学位論文（要約）

Studies on diversification of organelles and

membrane trafficking pathways using *Marchantia polymorpha*

(ゼニゴケを用いたオルガネラと膜交通経路の多様化の研究)

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Abstract

Membrane traffic is a fundamental system responsible for correct transport and localization of proteins, lipids, and polysaccharides in eukaryotic organisms including plants. Among key machinery components of membrane trafficking, Rab GTPases and SNARE proteins mediate tethering and fusion between transport vesicles and target membranes, respectively. Although the molecular framework is well conserved in eukaryotic lineages, it is also known that each eukaryotic lineage has acquired lineage-specific membrane trafficking pathways during evolution, which should be involved in lineage-specific biological functions. The diversification of membrane trafficking is considered to result from, at least partly, functional differentiation of the machinery components such as Rab GTPases and SNARE proteins. However, its detailed mechanisms remain almost unknown. In this study, I aimed to unveil how membrane trafficking pathways have diversified during land plant evolution using the liverwort, *Marchantia polymorpha,* which is an emerging model of basal land plants, with a special interest in SNARE molecules.

I identified 34 genes for SNARE proteins in *M. polymorpha* based on the genome and transcriptome information. I then examined subcellular localization of the majority of these SNARE molecules by expressing fluorescently tagged proteins in *M. polymorpha* thallus tissues. The results obtained and comparison with the subcellular localization of orthologous products in *Arabidopsis thaliana* indicated that the membrane trafficking system has increased its complexity during land plant evolution. Through this analysis, I also succeeded in establishing reliable endomembranous organelle markers in *M. polymorpha* (Chapter III).

I then carried out detailed analyses for the SYP1 group, which is remarkably

expanded in seed plants, in *M. polymorpha*. I found that one of four SYP1 members in *M. polymorpha* plays an essential role in cell plate formation during cytokinesis, while its close relative is specifically expressed in oil body cells and localized to the membrane of the oil body, an organelle unique to liverworts. Observation of various organelle marker proteins and a secretory cargo in dividing cells and in oil body cells indicated that targeting to these organelles is accomplished by transient redirection of the secretory pathway. Furthermore, I found that none of the known organelle markers are localized to the oil body membrane, although previous studies proposed several possible organelles as origins of this liverwort-specific compartment. These results indicated that functional diversification of SYP1 members accompanied with transient alteration of transport destinations should contribute to the acquisition of new organelles in the plant lineage (Chapter IV).

For insights into molecular mechanisms of biogenesis of the oil body in *M. polymorpha*, I then conducted forward genetic screening for mutant plants with altered oil body morphology or distribution patterns. I have successfully isolated several putative mutants from 16,000 T-DNA-tagged lines, which will be useful to unravel how and why liverworts attained the oil body during evolution (Chapter V).

Contents

Acknowledgements

Abbreviations

AP2/ERF: APETALA2/ethylene-responsive element binding factors

BF: bright field

BODIPY 493/503: 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-*s*-indacene

BSA: bovine serum albumin

CaMV: cauliflower mosaic virus

CCV: clathrin-coated vesicle

CRISPR/Cas9: clustered regularly interspaced short palindromic repeats-associated endonuclease Cas9 system

ER: endoplasmic reticulum

EYFP: enhanced yellow fluorescent protein

FM4-64: *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide

GA: glutaraldehyde

GEF: guanine nucleotide exchange factor

gRNA: guide RNA

mCitrine: monomeric Citrine

mGFP: monomeric green fluorescent protein

mRFP: monomeric red fluorescent protein

MSA: mitosis-specific activator

Myr-VAMP72: *N*-myristoylated VAMP72

ORF: open reading frame

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PFA: paraformaldehyde

PM: plasma membrane

PVC: prevacuolar compartment

RT-PCR: reverse transcription PCR

SNARE: soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor

SP: signal peptide

ST: sialyltransferase

TAIL-PCR: thermal asymmetric interlaced-PCR

T-DNA: transfer DNA

TEM: transmission electron microscope

TGN: *trans*-Golgi network

TMD: transmembrane domain

YFP: yellow fluorescent protein

Chapter I: **General Introduction**

Membrane traffic is a fundamental system responsible for precise transport and localization of proteins, lipids, and polysaccharides among single membrane-bounded organelles, the plasma membrane (PM), and the extracellular space, which is essential for homeostasis and precise functions of cells and organelles. The basic molecular framework underlying membrane trafficking is evolutionarily conserved among eukaryotes, which comprises four sequential processes: 1) sorting cargoes and forming transport vesicles on donor organelle membranes, 2) conveying transport vesicles, 3) tethering transport vesicles to target organelle membranes, and 4) fusing transport vesicles with target organelle membranes. Each of these processes is strictly controlled by evolutionarily conserved machinery components, which include coat protein complexes and Rab GTPases responsible for formation of transport vesicles at donor membranes and tethering of the transport vesicles to target organelle membranes, respectively (Fujimoto & Ueda, 2012). Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) is another evolutionarily-conserved component that functions at the final process of membrane trafficking, executing membrane fusion of organelles or transport vesicles with destination membranes (Sollner *et al.*, 1993a; Sollner *et al.*, 1993b). SNARE molecules are distinguished by the highly conserved helical region, referred to as the SNARE motif. SNARE molecules are divided into two classes, Q- and R-SNAREs according to the amino acid residue (Q, glutamine or R, arginine) at the distinctive position of the SNARE motif, referred to as the zero layer. In many cases, R-SNAREs are localized to transport vesicles and Q-SNAREs reside on target organelle membranes. Q-

SNAREs are further classified into Qa-, Qb-, Qc-, and Qb+Qc-SNAREs based on the similarity and the number of the SNARE motif (Fasshauer *et al.*, 1998; Bock *et al.*, 2001; Antonin *et al.*, 2002). Specific combinations of four SNARE motifs, each of which is supplied by Qa-, Qb-, Qc-, and R-SNARE proteins or by Qa-, Qb+Qc-, and R-SNARE proteins, assemble into a tight complex, which results in membrane fusion of transport vesicles with target organelles. The repertoire of SNARE proteins and their functions are well conserved among eukaryotic lineages, including animals, yeasts, and plants (Hong, 2005; Dacks & Field, 2007; Sanderfoot, 2007).

In addition to fundamental roles at the cellular level, membrane trafficking fulfills various higher-ordered functions in animals and plants. In land plants, various higher-ordered and plant-unique processes are reported to depend on the membrane trafficking system, including cell wall synthesis/remodeling, polar transport of auxin, embryogenesis, abiotic/biotic stress responses, and gravitropism (Lukowitz *et al.*, 1996; Geldner *et al.*, 2001; Kato *et al.*, 2002; Morita *et al.*, 2002; Collins *et al.*, 2003; Yano *et al.*, 2003; Leshem *et al.*, 2006; Petrasek *et al.*, 2006; Wisniewska *et al.*, 2006; Crowell *et al.*, 2009; Gutierrez *et al.*, 2009; Asaoka *et al.*, 2012; Uemura *et al.*, 2012; Hashiguchi *et al.*, 2014; Inada & Ueda, 2014; Kim & Brandizzi, 2014; Tanaka *et al.*, 2014; Uehara *et al.*, 2014). These diverse and specialized functions appear to result from land plants pioneer novel trafficking pathways accompanied by acquisition of new and land-plant-specific machinery components for membrane trafficking (Fujimoto & Ueda, 2012). For instance, VAMP727, an R-SNARE protein unique to seed plants with a typical insertion in its longin domain, plays significant roles in development and germination of the seeds in *Arabidopsis thaliana* (Ebine *et al.*, 2008). A member of RAB5 GTPases, ARA6, which is highly conserved in land plants and functions in endosomal trafficking, is also a notable

example uniquely acquired during plant evolution (Ueda *et al.*, 2001; Ebine *et al.*, 2011). Evolutionarily conserved machineries such as RAB5 and RAB7 are also shown to be recruited to different trafficking pathways between animals and land plants (Cui *et al.*, 2014; Ebine *et al.*, 2014; Singh *et al.*, 2014). Thus plants, especially seed plants, have acquired specific and novel membrane trafficking pathways.

It has been proposed that the diversification of components involved in membrane trafficking was achieved by reiterating multiplication of corresponding genes followed by accumulation of mutations leading to functional differentiation (Dacks & Field, 2007). In the green plant lineage, the remarkable expansion of genes involved in membrane trafficking has been considered to accompany terrestrialization and/or multicellularization of plants (Sanderfoot *et al.*, 2000; Rutherford & Moore, 2002; Dacks & Field, 2007; Sanderfoot, 2007). The diversification of SNARE molecules involved in the secretory pathway is especially apparent. The Qa-SNARE SYP1 group, which is composed of three subgroups (SYP11, SYP12, and SYP13) in seed plants and predominantly localized to the PM, is one of the most diversified SNARE molecules in land plants. SYP111/KNOLLE is specifically expressed in dividing cells and plays an essential role in formation of the cell plate to accomplish cytokinesis in *A. thaliana* (Lukowitz *et al.*, 1996; Lauber *et al.*, 1997). SYP121/PEN1/SYR1 is responsible for non-host penetration resistance against barley powdery mildew fungus in *A. thaliana* (Collins *et al.*, 2003; Kwon *et al.*, 2008). SYP121 is also reported to mediate the regulation of PM-resident ion channels (Sutter *et al.*, 2006; Honsbein *et al.*, 2009; Grefen *et al.*, 2010; Grefen *et al.*, 2015). For the SYP13 subgroup, SYP132 is ubiquitously expressed and is considered to execute constitutive secretion in *A. thaliana* plants, and also functions in tip-growing root hair cells in collaboration with SYP123, whereas its paralog SYP131 is

limitedly expressed in pollen grains (Enami *et al.*, 2009; Ichikawa *et al.*, 2014).

The relevance between the diversification of machinery components of membrane trafficking and terrestrialization and/or multicellularization in the green plant lineage has been mainly proposed by comparative genomics; the number of SNARE proteins encoded in the genome of the moss, *Physcomitrella patens,* is larger compared with unicellular green algal species (Sanderfoot, 2007). However, the knowledge regarding the organization, subcellular localization, and neofunctionalization of SNAREs in these species remains limited. *P. patens* has been shown to undergo large-scale genome duplications during evolution (Rensing *et al.*, 2007; Rensing *et al.*, 2008), which might imply that the increase of the number of SNARE genes in *P. patens* indicates the existence of paralogous genes without remarkable neofunctionalization. To gather more knowledge on the diversification of membrane trafficking in the evolutionary process of the green plant lineage, information from other basal land-plant lineages such as bryophytes (liverworts, mosses, and hornworts) and charophyte algae is apparently needed.

Marchantia polymorpha is a member of Marchantiophyta (liverwort), which are considered to occupy a basal position in phylogeny of terrestrial plants, while which lineage of the bryophytes   is basalmost remains debatable (Qiu *et al.*, 2006; Wickett *et al.*, 2014). In this study, *M. polymorpha* has been selected as a model of liverworts because genome and transcriptome information is available, and genetical and cell biological techniques, including agrobacterium-mediated transformation, gene targeting, and genome editing, have been established (Chiyoda *et al.*, 2007; Ishizaki *et al.*, 2008; Era *et al.*, 2009; Era *et al.*, 2013; Ishizaki *et al.*, 2013; Kubota *et al.*, 2013; Tsuboyama & Kodama, 2013; Sugano *et al.*, 2014; Ishizaki *et al.*, 2015; Kanazawa, 2015; Tsuboyama-Tanaka & Kodama, 2015). In order to acquire the knowledge on diversification of

organelle functions and membrane trafficking pathways, I systematically analyzed organization, subcellular localization, and functions of SNARE molecules in this emerging model plant, which provided key insights into diversification of membrane trafficking during land plant evolution.

Chapter II: **Materials and Methods**

Identification of genes of *M. polymorpha* and phylogenic analyses

Similarity search for *M. polymorpha* genes was executed using proteins of *A. thaliana* as queries as previously described in Kato *et al.* (2015) with version 3.1. Phylogenetic analysis in Figure 3-2 was performed as previously described (Banks *et al.*, 2011), with updated datasets. The dataset was as of Jan 17, 2015. Additional datasets of *Klebsormidium flaccidum* (Hori *et al.*, 2014, http://www.plantmorphogenesis.bio.titech.ac.jp/~algae_genome_project/klebsormidium /kf_download/131203_kfl_initial_genesets_v1.0_AA.fasta), *Physcomitrella patens* v1.6 (Zimmer *et al.*, 2013, https://www.cosmoss.org/physcome_project/linked_stuff/Annotation/V1.6/P.patens.V6_ filtered_cosmoss_proteins.fas.gz), and *Pinus taeda* (Neale *et al.*, 2014, http://loblolly.ucdavis.edu/bipod/ftp/Genome_Data/genome/pinerefseq/Pita/v1.01/Pita_ Annotation_v2/) were included. After retrieving 1000 similar sequences, the *M. polymorpha* SNARE sequences were merged and 1000 similar sequences were selected again, aligned with MAFFT v6.811b (Katoh & Toh, 2008), and a conserved sequence region was manually selected with MacClade 4 (Maddison & Maddison, 2000). The Neighbor Joining (Saitou & Nei, 1987) tree using maximum likelihood distance under the JTT model (Jones *et al.*, 1992) was constructed by using PHYLIP package 3.695 (Felsenstein, 2013). Bootstrap analysis was performed by resampling 1000 sets. After reviewing the large tree, sequences were further selected to retain the diversity but fit into a page and the phylogenetic analysis was repeated.

For Figure 5-3, sequences with AP2 domain were collected from *M. polymorpha*

genome database version 3.1 and *A. thaliana* genome database TAIR annotation version 10. Sequences were aligned with MUSCLE program version 3.8.31 (Edgar, 2004), and a conserved sequence region was manually selected, and the phylogenetic tree was constructed by PhyML program version 3.0 (Guindon *et al.*, 2010) under the LG model (Le *et al.*, 2008). Bootstrap analysis was performed by resampling 1000 sets.

Plant materials and transformation

The *M. polymorpha* male and female accessions Takaragaike-1 (Tak-1) and Takaragaike-2 (Tak-2), respectively (Ishizaki *et al.*, 2008), were used in this study. The gemmae and thalli were grown asexually on 1/2× Gamborg's B5 medium (Gamborg *et al.*, 1968) with 1.4% (w/v) agar at 22°C under continuous white fluorescent light. F1 spores were generated by crossing Tak-1 and Tak-2. The transition from the vegetative phase to the reproductive phase was induced by far-red illumination as previously described (Chiyoda *et al.*, 2008). Transformation of *M. polymorpha* was carried out according to previously described methods using the sporelings or excised thalli mediated by *Agrobacterium tumefaciens* strain GV2260 (Ishizaki *et al.*, 2008; Kubota *et al.*, 2013). Transgenic liverworts were selected on plates containing 10 mg/L hygromycin B and 100 mg/L cefotaxime or 0.5 μM chlorsulfuron and 100 mg/L cefotaxime (Ishizaki *et al.*, 2015).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA from 5-day-old thalli, antheridiophores, archegoniophores, and 7-day-old sporelings was isolated by the RNeasy Plant Mini Kit (Qiagen) and was used as templates for reverse transcription using SuperScript III Reverse Transcriptase (Invitrogen) and the oligo dT (18 mer) primer according to the manufacturer's instructions.

The cDNA was used for polymerase chain reaction (PCR) analyses. The primer sequences were listed in Table 2-1. The expression of Mp*EF1α* was used as a positive control.

Constructs

Open reading frames (ORFs) and genomic sequences of *M. polymorpha* genes were respectively amplified by PCR from cDNA and genome DNA prepared from the *M. polymorpha* accession Tak-1, and the PCR products were subcloned into pENTR™/D-TOPO (Invitrogen) according to the manufacturer's instructions. The oligonucleotide sequences used in PCR are listed in Table 2-1. To construct *mCitrine*-Mp*SYP12A*, *mCitrine*-Mp*SYP12B*, *mCitrine*-Mp*SYP13A*, *mCitrine*-Mp*SYP13B*, and *mCitrine*-Mp*SYP2*, genomic sequences comprising the 5′ flanking sequences (promoter + 5′ UTR), protein-coding regions, introns, and 3′ flanking sequences (7.0 kb for Mp*SYP12A*, 7.4 kb for Mp*SYP12B*, 9.8 kb for Mp*SYP13A*, 7.1 kb for Mp*SYP13B*, and 8.8 kb for Mp*SYP2*) were amplified and subcloned into the pENTR vectors. Next, the cDNA for mCitrine was inserted into the 5′ end of the protein-coding region using the In-Fusion HD Cloning System (Clontech) according to the manufacturer's instructions. To construct pMpGWB301-derived gateway vectors (*pro*Mp*SYP12A:mCitrine*-GW, *pro*Mp*CYCB1:mCitrine*-GW and *pro*Mp*SYP12B:mCitrine*-GW), the promoter:*mCitrine* sequences were amplified from genomic construct described above, and was inserted into the *Hin*dIII site of pMpGWB301 using the In-Fusion HD Cloning System. For the transformation of *M. polymorpha*, the resultant entry sequences were introduced into pMpGWB series (Ishizaki *et al.*, 2015) and modified pMpGWB (described above) for with Gateway LR Clonase™ II Enzyme Mix (Invitrogen) according to the manufacturer's instructions. For genome editing mediated by the clustered regularly interspaced short

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palindromic repeats-associated endonuclease Cas9 system (CRISPR/Cas9), target sequences were inserted into the *Bsa*I site of the pMpGE_En03 entry vector (Sugano *et al.*, unpublished). The resultant sequences were then introduced into pMpGE010 or pMpGE011 (Sugano *et al.*, unpublished) using the Gateway LR Clonase™ II Enzyme Mix. For transient expression in protoplasts of *A. thaliana*, subcloned cDNA or mutated sequences with fluorescent proteins were transferred into the p2GWY vector (Karimi *et al.*, 2005).

Transient expression in protoplasts of *A. thaliana* suspension cultured cells

Transient expression of chimeric proteins of the N-terminal sequences of VAMP72 members with enhanced yellow fluorescent protein (EYFP) in *A. thaliana* cells cultured in suspension was performed as previously described (Ueda *et al.*, 2001; Uemura *et al.*, 2004). Protoplasts of *A. thaliana* were prepared by incubation of approximately 2 g of the cultured cells in 25 mL enzyme solution (400 mM mannitol, 5 mM EGTA, 1% (w/v) cellulase Y-C, and 0.05% (w/v) Pectolyase Y-23). Protoplasts were washed twice by wash buffer (400 mM mannitol, 70 mM $CaCl_2$, and 5 mM MES-KOH (pH 5.7)) and resuspended in MaMg solution (400 mM mannitol, 15 mM $MgCl_2$, and 5 mM MES-KOH, pH 5.7). The suspended protoplasts, plasmid, and single-strand carrier DNA were mixed with DNA uptake solution (400 mM mannitol, 40% (w/v) polyethylene glycol 6000 and 100 mM $Ca(NO_3)_2$), placed on ice for 20 min, and diluted into dilution solution (400 mM mannitol, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose and 1.5 mM MES-KOH, pH 5.7). Protoplasts were collected and resuspended in MS medium supplemented with 400 mM mannitol and incubated with gentle rotation at 23°C for approximately 16 hours in the dark.

Confocal laser scanning microscopy

5-day-old thalli were used for observation unless otherwise defined. The samples were mounted in distilled water or dyeing solution and observed using an LSM 780 confocal microscope (Carl Zeiss) equipped with an oil immersion lens (63×, numerical aperture = 1.4) and an electrically driven stage. The samples were excited by laser at 488 nm (Argon 488) and 561 nm (DPSS 561-10), and the fluorescent emission was collected between 482-659 nm using twenty GaAsP detectors. Spectral unmixing, processing of the obtained images, and the construction of maximum intensity projection images were conducted using ZEN2012 software (Carl Zeiss). The images were processed digitally with ZEN2012 software and Photoshop software (Adobe Systems). For 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-*s*-indacene (BODIPY 493/503, Thermo Fisher) staining, thalli or gemmae were incubated in 200 nM BODIPY 493/503 dissolved in water for 10 min. For *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide (FM4-64, Thermo Fisher) staining, thalli were soaked in 10 μM FM4-64 solution for 2 min. Samples were then washed twice before observation. For observation by semi-super resolution microscopy, LSM 880 with Airy scan (Carl Zeiss) equipped with an oil immersion lens (63×, numerical aperture = 1.4) was used. The acquisition and calculation of images were conducted using ZEN 2 software (Carl Zeiss).

Electron microscopy

For the immunoelectron microscopic observation, 5-day-old Tak-1 thalli and the plant expressing mCitrine-MpSYP12B were used. The samples were fixed with 4% (w/v)

paraformaldehyde (PFA), 0.1% (w/v) glutaraldehyde (GA) (Distilled EM grade; Electron Microscopy Sciences, Hatfield, PA) and 0.5% (w/v) tannic acid in 0.05 M cacodylate buffer pH 7.4 at 4°C for 90 min, and then they were washed 3 times in 0.1 M cacodylate buffer for 15 min each. The samples were dehydrated in graded ethanol solutions (50% (v/v) and 70% (v/v)) at 4°C for 30 min each. The samples were infiltrated with a 50:50 mixture of ethanol and resin (LR white; London Resin Co. Ltd., Berkshire, UK) for 30 min each 3 times. After this infiltration, samples were incubated in 100% LR white three times at 4°C for 30 min each. The samples were transferred to a fresh 100% resin, and were polymerized at 50°C overnight. The polymerized resins were ultra-thin sectioned at 80 nm with a diamond knife using an ultramicrotome (Ultracut UCT; Leica, Vienna, Austria) and the sections were mounted on nickel grids. The grids were incubated with the primary antibody (rabbit polyclonal GFP pAb) in phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA) at 4°C overnight, then they were washed with PBS plus 1% (w/v) BSA 3 times for 1 min. They were subsequently incubated with the secondary antibody conjugated to 10 nm gold particles (goat anti rabbit IgG pAb) for 1 hour at room temperature. And after washing with PBS, the grids were placed in 2% (w/v) GA in 0.1 M cacodylate buffer. Afterwards, the grids were dried and then were stained with 2% (w/v) uranyl acetate for 15 min and in Lead stain solution (Sigma-Aldrich Co., Tokyo, Japan) at room temperature for 3 min. The grids were observed by a transmission electron microscope (TEM, JEM-1400Plus; JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 80 kV. Digital images (2048 × 2048 pixels) were taken with a CCD camera (VELETA; Olympus Soft Imaging Solutions GmbH, Münster, Germany).

For morphological observation by a TEM, 5-day-old Tak-1 thalli were used. The

samples were fixed with 2% (w/v) PFA and 2% (w/v) GA in 0.05 M cacodylate buffer pH 7.4 at 4°C overnight. After the fixation, samples were washed 3 times with 0.05 M cacodylate buffer for 30 min each, and were postfixed with 2% (w/v) osmium tetroxide in 0.05 M cacodylate buffer at 4°C for 3 hours. The samples were dehydrated in graded ethanol solutions (50% (v/v), 70% (v/v), 90% (v/v), and 100% (v/v)). The schedule was as follows: 50% (v/v) and 70% (v/v) for 30 min each at 4°C, 90% (v/v) for 30 min at room temperature, and 4 times of 100% for 30 min each at room temperature. After these dehydration processes, the samples were continuously dehydrated in 100% ethanol at room temperature overnight. The samples were transferred to a fresh 100% resin, and were polymerized at 60°C for 48 hours. The polymerized samples were ultra-thin sectioned at 70 nm with a diamond knife using an ultramicrotome (Ultracut UCT) and the sections were mounted on cupper grids. They were stained with 2% (w/v) uranyl acetate at room temperature for 15 min, and then they were washed with distilled water followed by being secondary-stained with Lead stain solution (Sigma-Aldrich) at room temperature for 3 min. The grids were observed with a TEM (JEM-1400Plus) at an acceleration voltage of 80 kV. Digital images (2048 × 2048 pixels) were taken with a CCD camera (VELETA).

Lightsheet microscopy

5-day-old thalli expressing 2×Citrine driven by the Mp*SYP12B* promoter were used for observation. The samples were embedded in low melt agarose gel and were observed using an Lightsheet Z.1 microscope (Carl Zeiss) equipped with a water immersion lens (5×, numerical aperture = 0.16). The samples were excited by laser at 488 nm (Argon 488). Acquisition of images and construction of three-dimension images from

multi-angle images were carried out using ZEN2014 software (Carl Zeiss). The images were processed digitally with Imaris software (Bitplane) and Photoshop software (Adobe Systems).

Thermal asymmetric interlaced-PCR (TAIL-PCR) and sequencing of TAIL-PCR products

TAIL-PCR was performed to identify flanking sequences of transfer DNA (T-DNA) insertions according to Liu *et al.*, 1995; Ishizaki *et al.*, 2008; Proust *et al.*, 2016 with minor modifications. Crude-extracted DNA was used as a template, flanking sequences were amplified using KOD FX neo DNA polymerase (Toyobo) and T-DNA-specific primers (TR1–3 and TL1–3 for the right border and the left border of T-DNA, respectively) and universal adaptor primers (AD1–6). The reaction cycles were shown in Table 2-2. After electrophoresis of the final TAIL-PCR products, bands were excised and DNA was purified from agarose gel using Wizard SV Gel and PCR Clean-Up System (Promega). Purified products were directly sequenced using TR3 or TL3 primer.

Table 2-1. The list of oligonucleotides used in the study.

Purpose	Gene name	primer 1 (5' to 3')	primer 2 (5' to 3')
RT-PCR	MpSYP8	CACCATGGCTACTGCCAAGGATGTAAC	TTATTGATACCAGTCCAAAAAC
	MpSYP3	CACCATGCCGGTGGCTCTGGGATCAGC	TCATGCTACGAAAACAACGAAAATTAAC
	MpSEC20	CACCATGGATCAAGATGTAGAAGAAGC	TTAGAGTTCATCATTGATTGGTAC
	MpMEMB1	CACCATGGCGATGATGGGGAGCG	CTATCCACGGGCCCATCTCC
	MpGOS11	CACCATGGCAGTTGCGAATGGCTG	CTATTTCGATATCCAGTACATG
	MpGOS12.1	ATGGAGGATGCGGACCCCGGATGGG	CTATCTTCCTGCGTCCGTTCGATATCC
	MpGOS12.2	ATGGAGGATGCGGACCCCGGATGGG	GTCAACGAGCATGAAGGGTGC
	MpUSE1A	CACCATGGGAATTTCGCAAGCGGAAG	CTAACCAGTGAGCCTTATCAAC
	MpUSE1B	CACCATGATACTTAGTAGAGCAGAG	TCAAGTGAGACGAATAATGAC
	MpBET1	CACCATGATGAACACTCGCCGAG	TCACTTTGTCAAGTAGTAAAC
	MpSFT1	CACCATGGCCAAGGGATCGAAGAGC	TTATCTAAGGAATTTAGCCC
	MpSEC22	CACCATGGTTAAATTAACCATTATTGC	TCAGCCGAAAATGTATCGC
	MpSYP4	CACCATGGCGACGCGCAATCAGACCG	TCAGAATAGAATTTTCTTCATGATATAC
	MpSYP2	CACCATGAGTTTTTTAGATCTAGAGG	TCACGCAAACATGACGATGATG
	MpVTI1	CTGTCTCGGAAATGTACATCCACC	CACCAATGATCCCTCCCATGATCC
	MpSYP6A	CACCATGTCGGCTTTAGATCCGTTTTAC	CTAGGCATTGAAAACAAGATAGGT
	MpSYP6B	CACCATGTCGCATCTCGATCCTTATTATC	TCAAGTGTAAAATATCAGCATG
	MpSYP5	CACCATGGCGCAAGCGGCGTCGG	TTACAAGGATTTGACCAAGC
	MpYKT6	CACCATGAAGATTACGGCCATTCTCC	TCACAATATCGAACAACACTGG
	MpVAMP71	CACCATGGCTATTTTATATGCGCTC	TTAAGAGTGGCAGCCATACAAG
	MpSYP12A	CACCATGAACGATCTTCTGCAGAAAACG	CTACTTCTTCAAACTCGTAGCTATG
	MpSYP12B	CACCATGAACGATCTGCTGGCAAG	TCACTTTGCCGTTGCAATGATGG
	MpSYP13A	CACCATGAACGATCTTTTAGGGGAG	TCATGCTTTGTTTGTTTTCCAAG
	MpSYP13B	CACCATGAACGATCTTCTGGGAGACTC	TCACTTTTGCCAGGGCTTGACG
	MpSNAP	CACCATGACCTCCGTGCATAGTAACC	TTATCGGTGGATGAGTCGTCGTG
	MpNPSN1	CACCATGGCCTCCCAAGGTCCCGAG	CTAGGACTCCAACAAAGCGAG
	MpSYP7A	CACCATGAGTGTTATAGATATCTTGAC	TCAGGCCAATAATTTGTATAG
	MpSYP7B	CACCATGAGCGTTACAGACTTGC	CTAGAAGAATTGATCTCCGAAG
	MpVAMP72A	CACCATGGGTGTGAACTCGTTGATTTAC	TTACTTGCACTTAAATCCCTTGC
	MpVAMP72B	CTACTGCTTCGTGGCTCGAGGAACGG	CGCCGACCGCCGGGAGGAGCAGTGCC
	MpVAMP72C	GTCACGTACACACGCGACAGCCACACC	GATTACAAGATGAGCTTGATTACAAGCG
	MpVAMP72D	GGTGGTGCTAGCGGAGTACAAGCCG	CGCCAAGTTGGCCAGAATCTCGGC
	MpVAMP72E	CACCATGGGGGCGAATCTTAGCAG	CTATATTGCAGTTTTAGCTTTTAG
	MpTOMOSYN11	GTACAGATTCGTTGCGTCTTTACGC	CATTTCCATTACACTCGACACCTCC
	MpTOMOSYN12	CACCATGTGTCTTACTGGCCGCTCC	TCACAGCTCGTACCACTTCCTG
	CUFF.11493	CGTGCCTCACGTCGGAATCTGATC	CTGGACATGGAGCTCAGCTTCGGG
	CUFF.21491	CACCATGCCTAGGACAATTGAAGTTGCC	TCATTCCCCCCTACATTGCACGAAC
	MpEF1α	TCACTCTGGGTGTGAAGCAGATGA	GCCTCGAGTAAAGCTTCGTGGTG

Table 2-1. (continued)

| Purpose	Gene name	primer 1 (5' to 3')	primer 2 (5' to 3')
pENTR for CDS	MpSYP8	CACCATGGCTACTGCCAAGGATGTAAC	TTATTGATACCAGTCCAAAAAC
	MpSYP3	CACCATGCCGGTGGCTCTGGGATCAGC	TCATGCTACGAAAACAACGAAAATTAAC
	MpSEC20	CACCATGGATCAAGATGTAGAAGAAGC	TTAGAGTTCATCATTGATTGGTAC
	MpMEMB1	CACCATGGCGATGATGGGGAGCG	CTATCCACGGGCCCATCTCC
	MpGOS11	CACCATGGCAGTTGCGAATGGCTG	CTATTTCGATATCCAGTACATG
	MpGOS12.1	CACCATGGAGGATGCGGACCCCGG	CTATCTTCCTGCGTCCGTTCG
	MpGOS12.2	CACCATGGAGGATGCGGACCCCGG	TCACTTGGAAATCCAGTAAAC
	MpUSE1A	CACCATGGGAATTTCGCAAGCGGAAG	CTAACCAGTGAGCCTTATCAAC
	MpUSE1B	CACCATGATACTTAGTAGAGCAGAG	TCAAGTGAGACGAATAATGAC
	MpBET1	CACCATGATGAACACTCGCCGAG	TCACTTTGTCAAGTAGTAAAC
	MpSFT1	CACCATGGCCAAGGGATCGAAGAGC	TTATCTAAGGAATTTAGCCC
	MpSEC22	CACCATGGTTAAATTAACCATTATTGC	TCAGCCGAAAATGTATCGC
	MpSYP4	CACCATGGCGACGCGCAATCAGACCG	TCAGAATAGAATTTTCTTCATGATATAC
	MpSYP2	CACCATGAGTTTTTTAGATCTAGAGG	TCACGCAAACATGACGATGATG
	MpVTI1	CACCATGTCTGAGATATTCGAAGGC	CTACTTACTACGACTGCTG
	MpSYP6A	CACCATGTCGGCTTTAGATCCGTTTTAC	CTAGGCATTGAAAACAAGATAGGT
	MpSYP6B	CACCATGTCGCATCTCGATCCTTATTATC	TCAAGTGTAAAATATCAGCATG
	MpSYP5	CACCATGGCGCAAGCGGCGTCGG	TTACAAGGATTTGACCAAGC
	MpYKT6	CACCATGAAGATTACGGCCATTCTCC	TCACAATATCGAACAACACTGG
	MpVAMP71	CACCATGGCTATTTTATATGCGCTC	TTAAGAGTGGCAGCCATACAAG
	MpSYP12A	CACCATGAACGATCTTCTGCAGAAAACG	CTACTTCTTCAAACTCGTAGCTATG
	MpSYP12B	CACCATGAACGATCTGCTGGCAAG	TCACTTTGCCGTTGCAATGATGG
	MpSYP13A	CACCATGAACGATCTTTTAGGGGAG	TCATGCTTTGTTTGTTTTCCAAG
	MpSYP13B	CACCATGAACGATCTTCTGGGAGACTC	TCACTTTTGCCAGGGCTTGACG
	MpSNAP	CACCATGACCTCCGTGCATAGTAACC	TTATCGGTGGATGAGTCGTCGTG
	MpNPSN1	CACCATGGCCTCCCAAGGTCCCGAG	CTAGGACTCCAACAAAGCGAG
	MpSYP7A	CACCATGAGTGTTATAGATATCTTGAC	TCAGGCCAATAATTTGTATAG
	MpSYP7B.1	CACCATGAGCGTTACAGACTTGC	TCATAAACGGAAGATCAAATTC
	MpSYP7B.2	CACCATGAGCGTTACAGACTTGC	CTAGAAGAATTGATCTCCGAAG
	MpVAMP72A	CACCATGGGTGTGAACTCGTTGATTTAC	TTACTTGCACTTAAATCCCTTGC
	MpVAMP72B	CACCATGGGGGCGAAGAATGGC	TCACAGGAGGCGCCGACCGC
	MpVAMP72C	CACCATGGGATCGATTCTTAGCAG	TCATAAAAAATCTATACAACTTTTG
	MpVAMP72C^{G2A}	CACCATGGCATCGATTCTTAGC	TCATAAAAAATCTATACAACTTTTG
	MpVAMP72D	CACCATGGGAGCGATTCTTAGCAG	CTAAAAATTTATACAAAGGTTAC
	MpVAMP72D^{G2A}	CACCATGGCAGCGATTCTTAGC	CTAAAAATTTATACAAAGGTTAC
	MpVAMP72E	CACCATGGGGGCGAATCTTAGCAG	CTATATTGCAGTTTTAGCTTTTAG
	MpVAMP72E^{G2A}	CACCATGGCGGCGAATCTTAGC	CTATATTGCAGTTTTAGCTTTTAG
	MpTOMOSYN11	CACCATGTTTATCAAGCGGTTTCTTCAG	TCAAAGTTCCCACCACTTCTTTGC
	MpTOMOSYN12	CACCATGTGTCTTACTGGCCGCTCC	TCACAGCTCGTACCACTTCCTG
	MpCLC	CACCATGGCGGAGTTCGAGTATGGGG	GGCAGTCACAGCTGCTGCCGC

Table 2-1. (continued)

Purpose	Gene name	primer 1 (5' to 3')	primer 2 (5' to 3')
pENTR for transient expression in A. thaliana cells	MpVAMP72A	GGCAAGCTTAAGGGTGGGCGCGCCGACCCAG	GGCAAGCTTCTCTGCCAGCACGACAGTACCTC
	MpVAMP72B	GGCAAGCTTAAGGGTGGGCGCGCCGACCCAG	GGCAAGCTTCACTCCCGGCCCCGTTCCTCGAG
	MpVAMP72C	GGCAAGCTTAAGGGTGGGCGCGCCGACCCAG	GGCAAGCTTCAAGCCATTCTTCGCCCGGGGCGAAGAATGGCTTGATCTACTGCTAAGAATCGATC
	MpVAMP72C^{G2A}	GGCAAGCTTAAGGGTGGGCGCGCCGACCCAG	GGCAAGCTTCAAGCCATTCTTCGCCCGGGGCGAAGAATGGCTTGATCTACTGCTAAGAATCGATC
	MpVAMP72D	GGCAAGCTTAAGGGTGGGCGCGCCGACCCAG	GGCAAGCTTCAAGCCATTCTTCGGCCCGGGC
	MpVAMP72D^{G2A}	GGCAAGCTTAAGGGTGGGCGCGCCGACCCAG	GGCAAGCTTCAAGCCATTCTTCGGCCCGGGC
	MpVAMP72E	GGCAAGCTTAAGGGTGGGCGCGCCGACCCAG	GGCAAGCTTGATCAAGTGATTCTTCACATCG
	MpVAMP72E^{G2A}	GGCAAGCTTAAGGGTGGGCGCGCCGACCCAG	GGCAAGCTTGATCAAGTGATTCTTCACATCG
pENTR for genomic sequences	MpSYP12A 5'	GCAGGCTCCGCGGCCAAATTTAATACTTATAGATTTG	GTGAAGGGGGCGGCCTTTGGCAGATCACTCCACCGTTG
	MpSYP12A CDS+3'	CACCCCCGGGGGCAGCGGCATGAACGATCTTCTGCAGAAAAC	AAGTGATTTCAATGTATGTCCCTC
	MpSYP12B 5'	GCAGGCTCCGCGGCCTCTGTACTTGCATTTAGAAAATC	GTGAAGGGGGCGGCCGACTGCTAAGCACAGAGTCGCAG
	MpSYP12B CDS+3'	CACCCCCGGGGGCAGCGGCATGAACGATCTGCTGGCAAGAG	ATCAGCCCCCCCGCGACGACAG
	MpSYP13A 5'	GCAGGCTCCGCGGCCAATTAGCAGATCCAGCTGCTTCC	GTGAAGGGGGCGGCCGATTGCCGCCTGCTTGGCTTACTG
	MpSYP13A CDS+3'	AAGAAGGGTGGGCGCATGAACGATCTTTTAGGGGTATG	GCTGGGTCGGCGCGCAGATGTGACAAGGTCAAGAAGAAC
	MpSYP13B 5'	GCAGGCTCCGCGGCCGATCATGGCGAGTGTGTCGTGC	GTGAAGGGGGCGGCCGATTGCGCGCTGCTGCTGCCTCC
	MpSYP13B CDS+3'	CACCGGATCCGGCGGCAGCGGCATGAACGATCTTCTGGGAGACTC	CATACAACTCAAAACAATTTTGATG
	MpSYP2 5'	GCAGGCTCCGCGGCCCACGAGCGAGTGAGACACCAGAGGAG	GTGAAGGGGGCGGCCCCTCCTGCTTCGTGGTAAATCCTCTTC
	MpSYP2 CDS+3'	CACCCCCGGGGGCAGCGGCATGAGTTTTTTAGATCTAGAGGC	GCAAGTGGTGATGAGCCTTGCGTGC
	mCitrine-*Sma*I	GCCCCCTTCACCCCCATGGTGAGCAAGGGCGAGGAG	CATGCCGCTGCCCCCCTTGTACAGCTCGTCCATGCC
	mCitrine-*Bam*HI	CCCCTTCACCGGATCATGGTGAGCAAGGGCGAGGAG	GCTGCCGCCGGATCCCTTGTACAGCTCGTCCATGCC

Table 2-1. (continued)

Purpose	Gene name	primer 1 (5′ to 3′)	primer 2 (5′ to 3′)
gRNA	MpSYP12A gRNA2	CTCGAGAAAGACACGACGGAGTCA	AAACTGACTCCGTCGTGTCTTTCT
	CUFF.11493 gRNA1	CTCGGCCGCCTTCTCCACCATCGT	AAACACGATGGTGGAGAAGGCGGC
	CUFF.11493 gRNA4	CTCGGTCAAATATATGTCTGGTAA	AAACTTACCAGACATATATTTGAC
Gateway vectors	proMpSYP12A: mCitrine	GGCCAGTGCCAAGCTAAATTTAATACTT ATAGATTTG	TTTGTACAAACTTGTCTTGTACAGCTC GTCCATGCCG
	proMpSYP12B: mCitrine	GGCCAGTGCCAAGCTTCTGTACTTGCAT TTAGAAAATC	TTTGTACAAACTTGTCTTGTACAGCTC GTCCATGCCG
	proMpCYCB1: mCitrine	GGCCAGTGCCAAGCTGAGGATGGTTTAA TCCTTTTTGC	TTTGTACAAACTTGTCTTGTACAGCTC GTCCATGCCG
Genotyping	CUFF.11493 a-b	CTTGCTAACAACGTGAGAAGCTGG	CATGTTCAATGATTATACTCACCTC
	CUFF.11493 c-d	CTGCCGTCATGCGATCTCGCTCGG	GATGCTTGGATACGCAGTTGGTCG
	CUFF.21491 e-f	CACCATGCCTAGGACAATTGAAGTTGCC	TCATTCCCCCCTACATTGCACGAAC
TAIL-PCR	AD1	NGTCGASWGANAWGAA	
	AD2	TGWGNAGSANCASAGA	
	AD3	AGWGNAGWANCAWAGG	
	AD4	GTNCGASWCANAWGTT	
	AD5	NTCGASTWTSGWGTT	
	AD6	WGTGNAGWANCANAGA	
	TR1	CCTGCAGGCATGCAAGCTTGG	
	TR2	GCTGGCGTAATAGCGAAGAGG	
	TR3	CCTGAATGGCGAATGCTAGAG	
	TL1	CAGATAAGGGAATTAGGGTTCCTATAGG	
	TL2	TATAGGGTTTCGCTCATGTGTTGAGC	
	TL3	AGTACATTAAAAACGTCCGCAATGTG	
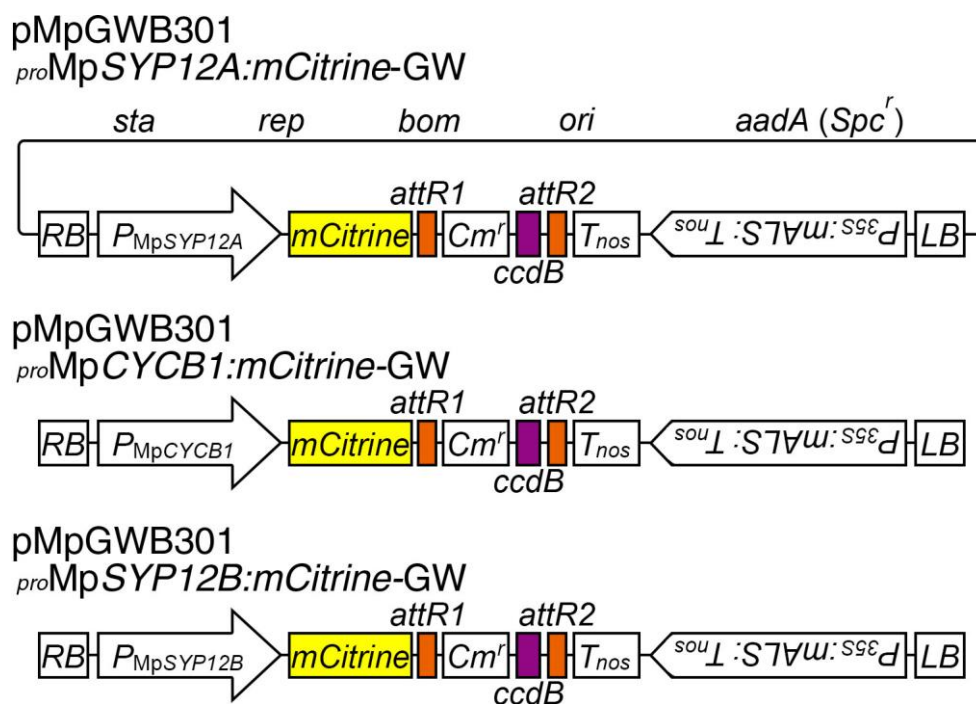
Figure 2-1. Gateway constructs

Schematic structures of gateway constructs prepared in this study. The vectors are derived from pMpGWB301 (Ishizaki *et al.*, 2015).

Table 2-2. Cycle setting for TAIL-PCR

Reaction	Cycle No.	Thermal settings
Primary	1	94°C 1 min, 95°C 1 min
	5	94°C 1 min, 65°C 1 min, 68°C 3 min
	1	94°C 1 min, 30°C 1min
		Ramping to 68°C 3 min, 68°C 3 min
	13	94°C 30 sec, 68°C 4 min
		94°C 30 sec, 68°C 4 min
		94°C 30 sec, 44°C 1 min, 68°C 3min
	1	68°C 5 min
Secondary	1	94°C 1 min, 95°C 1 min
	13	94°C 30 sec, 68°C 4 min
		94°C 30 sec, 68°C 4 min
		94°C 30 sec, 44°C 1 min, 68°C 3 min
	1	68°C 5 min
Tertiary	1	94°C 1 min, 95°C 1 min
	13	94°C 30 sec, 68°C 4 min
		94°C 30 sec, 68°C 4 min
		94°C 30 sec, 44°C 1 min, 68°C 3 min
	1	68°C 5 min

Chapter III: **Characterization of SNARE molecules of *Marchantia polymorpha***

Introduction

Membrane trafficking pathways and functions of organelles have been diversified among eukaryotic lineages, likely to fulfill functions specific to each lineage and/or organism, even though the basic molecular framework is highly conserved throughout eukaryotes. Completion of genome sequencing of Archaeplastida such as *Arabidopsis thaliana* (Arabidopsis Genome, 2000), *Populus trichocarpa* (Tuskan *et al.*, 2006), *Oryza sativa* (International Rice Genome Sequencing, 2005), *Selaginella moellendorffii* (Banks *et al.*, 2011), *Physcomitrella patens* (Rensing *et al.*, 2008), *Klebsormidium flaccidum* (Hori *et al.*, 2014), *Chlamydomonas reinhardtii* (Merchant *et al.*, 2007), *Ostreococcus tauri* (Palenik *et al.*, 2007), *Cyanidioschyzon merolae* (Matsuzaki *et al.*, 2004; Nozaki *et al.*, 2007), and many organisms of non-plant systems has enabled us to estimate and compare the complexity of the membrane trafficking system by counting numbers of machinery components of membrane trafficking such as Rab GTPases and SNARE molecules encoded in their genomes, which has provided insights into diversification and specification of membrane trafficking during eukaryotic evolution (Rutherford & Moore, 2002; Dacks & Field, 2007; Sanderfoot, 2007; Elias, 2008; Elias *et al.*, 2012).

In the green plant lineage, association between diversification of post-Golgi trafficking pathways and terrestrialization and/or multicellularization has been pointed out, which is based on increased numbers of genes for Rab GTPase, tethers, and SNARE proteins in land plants compared with algal species (Rutherford & Moore, 2002; Sanderfoot, 2007; Vukasinovic & Zarsky, 2016). Consistently, the evidence of unique

diversification of the post-Golgi trafficking system in plants has been obtained from studies using *A. thaliana* and tobacco. For example, the plant *trans*-Golgi network (TGN) has distinct functions from the TGN in animal cells; the TGN acts as the early endosome as well as functioning as a sorting hub in the secretory pathway in plant cells (Richter *et al.*, 2009; Uemura, 2016). The vacuolar transport is also organized in a plant-unique way, which acts as a fundamental basis of plant-specific vacuolar functions (Ebine *et al.*, 2014; Uemura & Ueda, 2014). Nevertheless, it is still possible that the increase in number of the machinery components reflects expansion of genes without functional differentiation because of recent whole or large-scale genome duplication, which is reported in several plant species (Tang *et al.*, 2008; Barker *et al.*, 2009). Thus, to gain more precise information on functional diversification of membrane trafficking during plant evolution, analyses in basal plant lineages including bryophytes and algal species are apparently needed.

To gather information regarding evolution and diversification of the membrane trafficking pathways in plants, I systematically analyzed SNARE molecules in the liverwort, *Marchantia polymorpha*. I identified 34 genes for SNARE proteins in *M. polymorpha* and subcellular localization of the majority of these SNARE molecules by expressing fluorescently tagged proteins in *M. polymorpha* thallus cells. The comparison of the subcellular localization of orthologous products between *M. polymorpha* and *A. thaliana* indicated that the membrane trafficking system has increased its complexity during land plant evolution, although *M. polymorpha* also seems to acquire specialized trafficking pathways unique to the organism.

Results

Genes for SNARE proteins encoded in the *M. polymorpha* genome

To gather information about the diversification of membrane trafficking pathways during the evolution of land plants, I comprehensively analyzed SNARE molecules in the liverwort *M. polymorpha*. I searched genes for proteins with the SNARE motif from the genome and transcriptome databases of *M. polymorpha* and discovered that 37 SNARE proteins in 34 loci were encoded in the *M. polymorpha* genome (Figure 3-1). These proteins were classified into 5 groups: 8 Qa-SNAREs, 7 Qb-SNAREs, 10 Qc-SNAREs, 1 Qb+Qc-SNARE, and 11 R-SNAREs. The majority of the SNARE genes in *M. polymorpha* lack paralogs in the genome, except Mp*GOS1*, Mp*USE1*, Mp*SYP6*, Mp*SYP1*, Mp*SYP7*, Mp*VAMP72*, and Mp*TOMOSYN1* (Figure 3-1). Homologs of all SNARE genes in *M. polymorpha* were also found in other land plants with a greater degree of redundancy, while the *SYP1* group was multiplicated also in *M. polymorpha*. Four *SYP1* genes were found in the genome of *M. polymorpha*, two each of which were categorized into the *SYP11/12* and *SYP13* groups. The phylogenic analysis estimated that the SYP1 group of the green plants is divided into two major groups: chlorophyte SYP1 and streptophyte SYP1, furthermore the SYP1 group of streptophytes is separated into two major groups: SYP13 and SYP11/12 groups (Figure 3-1 and 3-2). Among SYP11/12 group, SYP11 and SYP12 subgroups could diverge at the emergence of seed plants, and lycophytes and bryophytes including *M. polymorpha* possess the ancestral class of the SYP11/12 members (Figure 3-2). Each SYP1 member of *M. polymorpha* typically possessed a syntaxin domain, a Qa-SNARE domain, and a transmembrane domain (TMD), and these four SYP1 members were highly similar in their primary sequences aligned (Figure 3-3).

Splicing variants were detected for three loci, Mp*SYP7B*, Mp*VAMP72A*, and Mp*GOS12*. Two splicing variants at the Mp*SYP7B* locus were amplified by RT-PCR; Mp*SYP7B.1* encoded a canonical Qc-SNARE protein comprising of a syntaxin domain, a Qc-SNARE domain, and a TMD, while the protein translated from the other transcript, Mp*SYP7B.2* lacked the TMD (Figure 3-4A). Two different transcripts were amplified from the Mp*VAMP72A* locus with a consequence of alternative splicing. Each R-SNARE protein translated from the transcripts contained a longin domain, an R-SNARE domain, and a TMD, which R-SNARE proteins were comprised of different lengths of the longin domains (Figure 3-4B). Based on the transcriptome information, two different length of transcripts were amplified from the Mp*GOS12* locus. Both transcripts were predicted to be translated into Qb-SNARE proteins containing a Qb-SNARE domain and a TMD, with differences in the lengths of the carboxyl termini (Figure 3-4C).

Next, I examined the transcription profiles of the SNARE genes by RT-PCR in different developmental organs using Mp*EF1α* as a standard, the mRNA levels of which exhibit a constant accumulation in various tissues and under several environmental-stress conditions examined to date (Althoff *et al.*, 2013; Kanazawa *et al.*, 2013). Most of the SNAREs were ubiquitously transcribed in all of the demonstrated organs [5-day-old thalli, antheridiophores (male reproductive organs), archegoniophores (female reproductive organs), and 7-day-old sporelings], while several genes were detected to be transcribed in specific organs (Figure 3-5). One of the transcript variants at Mp*GOS12* locus, Mp*GOS12.2*, was detected only in thalli. Mp*USE1B* was transcribed in antheridiophores, archegoniophores, and sporelings but not in thalli, and the transcripts of both Mp*SYP6B* and Mp*SYP7B* were detected only in antheridiophores. Both Mp*SYP12B* and Mp*TOMOSYN12* were transcribed in thalli, antheridiophores, and archegoniophores but

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not in sporelings. The transcripts of Mp*VAMP72E* were detected in thalli, archegoniophores, and sporelings but not in antheridiophores. I could not detect the transcripts of Mp*VAMP72D* in any organs under the experimental condition. These results indicate that the genes without their paralogs are constitutively expressed in *M. polymorpha* plants during all developmental stages, in contrast several genes with their paralogs exhibit organ-specific transcription profiles, probably reflecting specific and/or additional requirements for development of specific cells.

Novel VAMP72 members with characteristic structures in *M. polymorpha*

In addition to the basal set of SNARE molecules that are conserved among land plants (Figure 3-1), I identified three VAMP72 members, with unique structures. VAMP7 belongs to the longin-type R-SNAREs. The longin domain of VAMP7 is usually positioned at the N-terminus followed by an R-SNARE domain and a TMD. In addition to these canonical three domains, MpVAMP72C, MpVAMP72D, and MpVAMP72E possessed extended sequences comprising 12-14 amino acid residues at their N-termini, which were predicted to contain the consensus sequence for *N*-myristoylation (predicted by NMT; http://mendel.imp.ac.at/myristate/SUPLpredictor.htm and Myristoylator; http://web.expasy.org/myristoylator/, Bologna *et al.*, 2004) (Figure 3-6A). R-SNARE proteins with potential *N*-myristoylation extension have not been reported to be identified in any other species, suggesting that this type of R-SNARE is uniquely acquired in liverworts. The other four VAMP7 proteins in *M. polymorpha*, MpVAMP71, two MpVAMP72A products derived from the same gene, and MpVAMP72B, did not contain the consensus for *N*-myristoylation. To verify whether the N-terminal sequences of MpVAMP72C–E were actually *N*-myristoylated in plant cells, I expressed the N-terminal

20 amino acids of MpVAMP72C–E tagged with enhanced yellow fluorescent protein (EYFP) in the protoplasts of *A. thaliana* cells cultured in suspension. The results showed that these chimeric proteins were localized to membrane vesicles, the endoplasmic reticulum (ER), and the plasma membrane (PM) (Figure 3-6B, D, and F). In contrast, the N-terminal 20 amino acid sequences of MpVAMP72A and MpVAMP72B and N-terminal sequences of MpVAMP72C–E, in which the glycine residue that was expected to undergo *N*-myristoylation was replaced with alanine (G2A), did not target EYFP to membranous compartments but revealed the dispersal of EYFP into the cytosol and nuclei (Figure 3-6C, E, G, H, and I). These results indicated that *M. polymorpha* possesses novel VAMP72 members that are *N*-myristoylated.

Markers of the Golgi apparatus and *trans*-Golgi network in *M. polymorpha*

To elucidate the functions of SNARE molecules, it is important and efficient to know their subcellular localization, and this endeavor should be preceded by the establishment of reliable organelle markers in *M. polymorpha*. To achieve this goal, I examined whether the Golgi marker used in *A. thaliana* and tobacco also works in *M. polymorpha*. Fluorescent proteins fused with the TMD of rat sialyltransferase (ST) are widely used as reliable Golgi markers (Boevink *et al.*, 1998; Ito, Y *et al.*, 2012). To verify whether the ST tagged with a fluorescent protein (XFP) is also useful in *M. polymorpha*, I expressed ST-Venus and ST-monomeric red fluorescent protein (mRFP) driven by the cauliflower mosaic virus (CaMV) 35S promoter in *M. polymorpha* thallus cells. These chimeric proteins were localized to punctate compartments in the cytoplasm and showed perfect overlap (Figure 3-7A). As a marker of the *trans*-Golgi network (TGN), I employed MpSYP6A because its orthologous product of *A. thaliana* is an established marker of the

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TGN (Sanderfoot *et al.*, 2001a; Uemura *et al.*, 2004; Robert *et al.*, 2008; Choi *et al.*, 2013). Fluorescence from Citrine-MpSYP6A and mRFP-MpSYP6A driven by the CaMV 35S promoter and Mp*EF1α* promoter, respectively, was observed on punctate compartments and the PM with perfect overlap (Figure 3-7B and C). Immune-electron microscopy using a polyclonal anti-GFP antibody demonstrated that punctate compartments visualized by ST-Venus or Citrine-MpSYP6A were *trans* cisternae of the Golgi apparatus and the TGN, respectively, in transgenic *M. polymorpha* expressing ST-Venus or Citrine-MpSYP6A (Era, 2013). These results indicated that ST-XFP is also useful as a Golgi apparatus marker in *M. polymorpha* cells. Thus, ST-mRFP and mRFP-MpSYP6A can be used as markers of the Golgi apparatus and TGN in *M. polymorpha*, respectively. Most of the TGN was observed to be associated with the Golgi apparatus, but Golgi-independent TGN was also observed (arrowheads in Figure 3-7D and E). Similar Golgi-associated and Golgi-independent TGN have also been reported in *A. thaliana* (Viotti *et al.*, 2010; Uemura *et al.*, 2014), which suggests that the organization and function of the TGN are conserved at least partially among land plant lineages.

Subcellular localization of fluorescently tagged SNARE proteins in *M. polymorpha*

Next, I analyzed the subcellular localizations of major members of the SNARE family in *M. polymorpha* by expressing fluorescently tagged SNARE molecules under the control of the CaMV 35S or their own promoters. The subcellular localization of these molecules was classified into several categories.

ER- and Golgi apparatus-localized SNAREs

I identified at least six ER-localized SNAREs: Qa-MpSYP8, Qb-MpSEC20, Qc-MpUSE1A, Qc-MpUSE1B, Qc-MpSYP7B.1, and R-MpSEC22. Citrine-fused

MpSYP7B.1, MpSEC20, MpUSE1A, and MpSEC22 driven by the CaMV 35S promoter were localized to reticulated membrane tubules and cisternal structures that were connected to the tubules (Figure 3-8A–D), which is the typical structure of the ER in both plant and animal cells (Voeltz *et al.*, 2002; Borgese *et al.*, 2006). The fluorescence of Citrine-MpUSE1B was scarcely detected in the reticulated pattern; it was observed on the nuclear envelope almost exclusively (Figure 3-8E), which is continuous with the ER. Nuclear-envelope localization was also observed in cells expressing other fluorescently tagged ER-localized SNAREs (for example, see Figure 3-8D). Citrine-MpSYP8 was localized to numerous punctate membrane domains, which were independent of and smaller in size than the Golgi apparatus visualized using ST-mRFP (Figure 3-8F). The punctate domains could be subdomains of the ER, as previously described for tobacco leaf cells (Bubeck *et al.*, 2008). In a consistent manner, Citrine-MpSYP8 was also localized to the reticulated network occasionally in addition to small punctate domains associated with the network (asterisk in Figure 3-8F).

Seven SNARE proteins were localized to the Golgi apparatus: Qa-MpSYP3, Qb-MpMEMB1, Qb-MpGOS11, Qb-MpGOS12.1, Qb-MpGOS12.2, and Qc-MpSFT1, and R-MpTOMOSYN11. These proteins driven by the CaMV 35S promoter were observed only on punctate organelles in the cytoplasm in *M. polymorpha* thallus cells, in which these SNAREs colocalized or tightly associated with ST-mRFP (Figure 3-9A, C, E, G, I, K, and M). Conversely, colocalization was not observed between these SNAREs and the TGN marker, mRFP-MpSYP6A (Figure 3-9B, D, F, H, J, L, and N).

These ER- or Golgi-localized SNARE molecules should be involved in the membrane trafficking pathways between the ER and Golgi apparatus and/or around the Golgi apparatus, such as the intra-Golgi transport and retrograde transport from post-

Golgi organelles in *M. polymorpha*.

TGN-localized SNAREs

I identified six TGN-localized SNAREs: Qa-MpSYP4, Qb-MpVTI1, Qc-MpBET1, Qc-MpSYP6A, Qc-MpSYP6B, and R-MpVAMP72B. Citrine-fused MpSYP4, MpVTI1, MpBET1, and MpSYP6B driven by the CaMV 35S promoter were localized to punctate compartments in *M. polymorpha* thallus cells, which also bore mRFP-MpSYP6A, the TGN marker (Figure 3-10A, C, E, and G). ST-mRFP, the Golgi apparatus marker, was not colocalized with these SNARE members (Figure 3-10B, D, F, and H).

Vacuolar membrane-localized SNAREs

Qa-MpSYP2, Qc-MpSYP5, and R-MpVAMP71 were localized to the vacuolar membrane in *M. polymorpha* thallus cells. Citrine-fused MpSYP2 and MpSYP5 driven by the CaMV 35S promoter were observed exclusively on the vacuolar membrane (Figure 3-11A and B), and Citrine-MpVAMP71 was also localized to punctate compartments (Figure 3-11C and D). To identify the punctate compartments, I coexpressed Citrine-MpVAMP71 and ST-mRFP or mRFP-MpSYP6A in *M. polymorpha* plants, and I discovered that Citrine-MpVAMP71 did not colocalize with either marker, although they are frequently observed in a close proximity (Figure 3-11C and D). These punctate compartments might represent multivesiculated endosomes, which have been occasionally observed close to the Golgi and TGN in *A. thaliana* cells (Richter *et al.*, 2007; Scheuring *et al.*, 2011; Singh *et al.*, 2014), or adaptor protein complex 3 (AP-3)-positive compartments, which are responsible for the transport of VAMP713 in *A. thaliana* (Ebine *et al.*, 2014).

In transgenic plants expressing Citrine-fused vacuolar membrane SNAREs, spherical structures with a strong fluorescence intensity, which were referred to as bulbs in *A. thaliana* (Saito *et al.*, 2002), were frequently observed in vacuoles (Figure 3-12A, C, and D). It has recently been reported that overexpression and a weak dimerizing nature of GFP lead to artificial enhancement of the accumulation of bulbs in *A. thaliana* (Segami *et al.*, 2014). To eliminate potential artificial effects of overexpression and dimerization of the fluorescent protein fused to MpSYP2 in *M. polymorpha*, I constructed a chimeric gene consisting of the Mp*SYP2* promoter, the cDNA for monomeric Citrine (mCitrine) to which the mutation leading to monomerization of GFP variants (Segami *et al.*, 2014) was introduced, and the genomic sequence of Mp*SYP2* starting from the start codon. In *M. polymorpha* plants expressing mCitrine-MpSYP2 under the regulation of its own promoter, I did not observe bulb-like spherical structures in the vacuole (Figure 3-12B). This result strongly suggests that overexpression and/or dimerization of fluorescently tagged vacuolar membrane SNAREs also results in an artificial enhancement of bulb-like structure formation in *M. polymorpha*.

PM- and oil body membrane-localized SNAREs

I then focused my interest on SNARE molecules on the PM. A majority of SYP1 members have been localized to the PM in *A. thaliana*, whose numbers have been reported to expand drastically during land plant evolution (Sanderfoot, 2007). It has also been reported that SYP111/KNOLLE, which is responsible for cell plate formation in dividing cells (Lukowitz *et al.*, 1996), is mislocalized to the PM when expressed in non-dividing cells (Völker *et al.*, 2001). Thus, to collect information on the authentic localization and expression of SYP1 members in *M. polymorpha*, I expressed SYP1

members tagged with mCitrine under the regulation of their own regulatory elements including 5′- and 3′-flanking regions and introns. Qa-MpSYP12A, Qa-MpSYP13A, and Qa-MpSYP13B were mainly localized to the PM in almost all cells in thalli (Figure 3-13A–C). Intriguingly, Qa-MpSYP13A was also localized to the oil body membrane in oil body cells (Figure 3-13D), which are a unique development in liverworts. Furthermore, Qa-MpSYP12B was specifically expressed in oil body cells and was localized to the oil body membrane with faint localization on the PM (Figure 3-13E). Oil body cells, a liverwort-specific cell containing the oil body, were recognized as dark-colored cells in the low magnification bright field (BF) images (Figure 3-14). Oil body cells were detected in 5-day-old thalli, antheridiophores, and archegoniophores, whereas they were not observed in sporelings (Figure 3-14), which is consistent with the absence of the Mp*SYP12B* transcript in sporelings, as demonstrated by RT-PCR (Figure 3-5). The oil body localization was not observed for the other MpSYP1 members (Figure 3-13F and G). These results indicate that the functions of SYP1 members have also diverged in *M. polymorpha*.

Regarding the SNARE molecules of subgroups other than Qa-SNAREs, I found that Citrine-tagged Qb-MpNPSN1 and R-MpVAMP72B were localized to the PM when they were expressed under the regulation of the CaMV 35S promoter (Figure 3-13H–J). Citrine-MpVAMP72B was also observed on punctate compartments, majority of which colocalized with the TGN marker, mRFP-MpSYP6A (Figure 3-13I). However, a part of the punctate compartments were independent of the TGN (arrowhead in Figure 3-13I), which could correspond to the intermediate compartments that are responsible for transport between the TGN and PM as reported in *A. thaliana* (Asaoka *et al.*, 2012). Colocalization between Citrine-MpVAMP72B and the Golgi marker, ST-mRFP, was not

observed (Figure 3-13J). Citrine-tagged Qb+Qc-MpSNAP, an *M. polymorpha* homolog of SNAP25, was dispersed into the cytosol following its expression under the regulation of the CaMV 35S promoter (Figure 3-13K). GFP-tagged orthologous products in *A. thaliana*, SNAP29, SNAP30, and SNAP33 were also shown to be dispersed into the cytosol in *A. thaliana* protoplasts (Uemura *et al.*, 2004), although these molecules mediate trafficking events to the PM, including a pathogen response (Kwon *et al.*, 2008) and cell plate formation (Heese *et al.*, 2001; El Kasmi *et al.*, 2013). Because the CaMV 35S promoter was inactive in the oil body cell (Figure 3-15), I could not verify the localization of Qb-, Qc-, and R-SNAREs on the oil body membrane.

Discussion

In Chapter III, I analyzed SNARE molecules in *M. polymorpha*, the genome of which is currently under detailed investigations. I showed that *M. polymorpha* has a conserved set of SNARE molecules with lower degrees of redundancy than the other land plant lineages. The genome of the moss *P. patens*, which also belongs to the bryophyte, encodes 57 SNARE proteins (Sanderfoot, 2007; Rensing *et al.*, 2008). This number is almost equivalent to the number of SNAREs in *A. thaliana*, which possesses 63 SNARE proteins (Sanderfoot, 2007). Conversely, smaller numbers of SNARE proteins are encoded in the genomes of unicellular algal species; 29 and 20 SNARE proteins have been identified in *C. reinhardtii* and *O. tauri*, respectively (Sanderfoot, 2007). Based on this evidence, it has been suggested that the expansion of the SNARE genes could be associated with the multicellularization and/or terrestrialization of green plants (Dacks & Field, 2007; Sanderfoot, 2007). However, my results and the recently unveiled genome of the filamentous charophytic algae, *K. flaccidum* (Hori *et al.*, 2014), did not firmly support this notion; the majority of the subgroups of SNARE protein in *M. polymorpha* consist of only one member that is expressed in all of the organs examined. Conversely, subgroups comprising two paralogous members, such as the Mp*GOS1*, Mp*USE1*, Mp*SYP6*, Mp*SYP7,* and Mp*TOMOSYN1* groups, exhibited distinct expression patterns between the subgroup members; one was expressed ubiquitously and the other was expressed in an organ-specific manner. The subcellular localization of GOS1, USE1, and SYP6 groups with fluorescently tagged SNARE proteins also revealed that the paralogous gene products exhibited a similar subcellular localization when expressed under the CaMV 35S promoter (summarized in Figure 3-16); this was largely identical to the localization of the orthologous products in *A. thaliana* (Uemura *et al.*, 2004). These

results indicated that the duplication of SNARE genes followed by the differentiation of regulatory elements such as the promoter resulted in the differentiation of expression patterns without changing the subcellular localization and presumably the molecular function in these subgroups.

SNAREs with distinct subcellular distributions in *A*. *thaliana* and *M. polymorpha*

Although the subcellular localization of a majority of SNARE proteins in *M. polymorpha* was comparable with that of the orthologous products of *A*. *thaliana*, several SNARE proteins exhibited distinct behaviors between *A*. *thaliana* and *M. polymorpha* (Figure 3-16).

Early secretory SNAREs

Several SNARE molecules that function in early secretory organelles such as the ER and Golgi apparatus exhibited good conservation in terms of their subcellular localization among land plants. For example, a putative set of cognate SNAREs, Qa-SYP8, Qb-SEC20, Qc-USE1, and R-SEC22, which are counterparts of the SNAREs that mediate retrograde transport from the Golgi apparatus to the ER in budding yeast (Lewis & Pelham, 1996; Lewis *et al.*, 1997; Dilcher *et al.*, 2003; Hong, 2005), were also localized to the ER and related compartments in both *A*. *thaliana* (Uemura *et al.*, 2004) and *M. polymorpha* (this study). These results suggest that the functions of these molecules are conserved. Among the *M. polymorpha* SNARE molecules that are homologous to yeast SNAREs mediating anterograde transport from the ER to the Golgi apparatus (Qa-Sed5p, Qb-Bos1p, Qc-Bet1p, and R-Sec22p, Newman *et al.*, 1990; Hardwick & Pelham, 1992; Banfield *et al.*, 1995; Nichols & Pelham, 1998), MpSYP3 (homologous to Sed5) and

MpMEMB1 (homologous to Bos1p) were also localized to the Golgi apparatus. However, MpBET1 was predominantly localized to the TGN in *M. polymorpha*, exhibiting good colocalization with MpSYP6A (Figure 3-10G). Intriguingly, the *A. thaliana* BET1 homolog BET11/BS14a is localized to *trans* cisternae of the Golgi apparatus in *A. thaliana* cells cultured in suspension (Uemura *et al.*, 2004) and tobacco leaf epidermal cells (Chatre *et al.*, 2005). These results may indicate diversified functions of BET1 among land plant lineages, although the effects of XFP tagging and overexpression on subcellular localization should be verified in future studies.

I also noticed a slight difference in the subcellular localization of Qc-SYP7 members between *A. thaliana* s and *M. polymorpha*. The SYP7 group is plant-specific Qc-SNARE (Sanderfoot *et al.*, 2000), and the genome of *A. thaliana* encodes three SYP7 members (SYP71–73). GFP-fused SYP71 is predominantly targeted to the PM, with slight localization at the ER in meristematic cells, when it is expressed under the regulation of its own promoter (Suwastika *et al.*, 2008). The PM localization of SYP71 is further supported by fractionation and co-immunoprecipitation studies (Suwastika *et al.*, 2008; El Kasmi *et al.*, 2013). However, Citrine-tagged MpSYP7B.1 driven by the CaMV 35S promoter was localized to the ER in *M. polymorpha* thallus cells (Figure 3-8A). These distinct localization patterns are most likely explained by the sensitivity of the subcellular localization of SYP7 members to overexpression or ectopic expression. It has been reported that transient expression of fluorescently tagged *A. thaliana* SYP7 members in *A. thaliana* protoplasts and tobacco leaf cells results in localization at the ER (Uemura *et al.*, 2004; Wei *et al.*, 2013), which suggests that the expression level must be strictly regulated for the proper localization of SYP7. As indicated in Figure 3-5, Mp*SYP7B* is not transcribed in thalli. However, I do not rule out the possibility that SYP7 members

also mediate membrane fusion at the ER in *M. polymorpha* (and also in *A. thaliana*). The occurrence of two Qc-SNAREs on the ER, MpUSE1 and MpSYP7, may represent the existence of two distinctive trafficking pathways to the ER. Further studies are needed to obtain complete information regarding the functions of the SYP7 group in land plants.

Vacuolar SNAREs

Qa-SYP2 represents the first group of SNAREs to be identified in plants and is known to act in vacuolar and endosomal trafficking pathways (Sanderfoot *et al.*, 1999; Sanderfoot *et al.*, 2000; Saito & Ueda, 2009). There are three *SYP2* genes in the genome of *A. thaliana* (*SYP21–23*). *SYP21/PEP12* and *SYP22/VAM3* were initially isolated as *A. thaliana* genes that rescue the deleterious effects of the yeast *pep12* and *vam3* mutations, respectively (Bassham *et al.*, 1995; Sato *et al.*, 1997), which suggests that the functions of the SYP2 members had already diverged in the common ancestor of yeasts and plants into the *SYP21/PEP12* and *SYP22/VAM3* groups. However, phylogenetic and comparative genomic analyses indicate that these genes diversified independently and convergently in fungal and plant lineages (Dacks *et al.*, 2008). In the present study, I found that *M. polymorpha* harbors only one *SYP2* member, supporting this notion. MpSYP2 resided on the vacuolar membrane, and no other Qa-SNAREs were localized to the vacuolar membrane. This result indicated that MpSYP2 is the sole vacuolar Qa-SNARE. In *A. thaliana*, SYP21/PEP12 is predominantly localized to the prevacuolar compartment (PVC), whereas SYP22/VAM3 is preferentially localized to the vacuolar membrane, although they have partially redundant functions (Sanderfoot *et al.*, 1999; Sanderfoot *et al.*, 2001b; Ohtomo *et al.*, 2005; Shirakawa *et al.*, 2010; Uemura *et al.*, 2010). A detailed comparison of MpSYP2 and SYP2 members of *A. thaliana* would

provide invaluable information on the diversification of the subcellular localization and functions of SYP2 members during plant evolution.

The Qb-VTI1 group would be another good target of study to unravel mechanisms underlying the functional diversification of SNARE proteins. There are four paralogous VTI1 proteins in *A. thaliana* (VTI11−14), which are diversified in their localization and functions. VTI11 is predominantly localized to the PVC and vacuolar membrane with a small population on the TGN in *A. thaliana* (Zheng *et al.*, 1999; Uemura *et al.*, 2004), and it mediates membrane fusion at the vacuole by forming a complex with SYP22/VAM3, SYP51, and VAMP727 or VAMP71 (Sanderfoot *et al.*, 1999; Zheng *et al.*, 1999; Sanderfoot *et al.*, 2001a; Yano *et al.*, 2003; Niihama *et al.*, 2005; Ebine *et al.*, 2008; Ebine *et al.*, 2011; Fujiwara *et al.*, 2014). Conversely, VTI12 is mainly localized to the TGN and PM (Uemura *et al.*, 2004; Niihama *et al.*, 2005), where it functions distinctly from VTI11 in vacuolar trafficking pathways and autophagy (Surpin *et al.*, 2003; Sanmartin *et al.*, 2007; Zouhar *et al.*, 2009) in a complex with SYP41, SYP51, and SYP61 (Bassham *et al.*, 2000; Sanderfoot *et al.*, 2001a; Niihama *et al.*, 2005; Zouhar *et al.*, 2009). In contrast, *M. polymorpha* harbors only one *VTI1* homolog. Intriguingly, Citrine-MpVTI1 was localized to the TGN almost exclusively in *M. polymorpha* thallus cells (Figure 3-10C). This result could indicate that ancestral VTI1 acted centrally in membrane trafficking around the TGN, and paralogous expansion of *VTI1* genes followed by the accumulation of mutations led to the diversification of their subcellular localization, binding partners, and functions during land plant evolution.

A similar paralogous expansion in *A. thaliana* was also observed in the VAMP71 group, for which the *M. polymorpha* genome contains only one gene. Four VAMP71 members are encoded in the genome of *A. thaliana* (VAMP711−714), among which

VAMP711, VAMP712, and VAMP713 are exclusively localized on the vacuolar membrane (Carter *et al.*, 2004; Szponarski *et al.*, 2004; Uemura *et al.*, 2004; Uemura *et al.*, 2005; Geldner *et al.*, 2009; Ebine *et al.*, 2014). However, VAMP714 is also observed on the Golgi apparatus (Uemura *et al.*, 2004). VAMP714 is detected in the vacuolar membrane fraction (Szponarski *et al.*, 2004) and interacts with SYP22 in co-immunoprecipitation experiments (Fujiwara *et al.*, 2014). These results may suggest that VAMP714 functions during transport from the Golgi apparatus to the vacuole.

Different localization of SYP1 members in *M. polymorpha*

The Qa-SYP1 family is one of the most diversified SNARE families in land plants, especially in seed plants (Sanderfoot, 2007). For example, close homologs of SYP111/KNOLLE, which is expressed only in dividing cells and mediates membrane fusion at forming cell plates in *A. thaliana* (Lukowitz *et al.*, 1996; Lauber *et al.*, 1997; Enami *et al.*, 2009), are found only in seed plant lineages (Sanderfoot, 2007); however, cytokinesis involving cell plate formation is observed throughout land plant lineages and in some algal species (Pickett-Heaps, 1967; Marchant & Pickett-Heaps, 1973; McIntosh *et al.*, 1995; Chapman *et al.*, 2001; Cook, 2004; Katsaros *et al.*, 2011). In *M. polymorpha*, I did not identify a Qa-SNARE that was specialized for cell plate formation, although I found one of SYP1 members required for cell plate formation during cytokinesis in *M. polymorpha* thallus cells, which will be presented in the next chapter.

Three of four SYP1 members in *M. polymorpha*, MpSYP12A, MpSYP13A, and MpSYP13B, were expressed in the whole tissues of 5-day-old thalli, and MpSYP12B was only expressed in oil body cells (Figure 3-13). The oil bodies in liverworts, which are formed in liverwort-specific oil body cells, are responsible for the synthesis and storage

of specific isoprenoids, phenolics, and bisbibenzyl compounds such as marchantin and its relatives (Asakawa, 1983; Suire *et al.*, 2000; Asakawa *et al.*, 2013; Tanaka *et al.*, 2016). The oil body is surrounded by a single unit membrane, which is reported to originate from the ER cisternae (Duckett, 1995; Suire, 2000). Interestingly, my results demonstrated that the surface membrane of the oil body and the PM shared the common Qa-SNAREs. In the Chapter IV and V, detailed mechanisms underlying oil body formation and the involvement of membrane trafficking in this process will be further investigated.

A novel type of R-SNARE

In this study, I identified novel R-SNARE members with a distinctive structural characteristic. MpVAMP72C–E were classified in the VAMP72 group; however, these molecules harbor the consensus sequence for *N*-myristoylation in addition to functional domains constituting canonical VAMP7 members. I confirmed that the N-terminal sequences of these proteins are indeed *N*-myristoylated in *A. thaliana* cells. Thus, these *N*-myristoylated VAMP72 (Myr-VAMP72) proteins should be attached to the membrane at two sites in the polypeptides: the myristoylated N-terminus and the C-terminal TMD. To my best knowledge, this type of R-SNARE has not been identified in other organisms including plants, which indicates that this type of SNARE was uniquely acquired in the liverwort during evolution. It is anticipated that uniquely acquired SNARE molecules could be involved in distinctive membrane trafficking pathways, underlying the specialized functions of the liverwort. The subcellular localization, effect of knock-out mutations, and identification of binding partners of these Myr-VAMP72 proteins would also be interesting for future projects to unravel the molecular function and physiological significance of Myr-VAMP72 in *M. polymorpha*.

SNARE molecules with distinct but unique structures in terms of membrane binding domains have also been reported in other systems. Animal Syntaxin 17, which possesses two tandem TMD-like structures, has been shown to mediate membrane fusion between the autophagosome and lysosome (Itakura *et al.*, 2012), in addition to its function in trafficking between the ER and ER-Golgi intermediate compartment (Steegmaier *et al.*, 2000; Muppirala *et al.*, 2011). SNARE molecules with two TMDs are also predicted in the malaria parasite *Plasmodium falciparum* (Ayong *et al.*, 2007), although the structural topology and functions of these proteins remain elusive. Molecular and functional analyses of these uniquely acquired SNARE proteins to each lineage, including Myr-VAMP72, will provide deeper insights into the diversification and evolution of membrane trafficking pathways that are associated with the diversification and/or acquisition of SNARE molecules during the evolution of eukaryotic cells.
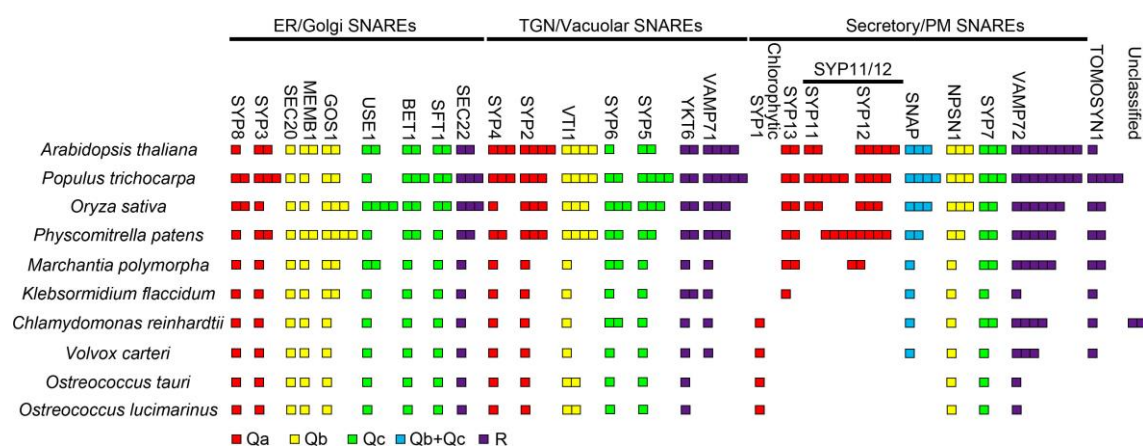
Figure 3-1. SNARE genes in green plants

SNAREs are classified into three major categories: ER/Golgi SNAREs, TGN/Vacuolar SNAREs, and Secretory/PM SNAREs, according to Sanderfoot (2007). The names given to the plant orthologs are shown below the classification. SNARE genes are indicated as individual boxes regardless of the presence of splicing variants, and each class of SNARE proteins is indicated in a different color (Qa, red; Qb, yellow; Qc, green, Qb+Qc, cyan; R, purple). The accession numbers of the SNARE genes for the listed organisms excluding *M. polymorpha* and *K. flaccidum* were retrieved from Sanderfoot 2007.
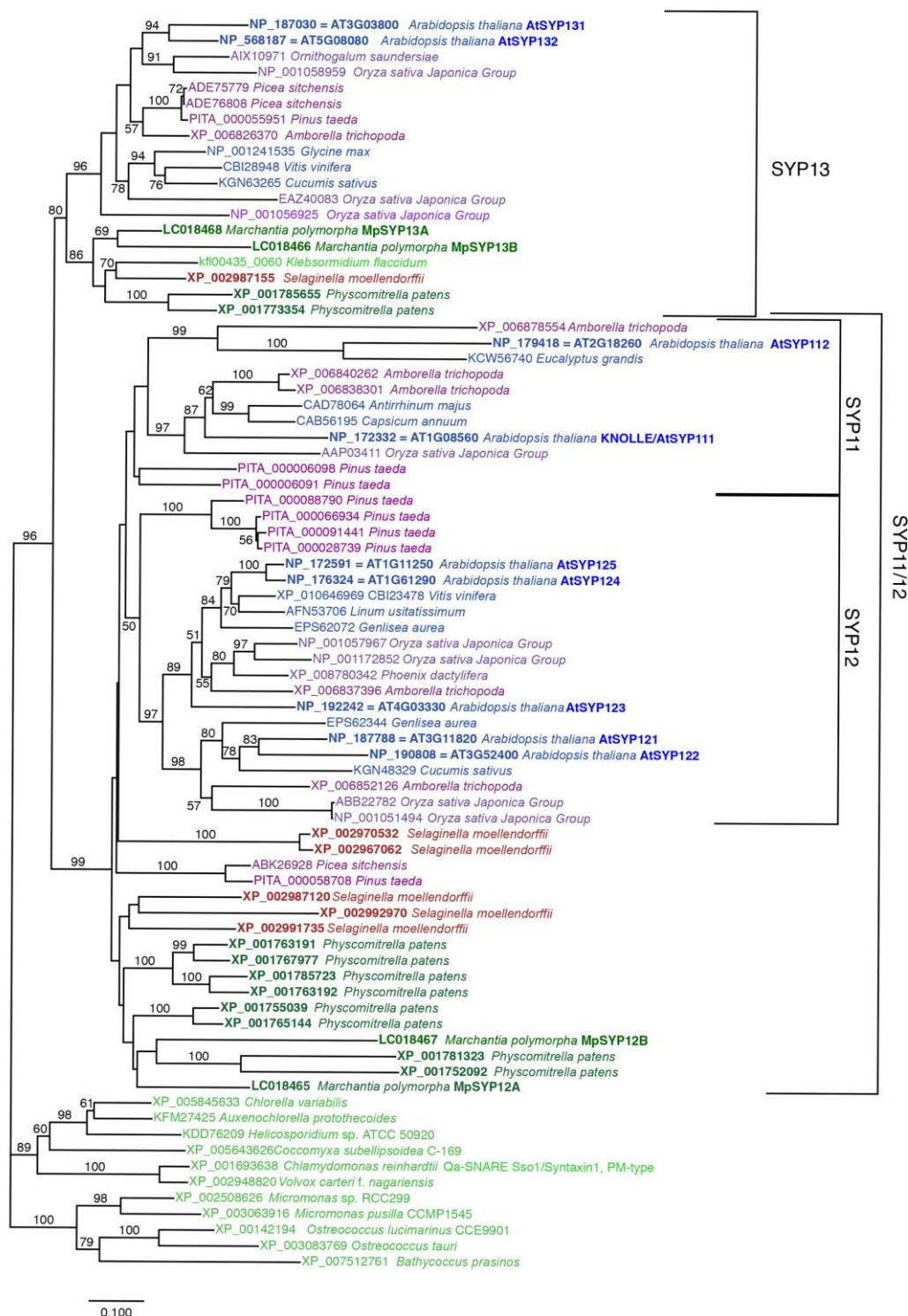
Figure 3-2. Neighbor Joining tree of the green plant SYP1 group

Amino acid substitution model of JTT (1992) was used. The tree is unrooted but drawn with proteins of chlorophytes as the outgroup to proteins of streptophytes. The branch lengths are proportional to the estimated number of substitution per site.

Figure 3-2. (continued)

Bootstrap probability is indicated as percentage on each branch with at least 50% support. Sequences from the NCBI database are labeled by their accession numbers. Organism names are given next to the accession number. For *A. thaliana* genes, the AGI codes and short names are given. Sequences from other sources have their own identifiers. The color of the label is according to the classification, blue: eudicots, blue violet: monocots, dark magenta: basal angiosperms (Amborella) and gymnosperms, brown: lycophytes (Selaginella), green: bryophytes, light green: green algae.

```
MpSYP13A   MNDLLGESGRHSTSAQDVTGNG---REKNGKDNN-DLEAG-PSGTPDGGADMLQFFNEVG  55
MpSYP13B   MNDLLGDSSREFSAAGGGTTAGSVEMSTNGKKKKRDVEAGREENEAEGDPGMDEFFREEA  60
MpSYP12A   MNDLLQKT---YSGGRGYLNMR------DVEKLGGEMEMQ----GIDPEKNLASFFDEVN  47
MpSYP12B   MNDLLARVFSRRDVVPDDVERG---------PAAFAMEEG---TSSEAGDELSGFFQEVD  48
           *****        .                          :*         :      :    ** *
```
Syntaxin domain
```
MpSYP13A   VIKTDMAQIRKNLAKLQDAHEETKTVTNAKAMKALKERMEKDIDEVSKVAQHIKGKIEAL  115
MpSYP13B   AIQGDLSQITSLLKKLEEAHQESQTLTNAKALKALKERMARDLDDVSKVVRHIKGKIEAL  120
MpSYP12A   VIKTDMERIKSLLAKLQDSNEESKTIHKVQAMKALRDRMDKDLAEVSKVARSVKQKLEEL  107
MpSYP12B   TLKKEMARVRDLLTKLQDAHEESKTARHTPALQELRDRINEDIGEVTKTSRLIKQGLEDL  108
           .::  ::  ::  .  *  **:::::*::*   :.  *::  *::*:  .*:  :*:*.  :  :*    :* *

MpSYP13A   DKSNIANRKKPNCGEGSSTDRTRMSMTATLKKKLKELMTEFQALRQKFTDEYREVVERRV  175
MpSYP13B   DRSNLASRKKPNCGEGTSTDRTRMGLTAGLKVKLKDLMTQFVNLRQSFNDEYRQVVERRV  180
MpSYP12A   DKANAASRRTKGCEEGTPTDRTRSSITNSLTKKLKDLMESFGTLRSKIMVEYRETIERRY  167
MpSYP12B   NKSNAASREIKGCEKGSSTDRTRIQLTNSLTESLKDLMHDFGTLRTRIVGEYREIIGRSY  168
           ::::*  *.*.    .*  :*:.*****   :*   *.  .**:**  .*   **   :   ***:  : *

MpSYP13A   FTVTGQKADEGTIDQLIETGDSEQIFQKAIQEQGRGQILDTIAEIQERHDAVKDIEKKLL  235
MpSYP13B   FTVTGQKADEEMIDQLIETGNSEQIFQKAIQAQGRGQILDTIAEIQERHDSVKDIEKKLL  240
MpSYP12A   YTVTGQKPDEETLEQIIDTGESENFLQKAIQEQGRGQIIETIKEIQERHDGVKEIEKSLL  227
MpSYP12B   YTVTGQRADETTIDRMVESGESETFIQRAIQEQGKGEVIDSLRDIQEQHEAVKDIERNLQ  228
           :*****:.**   :::::::*:** ::*:*** **:*::::::  :***:*:.**:**:.*
```
SNARE domain
```
MpSYP13A   ELHQIFLDMAVLVEAQGELLDNIETQVSKAVTYVQEGTVALQTAKKLQRGTRKCMCIAII  295
MpSYP13B   ELHQIFLDMAVLVEAQGELLDNISKNVSTAQDYVARGGVALGQARKLQKGTRKCMCYAVI  300
MpSYP12A   ELHQIFLDLAVLVESQGTVLDNIESQVNRAHSYVEKAGAHLTVAKKHQRNTRKWTCIAII  287
MpSYP12B   ELNQIFVDLSVLVEAQGALVNDIQTNVERADSYTKRAAVHLQVAKTHQRKNRLWMCAGVS  288
           **:***:*::****:** ::::*..:*. * *. ... . *  *:. *: .*     * .:
```
TMD
```
MpSYP13A   LLLIIIIIIVVAVVQPWKTNKA  317
MpSYP13B   LLLIIILIIVLATVKPWQK---  319
MpSYP12A   IVLIIIILVIVVPIATSLKK---  306
MpSYP12B   VMLIAAGVVAAIIATAK-----  305
           ::**    ::.    . .
```

Figure 3-3. Sequence alignment of SYP1 proteins of *M. polymorpha*
Amino acid sequences of four MpSYP1 members are aligned. The syntaxin domain (red line), SNARE domain (purple line), and transmembrane domain (TMD, light blue line) are indicated above the sequences. Identical (asterisk), highly-conserved (colon), and weakly-conserved (period) amino acid residues are indicated.
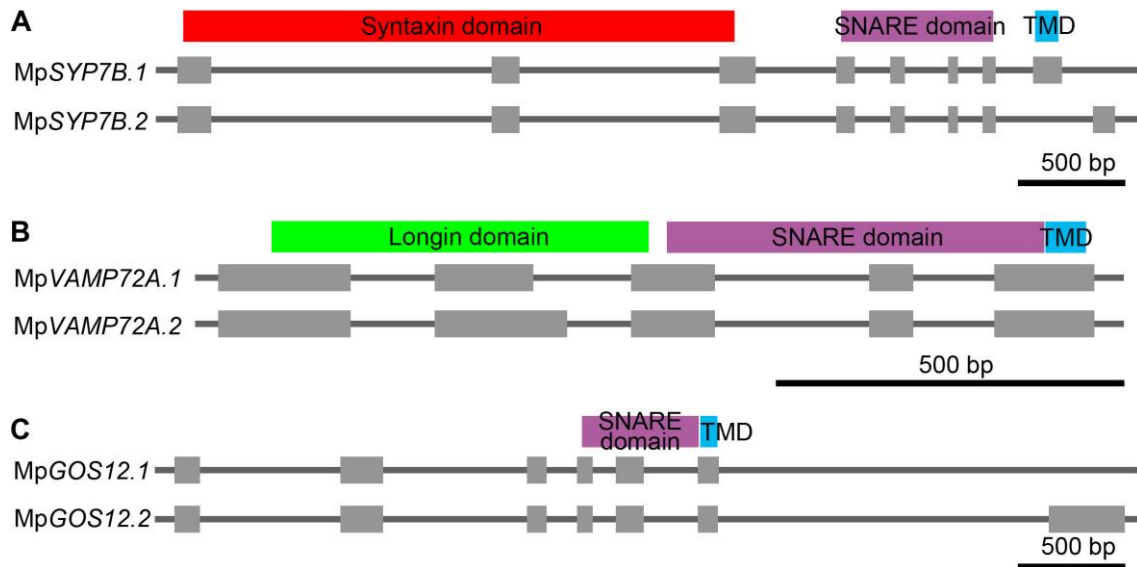
Figure 3-4. Schematic models of SNARE genes with splicing variants

(A) Two transcripts derived from the Mp*SYP7B* locus; one harbors a TMD, whereas the other lacks a TMD. (B) Two transcripts derived from the Mp*VAMP72A* locus; the lengths of the longin domain vary between the two products. (C) Two transcripts derived from the Mp*GOS12* locus; the lengths of the carboxyl termini vary between the two products. Gray boxes indicate protein-coding regions. Black bars = 500 bp.
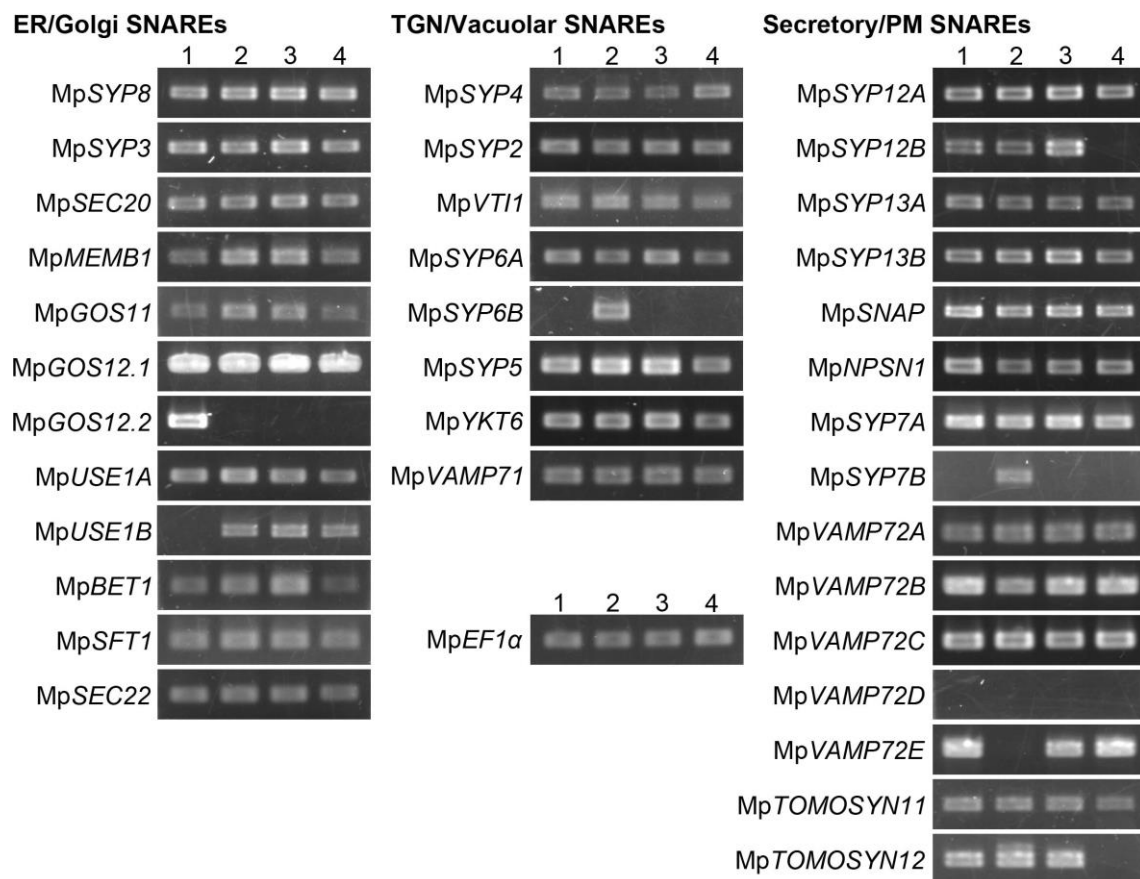
**Figure 3-5. Transcription of SNARE genes in *M. polymorpha***

Total RNA samples were extracted from 5-day-old thalli (lane 1), antheridiophores (lane 2), archegoniophores (lane 3), and 7-day-old sporelings (lane 4), which were subjected to RT-PCR. The amounts of template cDNA were adjusted based on the Mp*EF1α* expression.
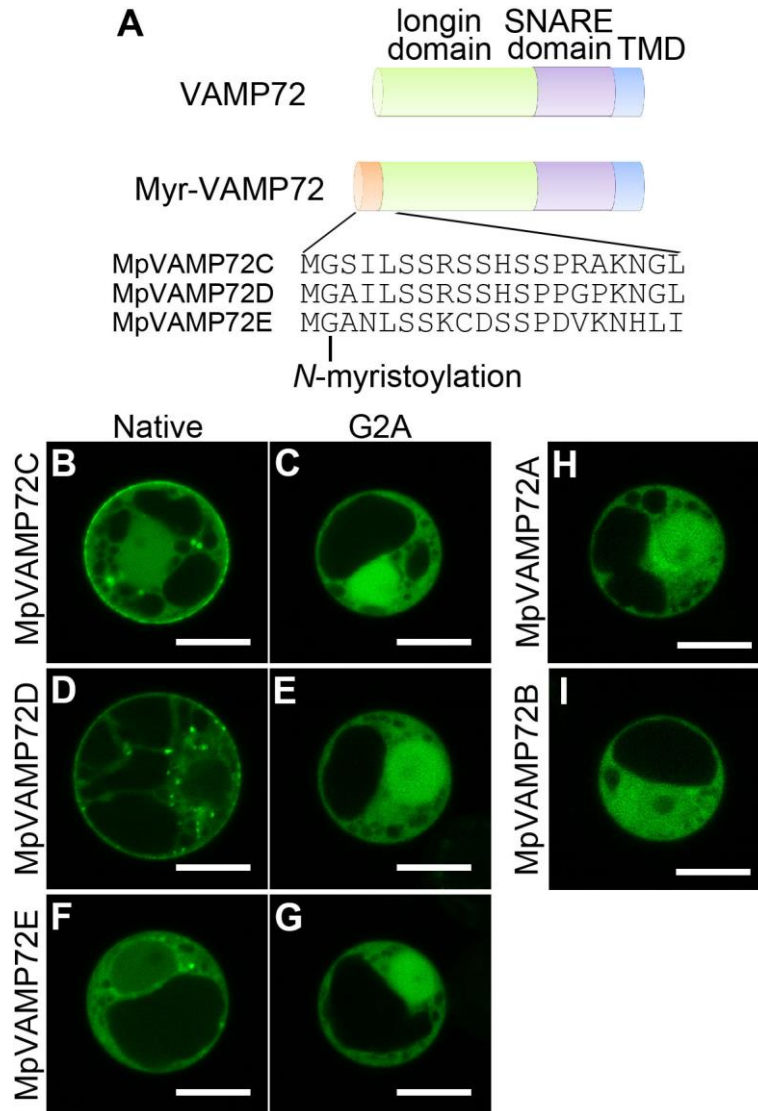
**Figure 3-6.** *M. polymorpha* **harbors unique VAMP72 members**

(A) Schematic primary structures of VAMP72 proteins in *M. polymorpha*. Canonical VAMP72 consists of the longin domain, SNARE domain, and transmembrane domain (TMD). In addition to these three domains, *N*-myristoylated VAMP72 (Myr-VAMP72) extends at the amino terminus, which contains the consensus for *N*-myristoylation. (B–I) Confocal images of protoplasts of *A. thaliana* cells cultured in suspension expressing YFP fused with the twenty N-terminal amino acid residues of the MpVAMP72 members. The N-terminal sequences of Myr-VAMP72 [MpVAMP72C (B), MpVAMP72D (D), and MpVAMP72E (F)] deliver YFP to the endomembrane compartments, although the N-terminal sequences of canonical VAMP72 [MpVAMP72A (H) and MpVAMP72B (I)] and mutated sequences of Myr-VAMP72 (G2A) [MpVAMP72C (C), MpVAMP72D (E), and MpVAMP72E (G)] do not. Scale bars = 10 μm.
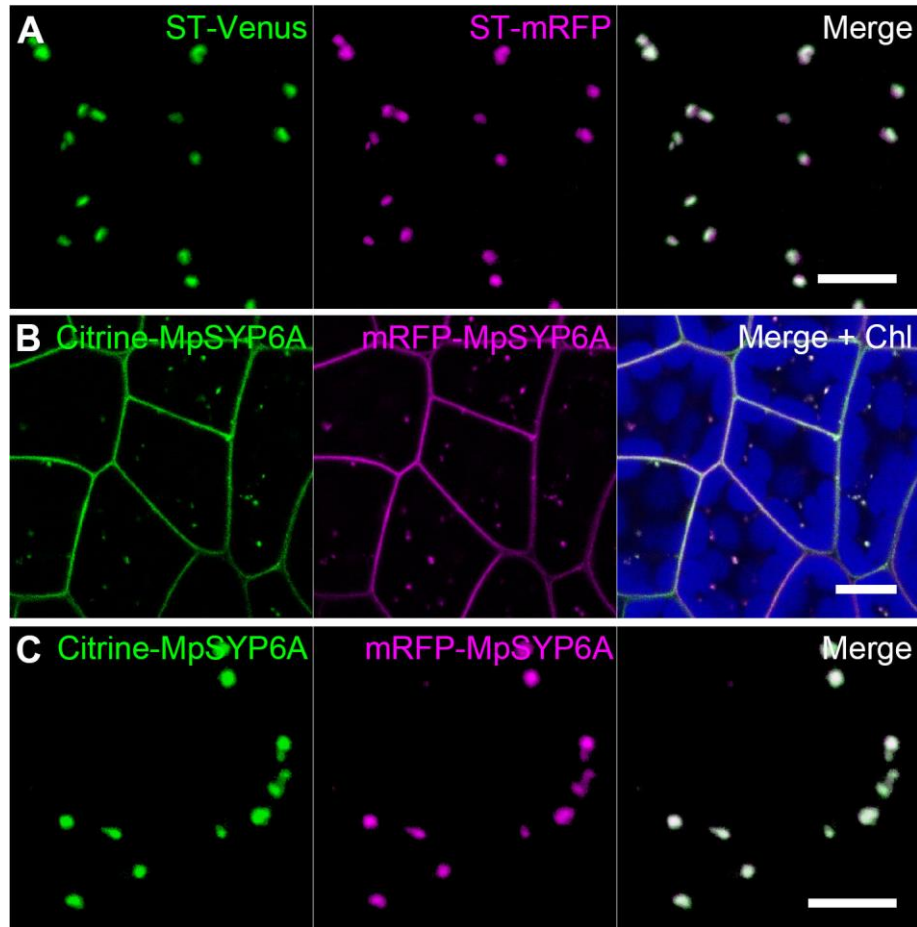
Figure 3-7. Markers of the Golgi apparatus and *trans*-Golgi network (TGN) in *M. polymorpha*
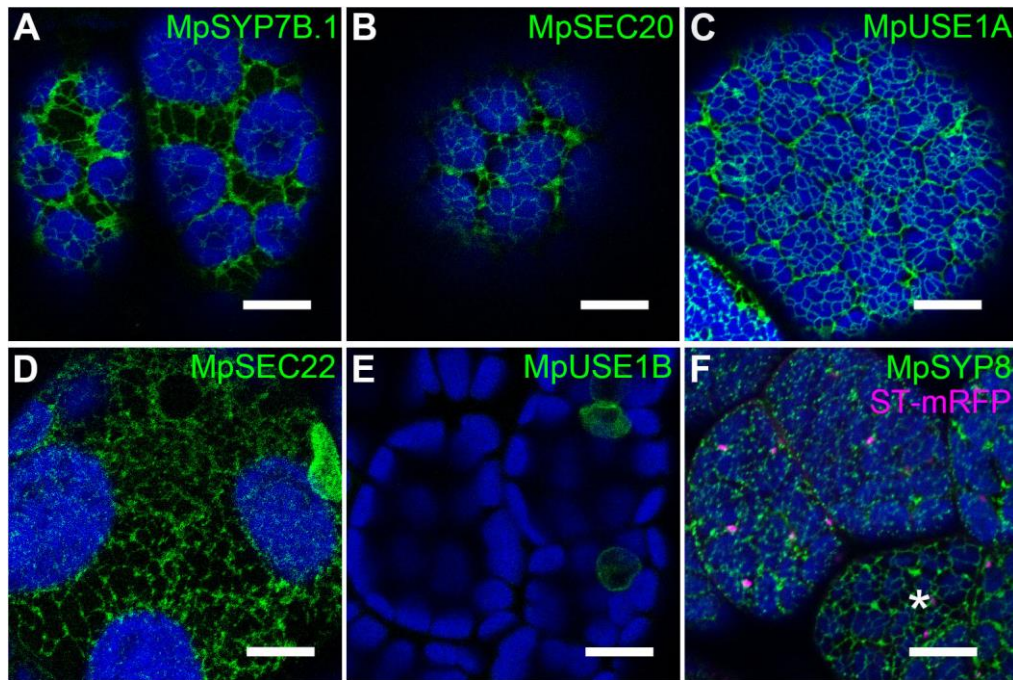
(A) Maximum intensity projection images of *M. polymorpha* thallus cells expressing ST-Venus and ST-mRFP. (B) Single confocal images of *M. polymorpha* thallus cells expressing Citrine-MpSYP6A and mRFP-MpSYP6A. (C) Maximum intensity projection images of *M. polymorpha* thallus cells expressing Citrine-MpSYP6A and mRFP-MpSYP6A.
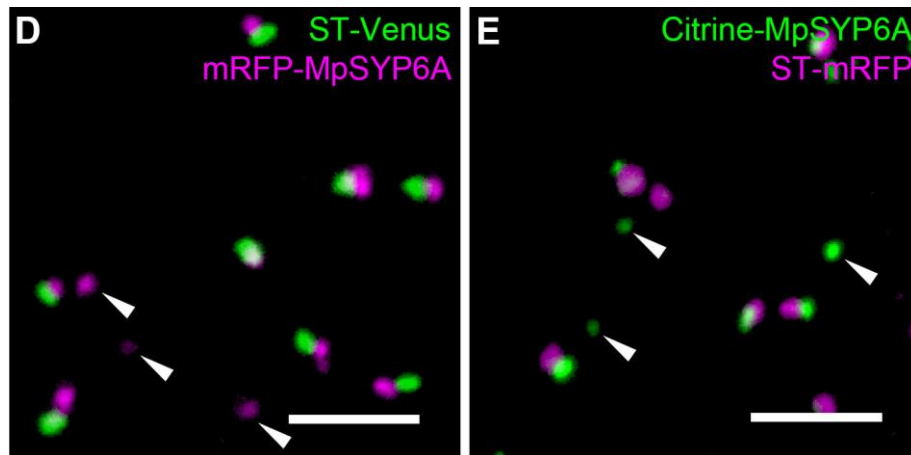
Figure 3-7. (continued)

(D) Maximum intensity projection images of *M. polymorpha* thallus cells expressing ST-Venus and mRFP-MpSYP6A. White arrowheads indicate the Golgi-independent TGN. (E) Maximum intensity projection images of *M. polymorpha* thallus cells expressing Citrine-MpSYP6A and ST-mRFP. White arrowheads indicate the Golgi-independent TGN. Green, magenta, and blue pseudo colors indicate fluorescence from YFP (Citrine or Venus), mRFP, and chlorophyll, respectively. Scale bars = 5 μm in (A), (C), (D), and (E), 10 μm in (B).

Figure 3-8. SNAREs localized to the ER

Single confocal images of *M. polymorpha* thallus cells expressing Citrine-MpSYP7B.1 (A), Citrine-MpSEC20 (B), Citrine-MpUSE1A (C), Citrine-MpSEC22 (D), Citrine-MpUSE1B (E), or Citrine-MpSYP8 and ST-mRFP (F). Green, magenta, and blue pseudo colors indicate fluorescence from Citrine, mRFP, and chlorophyll, respectively. Scale bars = 10 μm.
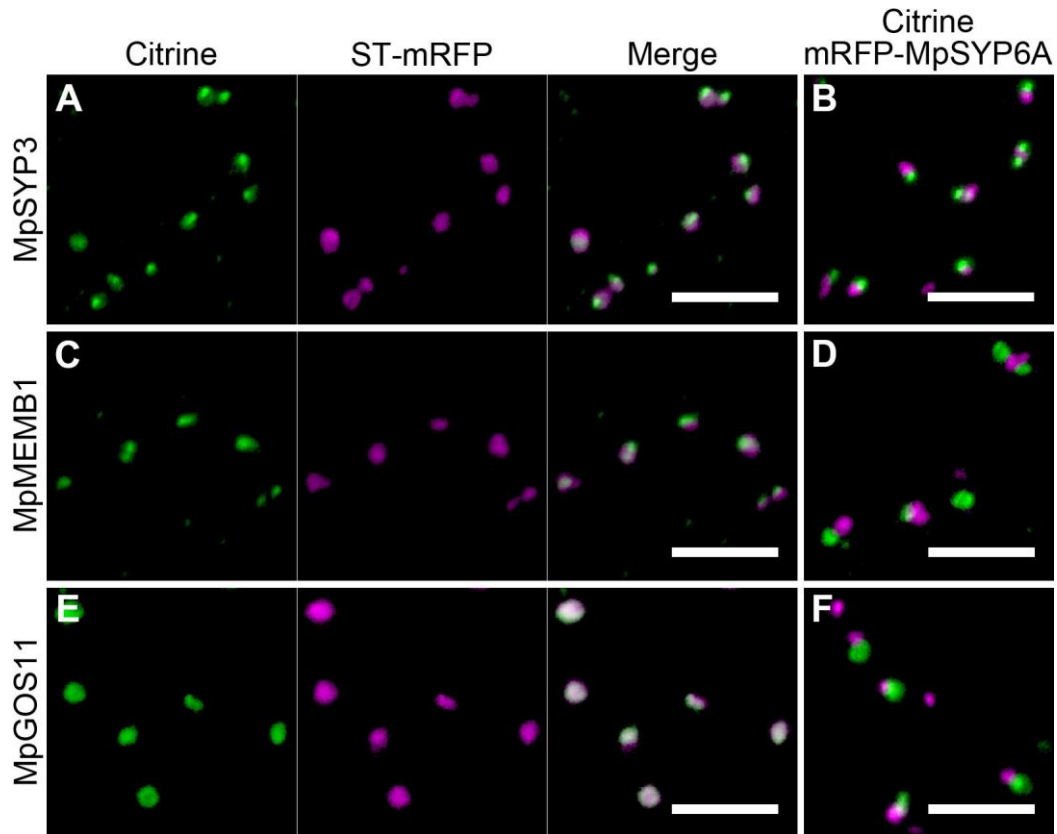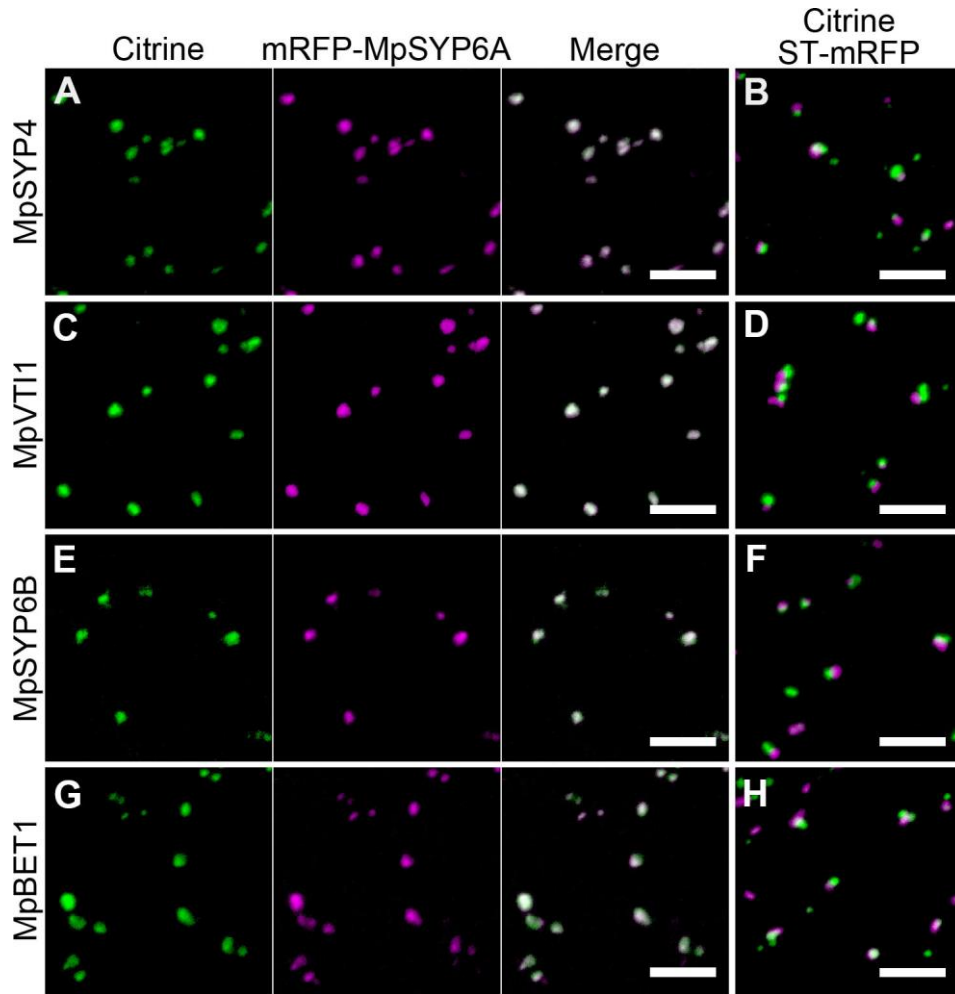
Figure 3-9. Golgi apparatus-localized SNAREs

Colocalization of Golgi-localized SNAREs with the marker for the Golgi apparatus (ST-mRFP) or the TGN (mRFP-MpSYP6A). (A, C, E, G, I, K, and M) Maximum intensity projection images of *M. polymorpha* thallus cells expressing ST-mRFP and Citrine-MpSYP3 (A), Citrine-MpMEMB1 (C), Citrine-MpGOS11 (E), Citrine-MpGOS12.1 (G), Citrine-MpGOS12.2 (I), Citrine-MpSFT1 (K), or Citrine-MpTOMOSYN11 (M). (B, D, F, H, J, L, and N) Maximum intensity projection images of *M. polymorpha* thallus cells expressing mRFP-MpSYP6A and Citrine-MpSYP3 (B), Citrine-MpMEMB1 (D), Citrine-MpGOS11 (F), Citrine-MpGOS12.1 (H), Citrine-MpGOS12.2 (J), Citrine-MpSFT1 (L), or Citrine-MpTOMOSYN11 (N). The green and magenta pseudo colors indicate fluorescence from Citrine and mRFP, respectively. Scale bars = 5 μm.
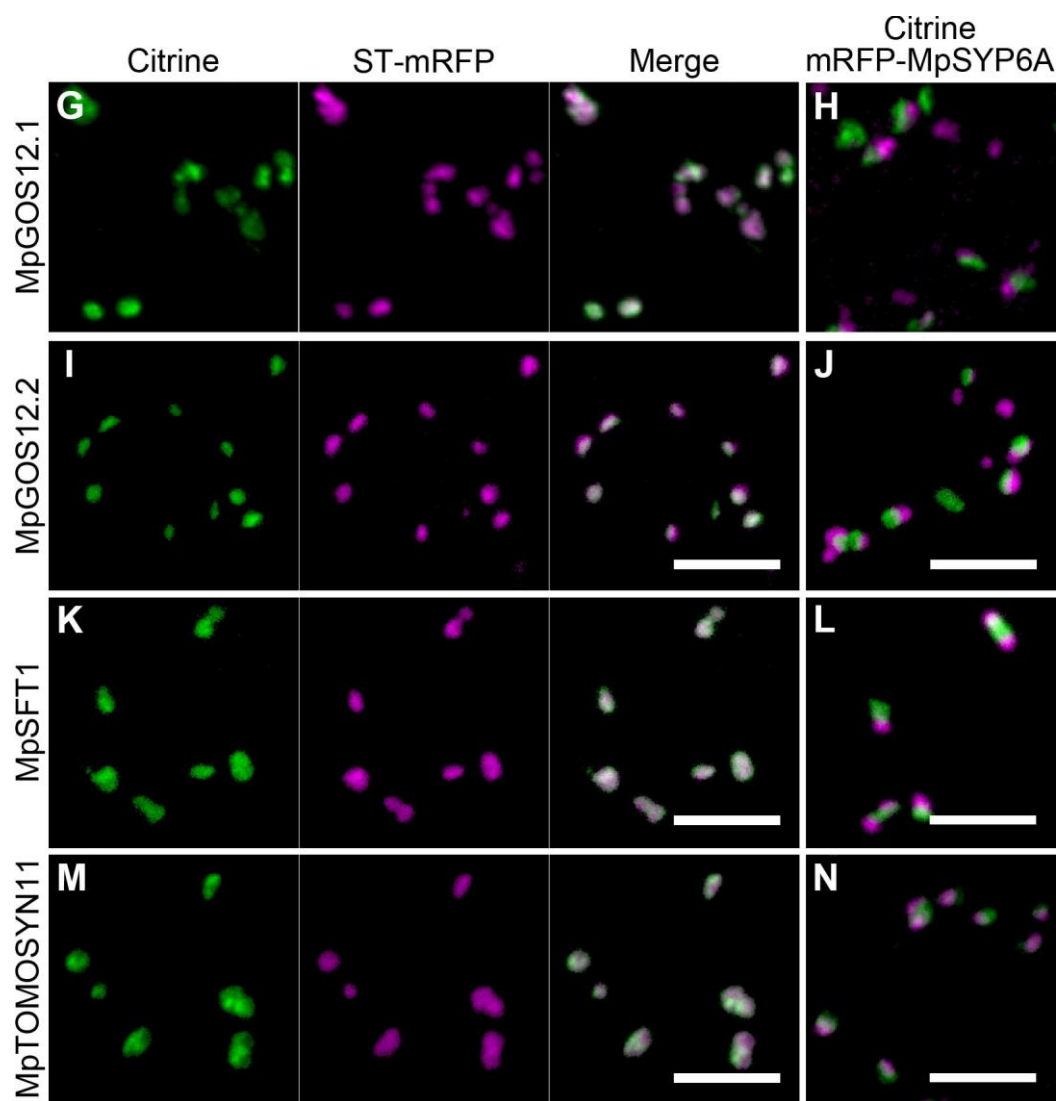
Figure 3-9. (continued)

Figure 3-10. TGN-localized SNAREs

Colocalization of TGN-localized SNAREs with the marker for the TGN (mRFP-MpSYP6A) or the Golgi apparatus (ST-mRFP). (A, C, E, G) Maximum intensity projection images of *M. polymorpha* thallus cells expressing mRFP-MpSYP6A and Citrine-MpSYP4 (A), Citrine-MpVTI1 (C), Citrine-MpSYP6B (E), or Citrine-MpBET1 (G). (B, D, F, H) Maximum intensity projection images of *M. polymorpha* thallus cells expressing ST-mRFP and Citrine-MpSYP4 (B), Citrine-MpVTI1 (D), Citrine-MpSYP6B (F), or Citrine-MpBET1 (H). The green and magenta pseudo colors indicate fluorescence from Citrine and mRFP, respectively. Scale bars = 5 μm.
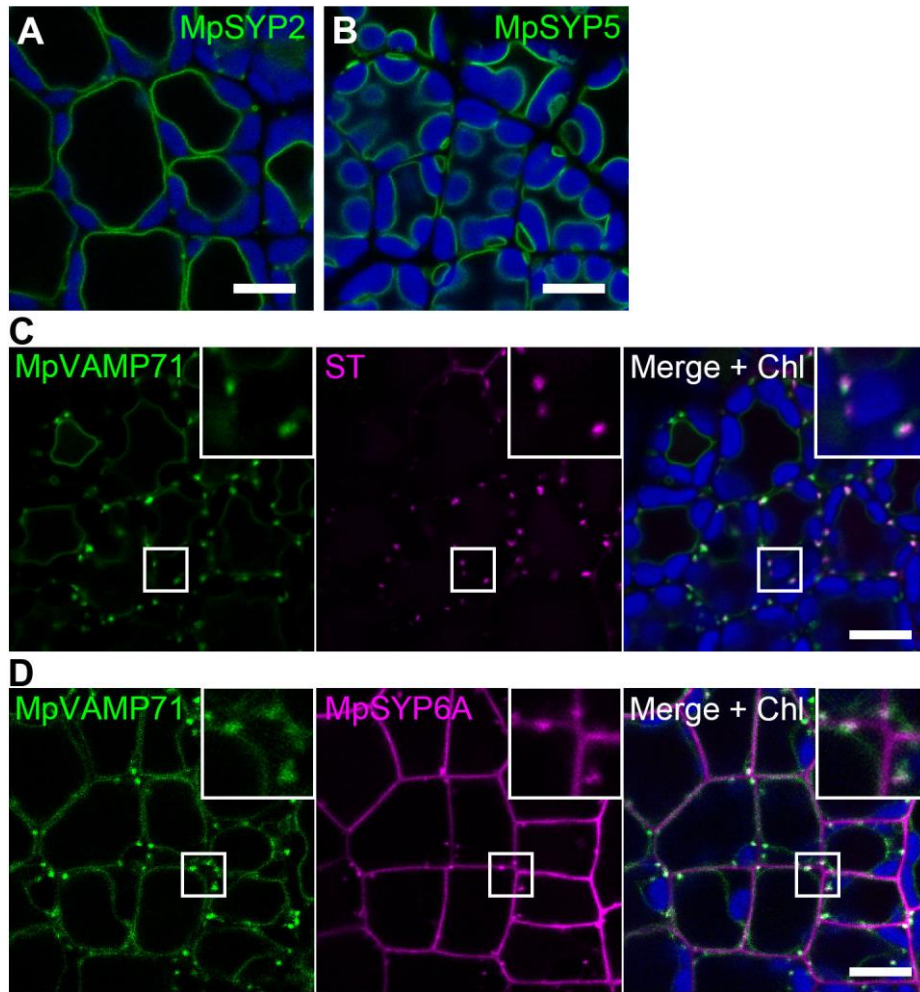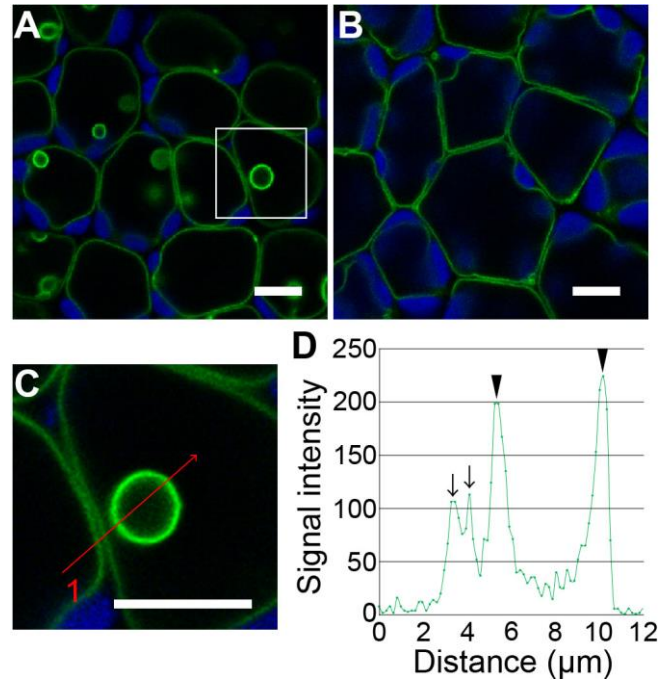
Figure 3-11. Vacuolar membrane-localized SNAREs

(A and B) Single confocal images of *M. polymorpha* thallus cells expressing Citrine-MpSYP2 (A) or Citrine-MpSYP5 (B) under the regulation of the CaMV 35S promoter. (C and D) Single confocal images of *M. polymorpha* thallus cells expressing Citrine-MpVAMP71 and ST-mRFP (C) or mRFP-MPSYP6A (D). The insets are magnified images of the boxed regions in (C) and (D). Green, magenta, and blue pseudo colors indicate fluorescence from Citrine, mRFP, and chlorophyll, respectively. Scale bars = 10 µm.

Figure 3-12. Potential effect of dimerization and overexpression on the localization of fluorescently tagged MpSYP2

(A) A single confocal image of *M. polymorpha* thallus cells expressing MpSYP2 tagged with Citrine under the regulation of the CaMV 35S promoter. (B) A single confocal image of *M. polymorpha* thallus cells expressing MpSYP2 tagged with mCitrine under the regulation of the Mp*SYP2* promoter. (C) Magnified image of the boxed region in B. (D) Fluorescence intensity of Citrine along the red arrow (1) in (C). Arrows indicate vacuolar membranes, and arrowheads indicate the membrane of the bulb. Scale bars = 10 μm.

Figure 3-13 PM- and oil body membrane-localized SNAREs

(A–C) Single confocal images of *M. polymorpha* thallus cells expressing mCitrine-MpSYP12A (A), mCitrine-MpSYP13A (B), or mCitrine-MpSYP13B (C) under the regulation of their own promoters. (D–G) Single confocal images of *M. polymorpha* thallus cells expressing mCitrine-MpSYP13A (D), mCitrine-MpSYP12B (E), mCitrine-MpSYP12A (F), or MpSYP13B (G) driven by their own promoters. BF, bright field images.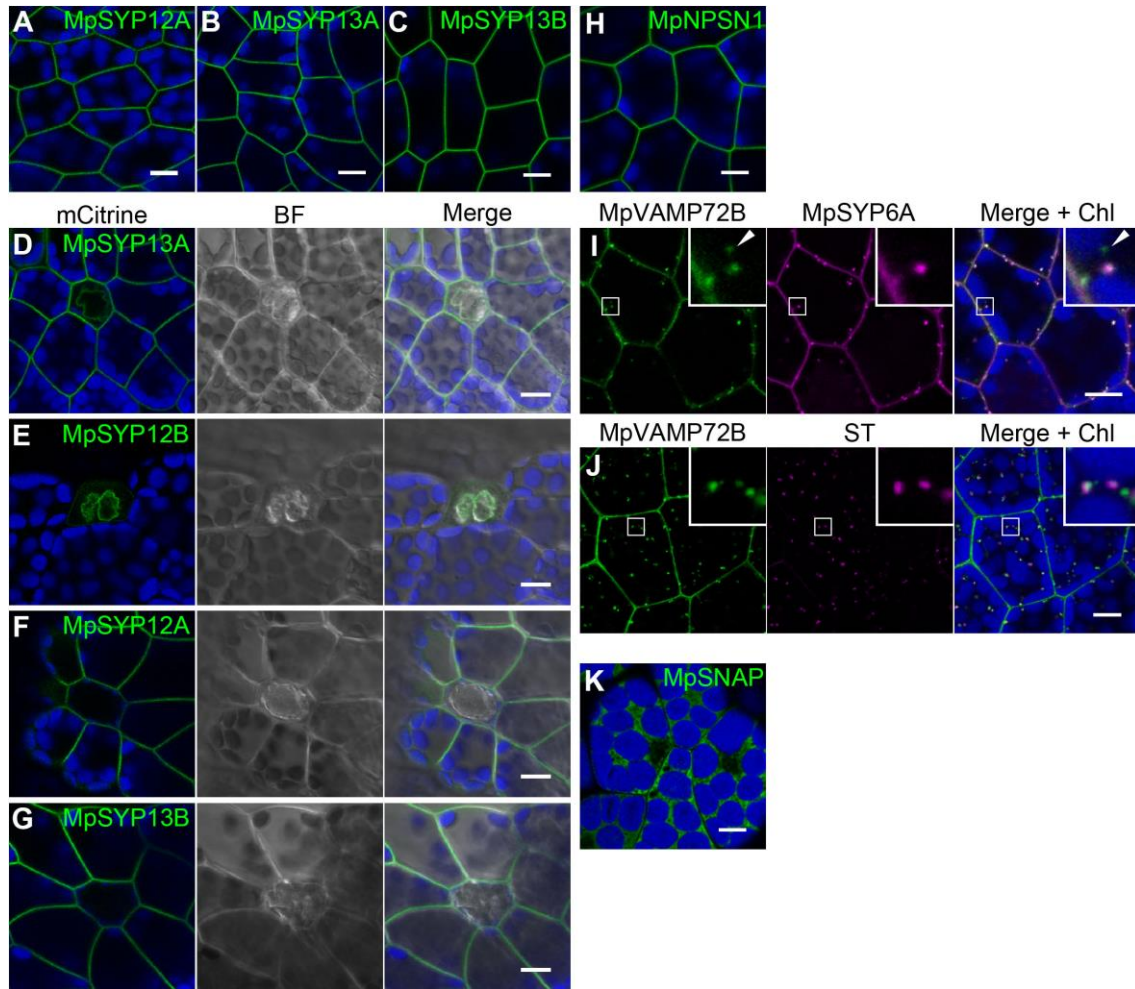 (H) A single confocal image of *M. polymorpha* thallus cells expressing Citrine-MpNPSN1 under the regulation of the CaMV 35S promoter. (I and J) Single confocal images of *M. polymorpha* thallus cells expressing Citrine-MpVAMP72B and mRFP-MpSYP6A (I), or Citrine-MpVAMP72B and ST-mRFP, under the regulation of the CaMV 35S promoter (J). The insets are magnified images of the boxed regions in (I) and (J). (K) A single confocal image of *M. polymorpha* thallus cells expressing Citrine-MpSNAP under the regulation of the CaMV 35S promoter. Green, magenta, and blue pseudo colors indicate fluorescence from (m)Citrine, mRFP, and chlorophyll, respectively. Scale bars = 10 μm.

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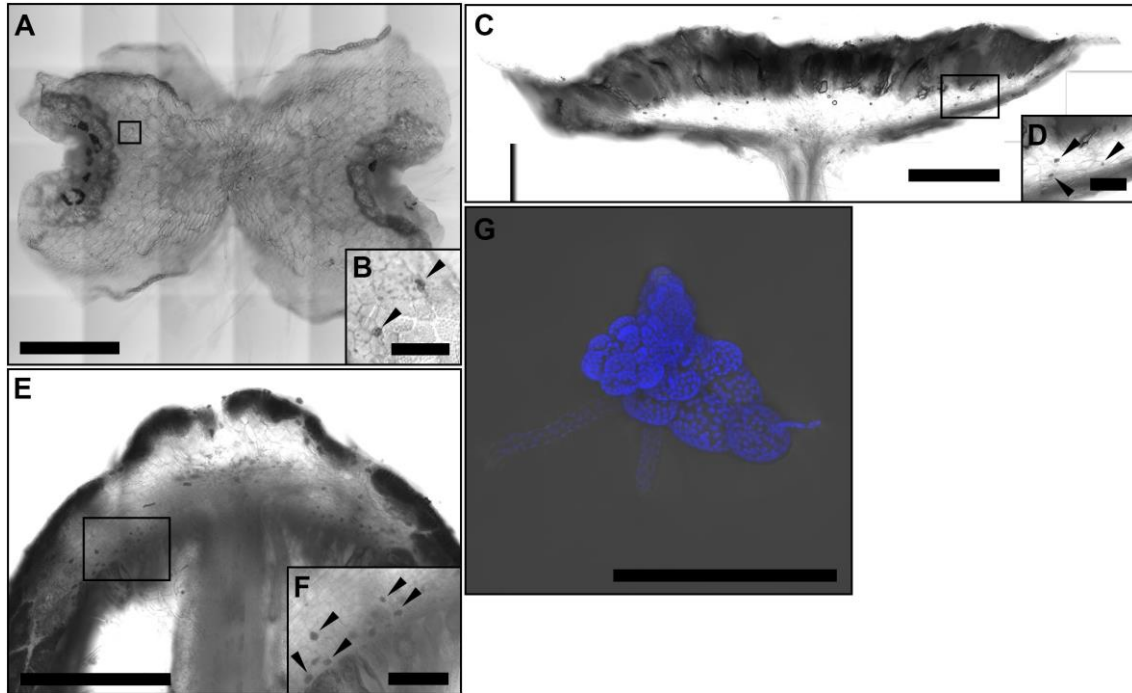
Figure 3-14 Distribution of oil body cells in *M. polymorpha*

(A–F) Bright field (BF) images of a 5-day-old thallus (A and B), an antheridiophore (C and D), and an archegoniophore (E and F). Magnified images of the boxed regions in (A), (C), and (E) are presented as (B), (D), and (F), respectively. Arrowheads indicate oil bodies. (G) A maximum intensity projection image of a 7-day-old sporeling overlaid on the BF image. The blue pseudo color indicates autofluorescence from chlorophyll. Scale bars = 1 mm in (A), (C), and (E), 100 μm in (B), and 200 μm in (D), (F), and (G).
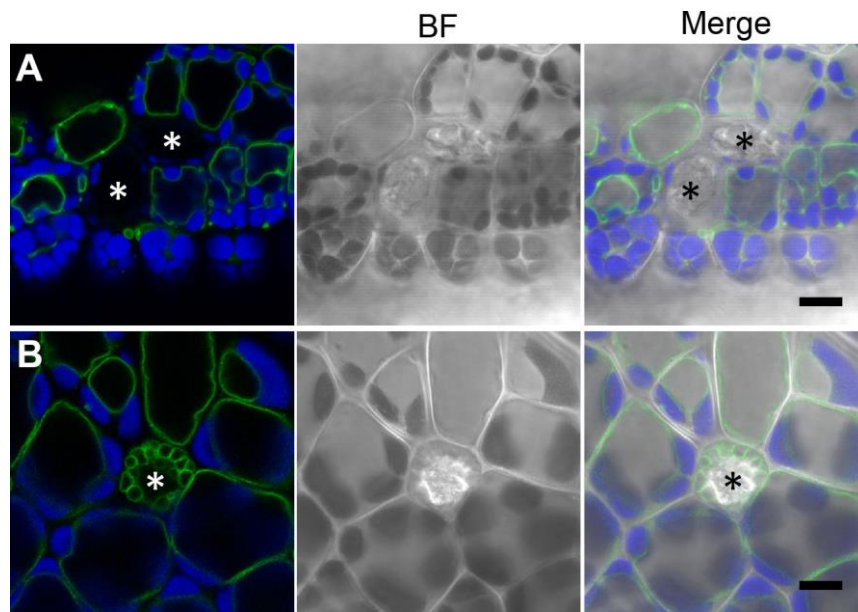
Figure 3-15 Inactivation of the CaMV 35S promoter in oil body cells.

(A) Single confocal images of *M. polymorpha* thallus cells expressing Citrine-MpSYP2 under the regulation of the CaMV 35S promoter. The Citrine signal is not visible in oil body cells (asterisks). (B) Single confocal images of *M. polymorpha* thallus cells expressing mCitrine-MpSYP2 under the regulation of the Mp*SYP2* promoter. The vacuolar membrane in an oil body cell (asterisk) is visualized using mCitrine. Scale bars = 10 μm.
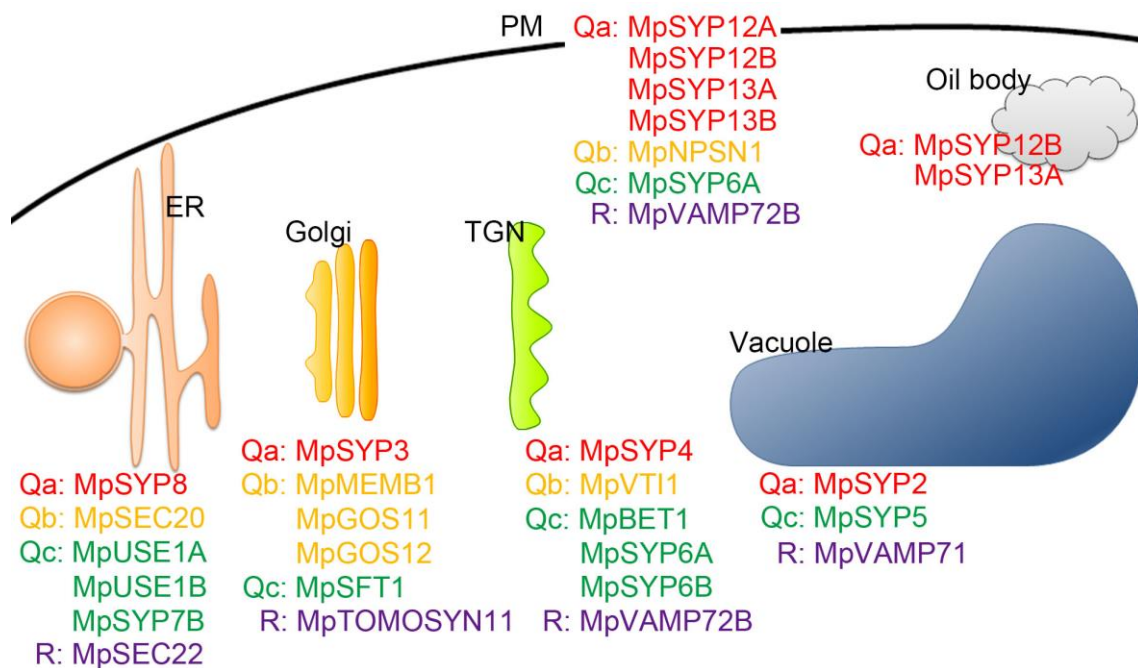
Figure 3-16 Schematic representation of the SNARE distribution in _M. polymorpha_ Subcellular localization of SNARE proteins determined under my experimental conditions.

Chapter IV: **Cell-specific redirection of the secretory trafficking pathway lead to acquisition of new organelles during land plant evolution**

第４章
本章については、５年以内に雑誌等で刊行予定のため、非公開。

Chapter V: **Screening for mutants of oil body biogenesis and morphogenesis**

第５章
本章については、５年以内に雑誌等で刊行予定のため、非公開。

Chapter VI: **Conclusion**

第 6 章
本章については、５年以内に雑誌等で刊行予定のため、非公開。

The main part of Chapter II and III has been published on Plant and Cell Physiology (Oxford University Press) as an article entitled "SNARE Molecules in *Marchantia polymorpha*: Unique and Conserved Features of the Membrane Fusion Machinery." by T. Kanazawa, A. Era, N. Minamino, Y. Shikano, M. Fujimoto, T. Uemura, R. Nishihama, K.T. Yamato, K. Ishizaki, T. Nishiyama, T. Kohchi, A. Nakano, and T. Ueda (2016, volume 57, Issue 2, pages 307-324).

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