypic fusion is specifically affected by the *sgr3-1* mutation.

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

2-1 Functions of ANTH-domain proteins in plant physiology

AP180 N-terminal homology domain-containing proteins (ANTH proteins) are thought to act as adaptors bridging the clathrin coat and cargo proteins during clathrin-coated vesicle formation. ANTH proteins have been remarkably expanded during land plant evolution, and we are investigating how this protein family has been functionally diversified in *A. thaliana*. We found that a pair of ANTH proteins, PICALM1a and PICALM1b, are required for retrieving a secretory SNARE protein, VAMP721, from the plasma membrane. This function is required for normal vegetative development of *A. thaliana* (Figure. 2). This finding also highlighted the divergent mechanisms of VAMP7 recycling from the plasma membrane between plants and animals.

We also found that another paralogous set of PICALM proteins, PICALM5a and 5b is required for tip-localization of ANXUR receptor kinases acting in an autocrine signaling pathway required for pollen tube integrity in *A. thaliana* (Figure 2). Thus, functionally differentiated ANTH proteins underpin various physiological processes in *A. thaliana*. Consequently, we are now investigating the molecular function of PICALMs with a special focus on the mechanisms of cargo recognition by PICALMs in *A. thaliana*.

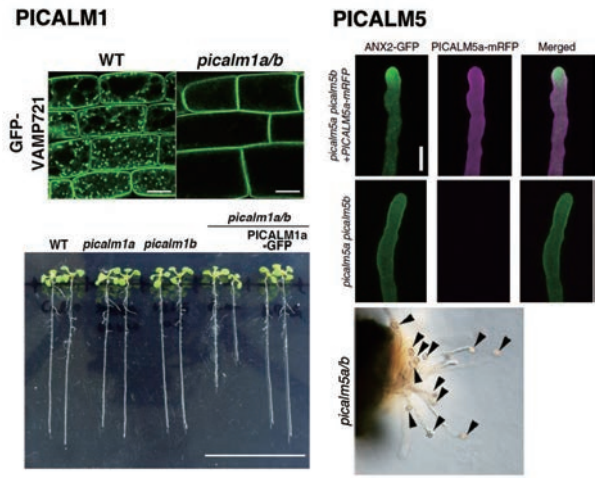

Figure 2. PICALM1 and PICALM5 mediate endocytosis of distinct plasma membrane proteins, which are required for normal vegetative development and pollen tube integrity, respectively. (Left: Fujimoto *et al*., 2020, modified, right: Muro *et al*., 2018, modified)

2-2 Membrane trafficking in plant gametogenesis

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

Cytokinesis in land plants is achieved by the re-direction of the secretory pathway. As such, KNOLLE/SYP111 plays an important role in membrane fusion in the formation of cell plates in *A. thaliana* somatic cells. Conversely, no deleterious effects on gametogenesis have been reported regarding mutations in KNOLLE. We found that KNOLLE and other SYP1 members were highly expressed during cytokinesis in gametogenesis (Figure 3). Mutant analyses of *syp1* members also supported that KNOLLE and other SYP1 regulate cytokinesis during gametogenesis in *A. thaliana*.

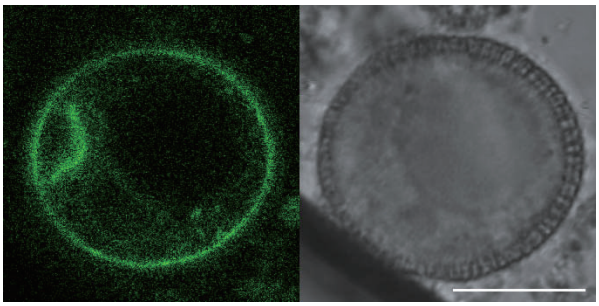

Figure 3. Expression and subcellular localization of GFP-KNOLLE during pollen mitosis I. GFP-KNOLLE accumulates at the cell plate. Bar = 10 μm.

Distinct from seed plants, basal land plants including *M. polymorpha* utilize the spermatozoid with two (or more) motile flagella as the male gamete during sexual reproduction. We visualized the spermatozoid formation process, especially spermiogenesis, using fluorescently-tagged organelle markers

in *M. polymorpha*. 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 endosome, vacuole, and autophagosome, is transiently increased during this process. To reveal the molecular mechanisms of cytoplasm removal and organelle remodeling, we have established the analytical tools of autophagy in *M. polymorpha* (Norizuki *et al.*, 2019). *M. polymorpha* possesses core machineries of autophagy with lower degrees of redundancy. The mutations in Mp*ATG5* and Mp*ATG7*, which are key factors for autophagosome formation, affected the transportation of cytosolic components to the vacuole for degradation (Figure 4).

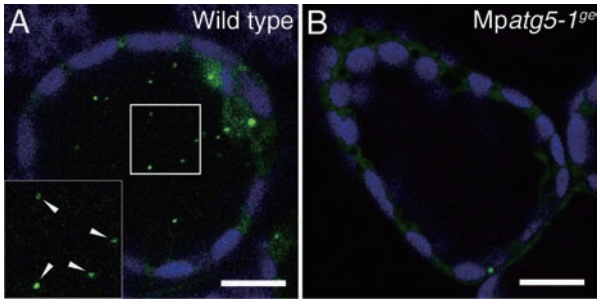

Figure 4. Establishment of analytical tools for the autophagy study in *M. polymorpha*. YFP-tagged MpATG8a is targeted to vacuole in wild type (A), but not in the Mp*atg5-1*^{ge} mutant (B). Bars = 10 μm. (Norizuki *et al*., 2019).

Autophagy-defective mutants exhibited defects regarding cytoplasm removal, spermatozoid motility, and fertility. Although a majority of organelles are removed during spermiogenesis, a specific set of organelles persists in mature spermatozoids, which implies that there should be a mechanism for selective removal of unneeded organelles, which we are also investigating. 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 (Figure 5).

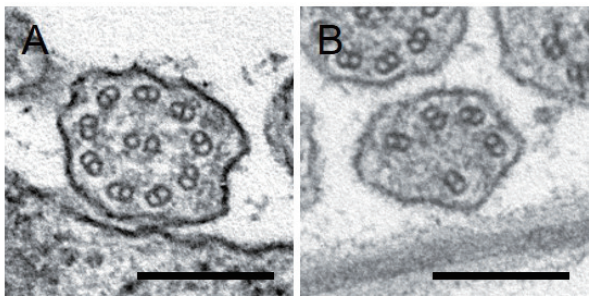

Figure 5. Transverse sections of flagella in wild-type (A) and mutant (B) spermatids. The microtubule-based "9 + 2" axoneme structure is severely compromised in the mutant. Bars = 200 nm.

Publication List:

〔Original papers〕

- Duan, Z., Tanaka, M., Kanazawa, T., Haraguchi, T., Takyu, A., Era, A., Ueda, T., Ito, K., and Tominaga, M. (2020). Characterization of ancestral myosin XI from *Marchantia polymorpha* by heterologous expression in *Arabidopsis thaliana*. Plant J. *104*, 460–473. DOI: 10.1111/tpj.14937
- Fujimoto, M., Ebine, K., Nishimura, K., Tsutsumi, N., and Ueda, T. (2020). Longin R-SNARE is retrieved from the plasma membrane by ANTH domain-containing proteins in Arabidopsis. Proc. Natl. Acad. Sci. U.S.A. *117*, 25150–25158. DOI: 10.1073/pnas.2011152117
- Kanazawa, T., Morinaka, H., Ebine, K., Shimada, T.L., Ishida, S., Minamino, N., Yamaguchi, K., Shigenobu, S., Kohchi, T., Nakano A., Ueda, T. (2020). The liverwort oil body is formed by redirection of the secretory pathway. Nat. Commun. *11*. DOI: 10.1038/s41467-020-19978-1
- Lupanga, U., Roehrich, R., Askani, J., Hilmer S., Kiefer, C., Krebs, M., Kanazawa, T., Ueda, T., and Schumacher, K. (2020). The Arabidopis V-ATPase is localized to the TGN/EE via a seed plant-specific motif. eLife *9*. DOI: 10.7554/eLife.60568
- Otsuka, K., Mamiya, A., Konishi, M., Nozaki, M., Kinoshita, A., Tamaki, H., Arita, M., Saito, M., Yamamoto, K., Hachiya, T., *et al.* (2021). Temperature-dependent fasciation mutants provide a link between mitochondrial RNA processing and lateral root morphogenesis. eLife *10*. DOI: 10.7554/eLife.61611
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- Shimada, T.L., Ueda, T., and Hara-Nishimura, I. (2021). Excess sterol accumulation affects seed morphology and physiology in *Arabidopsis thaliana*. Plant Signal. & Behav. *16*. DOI: 10.1080/15592324.2021.1872217
- Shimizu, Y., Takagi, J., Ito, E., Ito, Y., Ebine, K., Komatsu, Y., Goto, Y., Sato, M., Toyooka, K., Ueda, T., *et al*. (2021). Cargo sorting zones in the trans-Golgi network visualized by super-resolution confocal live imaging microscopy in plants. Nat. Commun. *12*. DOI: 10.1038/s41467-021-22267-0
- Suzuki, R., Ueda, T., Wada, T., Ito, M., Ishida, T., and Sawa. S. (2021). Identification of genes involved in *Meloidogyne incognita*-induced gall formation processes in *Arabidopsis thaliana*. Plant Biotech. *38*. DOI:10.5511/plantbiotechnology.20.0716a

〔Review article〕

- Norizuki, T., Minamino, N., and Ueda, T. (2020). Role of autophagy in male reproductive processes in land plants. Front. Plant Sci. *11*. DOI: 10.3389/fpls.2020.00756

〔Book Chapter〕

- Ito, E., Choi, S.W., and Ueda, T. (2020). Purification and interaction analysis of a plant-specific RAB5 effector by in vitro pull-down assay. Methods Mol Biol *2177*, 183-197. DOI: 10.1007/978-1-0716-0767-1_15