Division of Cellular Dynamics

Professor
UEDA, Takashi

Assistant Professor:	*EBINE, Kazuo*
	KANAZAWA, Takehiko
Technical Staff:	*HAYASHI, Kohji*
NIBB Research Fellow:	*MINAMINO, Naoki*
Postdoctoral Fellow:	*MURO, Keita*
SOKENDAI Graduate Student:	*HACHINODA, Shou*
Visiting Graduate Student:	*NORIZUKI, Takuya*
	KAJIWARA, Keiji
Technical Assistant:	*YAMAMOTO, Mayuko*
	OHARA, Satomi
Secretary:	*OKUBO, Masayo*

Membrane traffic between single membrane-bounded organelles plays an integral role in various activities in eukaryotic cells. Recent comparative genomics have 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 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. 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 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.

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

Through analyses of SNARE members in *M. polymorpha*, a member of the SYP1 group was localized to the membrane of the oil body, an organelle specific to liverworts, whose origin and biogenesis remain unclear.

Furthermore, we discovered an oil body formation master regulator, whose molecular function is currently being investigated. We also characterized the membrane trafficking pathway responsible for oil body biogenesis, and found that the trafficking pathway to the oil body should be a redirected secretory pathway.

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* (Figure 1), which would lead to identification of unknown factors involved in oil body biogenesis.

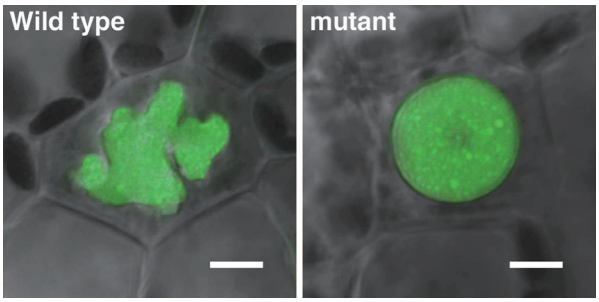

Figure 1. Oil bodies in wild-type and mutant *M. polymorpha*. The oil body exhibits complex morphology in the wild-type plant, whereas spherical in the mutant. Bars = 5 μm.

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 Reorganization of the membrane trafficking system during pathogenic fungus invasion

Membrane traffic also plays important roles in plant-microbe interaction. Phosphoinositides, which are minor phospholipids in endomembranes, function within membrane traffic and signaling. Systematic observation of phosphoinositides during the invasion of the pathogenic fungi, Colletotrichum, revealed the strong accumulation of PtdIns(4,5)P2 in the extra-invasive hyphal membrane (EIHM) (Figure 2). During Colletotrichum infection, an exocytic factor was recruited to the EIHM, but endocytic factors were eliminated. Furthermore, overexpression of Arabidopsis PIP5K, which catalyzes PtdIns4P into PtdIns(4,5)P2, increased the Colletotrichum invasion, suggesting that the pathogenic fungi, Colletotrichum, could modify PtdIns in the EIHM in the successful infection (Shimada *et al*., 2019).

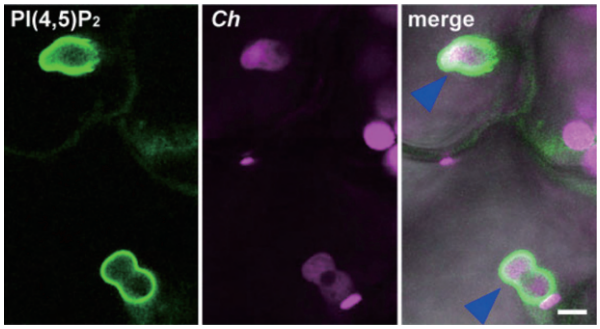

Figure 2. Accumulation of PtdIns(4,5)P2 in the EIHM formed by Colletotrichum (*Ch*). Bar = 5 µm. (Shimada *et al*., 2019)

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 important roles in membrane fusion in the forming 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* .

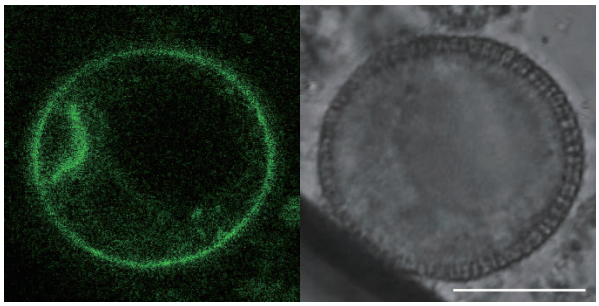

Figure 3. Expression and subcellular localization of GFP-KNOLLE during pollen mitosis I. GFP-KNOLLE accumulates at the cell plate. Bar = 10 µm. t

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, was 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).

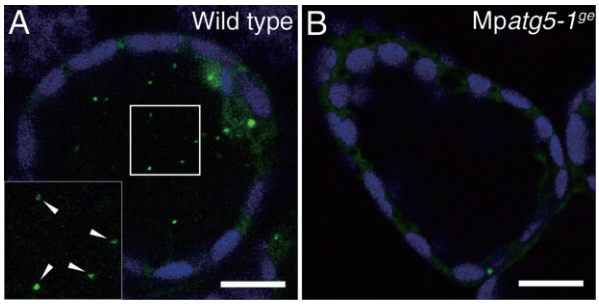

Figure 4. Establishment of analytical tools for the autophagy study in *M. polymorpha*. YFP-tagged MpATG8a is targeted to vacuole in wild type (A), but not in the Mp*atg5-1*^{ge} mutant (B). Bars = 10 µm. (Norizuki *et al*., 2019).

Autophagy-defective mutants exhibited defects regarding cytoplasm removal, spermatozoid motility, and fertility. 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).

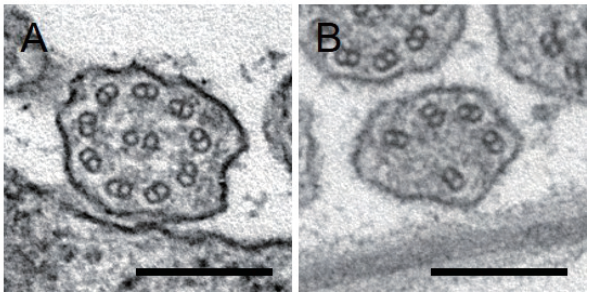

Figure 5. Transverse sections of flagella in wild-type (A) and mutant (B) spermatids. The microtubule-based "9 + 2" axoneme structure is severely compromised in the mutant. Bars = 200 nm.

2-3 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 exhibit remarkable expansion during land plant evolution, and we examined how this protein family has been functionally diversified in *A. thaliana*. We found that a pair of 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*, whereas another receptor kinase PRK6 acting in pollen tube guidance is not affected (Muro *et al*., 2018). Now we are looking for PICALM members regulating localization of PRK6 and other receptors in Arabidopsis pollen tube.

We also found that another paralogous set of PICALM proteins is required for retrieving secretory SNARE proteins from the plasma membrane (under revision), which itself is required for normal vegetative development. These lines of evidence indicated that ANTH proteins are functionally differentiated, which in turn underpin various physiological processes in *A. thaliana*.

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- Shimada, T.L., Betsuyaku, S., Inada, N., Ebine, K., Fujimoto, M., Uemura, T., Takano, Y., Fukuda, H., Nakano, A., and Ueda, T. (2019). Enrichment of phosphatidylinositol 4,5-bisphosphate in the extra-invasive hyphal membrane promotes colletotrichum infection of *Arabidopsis thaliana*. Plant Cell Physiol. *60*, 1514-1524. doi: 10.1093/pcp/pcz058
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- Uemura, T., Nakano, T.R., Takagi, J., Wang, Y., Kramere, K., Finkemeier, I., Nakagami, H., Tsuda, K., Ueda, T., Schulze-Lefert, P. and Nakano, A. (2019). A Golgi-released subpopulation of the trans-Golgi network mediates constitutive and pathogen-inducible protein secretion in Arabidopsis. Plant Phys. *179*, 519-532. doi:10.1104/pp.18.01228

〔Review article〕

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