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Biogenesis and function in plant cells

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Chapter 1

General introduction

Lysosomes, vacuoles and additional vacuoles: different organelles or just more of the same?

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Abbreviations

AAA ATPase: ATPase associated with various cellular activities

ABA: abscisic acid

ALIX: apoptosis-linked gene-2 interacting protein X

ALP: alkaline phosphatase

AMSH: associated molecule with the SH3-domain of STAM

APA1: aspartic protease A1

APs: adapter proteins

ARL8: ADP-ribosylation factor-like protein 8B

ASM: acid sphingomyelinase

CAP2: calcium-dependent protein kinase 1 adaptor protein 2

CCVs: clathrin-coated vesicles

CCZ1: calcium caffeine zinc sensitivity1

CHMP: charged multivesicular body protein

CLEAR: coordinated lysosomal expression and regulation

COPs: coat proteins

CORVET: class C core vacuole/endosome tethering

CV: central vacuole

Doa4: degradation of alpha-4

DUB: deubiquitinating enzymes

DVs: dense vesicles

EEA1: early endosome antigen 1

EEs/LEs: early/late endosomes

ER: endoplasmic reticulum,

ESCRT: endosomal sorting complex required for transport

FREE: FYVE domain protein required for endosomal sorting

FYVE: Fab1, YOTB, Vac1 and EEA1 domains containing protein

GC: β -glucocerebrosidase

GEF: guanine-nucleotide exchange factors

GGA: Golgi localized, γ -ear-containing Arf-binding family proteins

GM2AP: GM2 activator protein

HOPS: homotypic fusion and protein sorting,

HRS: hepatocyte growth factor-regulated Tyr kinase substrate

IGF2R: Insulin-like growth factor 2 receptor

ILVs: intraluminal vesicle

IST1: increased salt tolerance1

KCO: Potassium channel opener

LAMP: lysosomal-associated membrane protein

LECA: last eukaryotic common ancestor

LIMP: lysosomal integral membrane protein

LROs: lysosome-related organelles

Lrp1: LDL receptor-related protein 1

LV: lytic vacuole

MITF: Microphthalmia-associated basic helix-loop-helix (bHLH) leucine zipper transcription factors

Mon1: monensin sensitivity1

MVB12: MVB sorting factor 12

MVBs: multivesicular bodies

NE: nuclear envelope

NHX5/NHX6: sodium-proton exchanger 5/6

PAC: precursor-accumulating vesicles

PAT10: protein S-ACYL transferase10

PBII: protein body II

PI(3)P: Phosphatidylinositol 3-phosphate

PI3P5Ks: phosphatidylinositol 3-phosphate 5-kinase

PLEKHM1: pleckstrin homology domain-containing family M member 1

PM: plasma membrane

PROS: positive regulator of SKD1

PrP: prion protein

PSV: protein storage vacuole

PVCs: prevacuolar compartment

RILP: RAB interacting lysosomal protein

RMR: transmembrane-RING H2 motif receptor protein

SAND-1: Sp100, AIRE-1, NucP41/75, DEAF-1 domain containing protein

SAPs: sphingolipid activator proteins

SCU4: sucrose transporter 4

SKD1: suppressor of K1 transport growth defect 1

SKIP: SifA and kinesin-interacting protein

SNARE: soluble N-ethylmaleimide-sensitive factor activating protein receptor

SNF: sucrose nonfermenting proteins

STAM: signal transducing adaptor molecule

STAM: signal transducing adaptor molecule

SYP22: syntaxin-22

TFE3: transcription factor binding to IGHM enhancer 3

TFEB: transcription factor EB

TGN: trans-Golgi network

TIPs: tonoplast intrinsic proteins

TOL: TOM like

TOM1: target of Myb 1

TSG101: tumor susceptibility gene-101

VAMP: vesicle associated membrane proteins

VCL1: vacuoleless1

VHA-a3: V-type proton ATPase subunit a3

VPE: vacuolar processing enzyme

VPS: vacuolar protein sorting-associated protein

VSR: vacuolar sorting receptor

VTI12: VPS 10 interacting 12 protein

Introduction

It is well known that vacuoles and lysosomes play a crucial role in cells of very diverse organisms as key components of the endomembrane system. The physiological function and biogenesis of these endomembrane compartments have been extensively investigated since the discovery of vacuoles in plants by Antonie van Leeuwenhoek in 1676 and of lysosomes in liver cells by Christian de Duve in 1976¹. Lysosomes and vacuoles share lots of common ground considering the similarities in their biological function and their crucial role in endocytic and secretory pathways. However they are also clearly different from each other in dimension and numbers in a single cell¹.

The long standing consensus that a single plant cell possesses one single vacuole with multiple functions² was overthrown some 20 years ago by the identification of protein storage vacuoles (PSVs) coexisting with the lytic vacuole (LV) in the same cells. The different identity of these compartments is illustrated by different tonoplast intrinsic proteins (TIPs) found in the membranes of the two types of vacuole. These observations generated the idea that functionally distinct vacuoles can co-exist in a single cell³⁻⁵ and resulted in the study/search of additional vacuoles in other plant cell types, bringing to their identification in a number of different cell types. These included secondary vacuoles in barley aleurone protoplasts⁶; distinct vacuoles in mesophyll cells of *Mesembryanthemum crystallinum* induced by salt stress⁷; senescence-associated vacuoles in leaf mesophyll and vacuoles involved in the contraction of guard cells in *Arabidopsis* and soybean^{8, 9}. In spite of the increasing evidence that multiple vacuoles can co-exist within a single cell, the number of cell types in which they are described remains limited opening the debate on whether they are widespread in the plant kingdom or limited to a few cell types cells fulfilling some specific functions¹⁰. Recently it was found that cells in the petal epidermis of petunia and rose flowers possess besides the large central vacuole (CV)

containing pigments (anthocyanins), numerous small vacuoles, called vacuolinos⁹. The presence of multiple, functionally distinct vacuoles in a single plant cell raises numerous questions regarding, for example, the mechanisms by which they are generated and how they “differentiate” with regard to their content and biological functions.

Similarly, in animal cells, lysosome-related organelles (LROs), in particular melanosomes¹¹ and platelet dense granules¹², are described as specialized lysosome types. These organelles are an example of additional lysosomes coexisting with conventional lysosomes and add to the idea that diverse vacuoles/lysosomes can co-exist in one single cell¹³.

Although much effort has been devoted to unravel the mechanisms underlying the biogenesis and maturation of lysosomes/vacuoles, our understanding on these processes remains limited. In this review, we discuss the similarity and diversity of the protein sorting pathways involving lysosomes and vacuoles, the evolution of lysosome/vacuole and additional vacuoles (like vacuolinos). Based on conserved components of the sorting pathway reported in literature, we propose scenarios how lysosomes/vacuoles and vacuolinos may have acquired their unique functions during evolution and we try to understand the evolutionary relationship among these compartments.

Overview on the endomembrane trafficking system

The endomembrane trafficking system consist of multiple subcellular organelles, including the plasma membrane (PM), nuclear envelope (NE), endoplasmic reticulum (ER), Golgi apparatus, trans-Golgi network (TGN), early/late endosomes (EEs/LEs), prevacuolar compartments/multivesicular bodies (PVCs/MVBs), autophagosomes and lysosomes/vacuoles, is driven by the coordinated action of evolutionarily conserved regulators such as Ras small GTPase, the class C core vacuole/endosome tethering complex (CORVET) and the homotypic fusion and

protein sorting tether complex (HOPS), soluble N-ethylmaleimide-sensitive factor activating protein receptors (SNAREs), vesicle coat proteins (COPs), adapter proteins (APs), and endosomal sorting complex required for transport (ESCRT) machinery.

Many proteins are synthesized initially at the rough ER interface and subsequently delivered by COPII-dependent or -independent vesicle trafficking to the Golgi apparatus for further post-translational modification. From TGN or TGN/EEs secretory proteins are sequestered into secretory vesicles and delivered to PM, whereas lysosomal/vacuolar cargos are sorted into clathrin-coated vesicle (CCVs) by a receptor-dependent or -independent manner to reach lysosomes/vacuoles via the intermediate compartment LEs/PVCs/MVBs, described as the canonical protein sorting pathway. In addition to newly synthesized proteins, proteins for degradation in degradative lysosome/vacuole are recognized and sorted into intraluminal vesicle (ILVs) of MVBs, mainly under control of ESCRT machinery, to merge into post-Golgi trafficking pathway.

Post-Golgi trafficking is a common route in multiple protein trafficking pathways and has been well documented, which is well known as canonical protein sorting pathway. In general, multiple members of RAB GTPase that are activated by its guanine-nucleotide exchange factors (GEFs) play role in recruiting downstream effector proteins and tether complexes on the membrane of EEs/TGN, PVCs/MVBs/LEs and vacuole/lysosome, which are then associated with the SNAREs to mediate the fusion event between different compartments, and subsequently accomplish the delivery of vacuolar/lysosomal proteins to their destination organelle. Although the core regulators of canonical protein sorting pathway are deeply conserved (from plants, to fungi and animals), unique diversifications in mechanism of PVCs/MVBs/LEs maturation and their fusion with lysosome/vacuole arose during the evolution, which are discussed in detail in the

following sections. All these suggesting that canonical protein sorting pathway may not be the only sorting mechanism, lead to the uncovering of other Golgi-dependent protein trafficking pathways, including RAB5-dependent RAB7-independent and AP-3-dependent pathway.

As a shortcut for lysosomal/vacuolar proteins to reach lysosome/vacuole by comparing with Golgi-dependent protein trafficking pathway, the Golgi-independent pathway, also known as the direct ER-to-Vacuole trafficking pathway, have enriched our knowledge on the diversity and complexity of endomembrane traffic systems.

Sorting of proteins to the lysosomes

Lysosomes are dynamic membrane-bound organelles with multiple functions. In particular lysosomes are the place of degradation, by acid-dependent hydrolases, of macromolecules delivered by the secretory, endocytic, autophagic and phagocytic trafficking pathways¹⁴. In recent years, evidence accumulated^{15, 16} which indicated additional roles for lysosomes in nutrient sensing, transcriptional regulation, and metabolic homeostasis. To perform these diverse biological functions, certain proteins or cargos are delivered to lysosomes by conventional or specific diversified pathways taking advantage of the flexibility of the endomembrane trafficking system. We report here a short description of the known pathways contributing to the final protein set of the lysosome.

Mannose-6-phosphate (M6P) dependent pathway

The M6P dependent pathway is the best characterized pathway for the sorting of newly synthesized acid hydrolases to lysosomes. When passing through the *cis*-Golgi, newly synthesized acid hydrolases are modified with mannose-6-phosphate moiety¹⁷, which is subsequently recognized in the *trans*-Golgi network (TGN) by the mannose 6-phosphate receptors (MPRs), which are type I transmembrane

glycoproteins belonging to the P-type lectin family¹⁸. The MPR bound hydrolases are with the help of the Golgi localized, γ -ear-containing Arf-binding family proteins (GGA) and in sequential adaptor protein 1 (AP-1)¹⁹ sorted into clathrin-coated vesicles(CCVs)²⁰⁻²², which bud from TGN²³ and subsequently fuse with endosomes to merge into the post-Golgi endosomal/lysosomal trafficking pathway.

Multiple regulators have been identified as the major players of post-Golgi trafficking including small GTPase RAB5 and RAB7, GEFs of RAB5 and RAB7, the tether complexes CORVET(comprising the core subunits VPS11, VPS18, VPS16, VPS33, and the CORVET-specific subunits VPS3 and VPS8) and HOPS (comprising core subunits VPS11, VPS18, VPS16, VPS33, and the HOPS-specific subunits VPS39 and VPS41)²⁴ and SNARE proteins. The membrane bound portion of RAB5 mainly localizes to EEs where it is activated by a complex of Rabex5, a homolog of yeast VPS9 (ref^{25,26}), and Rabaptin, which is also an effector of RAB5 (ref²⁵). Activated (GTP-bound) RAB5 interacts with multiple effectors including early endosome antigen 1 (EEA1)²⁷⁻²⁹ and the CORVET tether complex³⁰, which mediate the homotypic fusion of EEs. Maturation of EEs towards LEs requires the sequential activates known as RAB5-to-RAB7 conversion³¹. SAND-1/Mon1, in coordination with Ccz1 forming a complex to serve as the GEF of RAB7, has been identified as the crucial switch for the RAB conversion³²⁻³⁴. By interacting with RAB5-GTP, SAND-1/Mon1-Ccz1 complex displaces Rabex5 from the membrane of EEs, in the meanwhile promotes the recruitment of RAB7 and its tethering effector HOPS complex^{34,35}, which coordinates with ESCRT machinery resulting in the maturation of endosomes to form MVBs/LEs³⁶. After the maturation of endosomes, RAB7, HOPS, and SNAREs take over the control of fusion of MVBs/LEs with lysosomes³⁷. Unlike in yeast, HOPS recruits on the MVBs/LEs by the direct interaction of Vps41 and Vps39 subunits with GTP-bound activated RAB7 (ref^{38,39}), in mammalian instead, RAB7 might binds HOPS complex in an indirect way with the help of PLEKHM1/SKIP and RILP, which have been proved

as RAB7-binding proteins and possess multiple binding sites for HOPS⁴⁰⁻⁴³. Besides a small GTPase Arl8b, instead of RAB7, has been identified as a key player to recruit human VPS41 and subsequently assembling of the HOPS complex on the lysosomal membrane⁴⁴. A trans-SNAREs complex comprised with Vti1b, syntaxin 6, VAMP7 and VAMP8 have been identified as a key player for the heterotypic fusion of MVBs/LEs with lysosomes⁴⁵.

M6P-independent pathway

Next to the M6P-dependent pathway characterized by the function of MPRs for the Golgi-to-lysosome trafficking of lysosomal hydrolases, there is a M6P-independent pathway, instead of relying on mannose 6-phosphate receptor, engaging two different receptors, namely the lysosomal integral membrane protein (LIMP-2)⁴⁶ and the VPS10P-domain receptor, sortilin^{47, 48}. This pathway delivers multiple types of lysosomal proteins, including β -glucocerebrosidase (GC)^{49, 50}, the sphingolipid activator proteins (SAPs), the GM2 activator protein (GM2AP)⁵¹, acid sphingomyelinase (ASM)⁵² and cathepsins D and H (ref⁵³). The M6P-independent pathway has been well-reviewed by MF Coutinho *et al.* in 2012 (ref⁵⁴). Growing evidence has put in doubt whether LIMP-2 is indeed a receptor in the M6P-independent trafficking of GC, because an M6P moiety in LIMP-2 protein been identified based on the crystal structure study of LIMP-2(ref⁵⁵). Further analysis confirmed a weak M6P- and glycosylation-dependent interaction between the soluble LIMP-2 and CI-MPR domain⁵⁵. However, Blanz *et al.* (ref⁵⁰) recently claimed that in mouse embryonic fibroblasts with impairments in either MPRs or the M6P-forming N-acetylglucosamine (GlcNAc)-1-phosphotransferase, LIMP-2 still localizes to lysosomes. Interestingly, the role of sortilin as a cargo receptor in the M6P-independent pathway for the sorting of non-phosphorylated cathepsin D and cathepsin B to lysosome has been challenged as well. Markmann *et al.*(ref⁵⁶) found that instead of sortilin, a LDL-receptor and LDL receptor-related protein 1 (Lrp1)

are involved in the targeting of these enzymes to lysosomes in an M6P- and sortilin-independent pathway, suggesting a large complexity of cargo receptors are involved in M6P-independent pathway. These findings indicate that our understanding of the machinery mediating the M6P-independent pathway is still incomplete, and requires more work to extend our knowledge in this area.

AP-3 dependent pathway

The adaptor protein-3 (AP-3) dependent trafficking pathway was described as the direct route that delivers, amongst others, alkaline phosphatase (APL) and the syntaxin-like protein Vam3p from the TGN to the vacuole by AP-3 clathrin coated vesicles in yeast⁵⁷⁻⁵⁹. The AP-3 clathrin coated vesicles are formed at TGN mediating by the TGN localized AP-3 adapter protein. In animal cells, the sorting pathway driven by the AP-3 adaptor complex is less characterized, where it is unclear whether AP-3-containing vesicles are formed at the TGN or in early endosomes (EE), and whether AP-3 dependent pathway is involved in direct or indirect lysosomal sorting. Immunofluorescence and EM localization studies show localization of the AP-3 complex in both the TGN and lysosomes^{58, 60}. Further research claimed that proteins are delivered to the lysosome membrane from the tubular sorting endosomes. These are domains of the EE which seem to specifically contain proteins destined for the lysosome to be degraded. The observation that lysosomal-associated membrane protein 1(LAMP-1) and LAMP-2, two membrane proteins, which represent a large percentage of lysosome proteins in animals, are concentrated in AP-3 buds on endosomal tubules⁶¹ supports the role for tubular EE structures in lysosomal traffic. Lysosomal proteins that enter the AP-3 dependent pathway have a specific acidic di-leucine targeting motif. They may use both the TGN and early endosomes as exit sites to traffic to lysosomes⁶². Interestingly, newly synthesized LAMPs (including LAMP-1 and -2), are delivered to late endosome directly from TGN by 'LAMP carriers'. In this protein sorting pathway hVps41 (a

subunit of the HOPS complex) and VAMP7 (v-SNARE)⁶³ are required for the fusion of LAMP carriers with late endosomes.

ESCRT complex dependent pathway

The pathway mediated by the Endosomal Sorting Complex Required for Transport (ESCRT) has been extensively studied for its pivotal role in biogenesis of MVBs, as well as the recognition and sorting of ubiquitinated proteins or non-ubiquitin modified cargos to lysosome/vacuole for degradation. ESCRT is primarily involved in membrane remodeling processes⁶⁴⁻⁶⁸. The interaction of ESCRT subunits and phospholipids results in different ESCRTs complexes with distinct functions: ESCRT-0, -I, -II and -III⁶⁹, which are ancient machineries as some of their components are present in *Archeae*, some fungus, plants and animals⁴⁹. These complexes, consisting of several subunits, facilitate the sorting of ubiquitinated membrane proteins into intraluminal vesicles (ILVs) and subsequently into lysosomes.

ESCRT-0, consisting of HRS (hepatocyte growth factor-regulated Tyr kinase substrate)⁷⁰ and STAM (signal transducing adaptor molecule) subunits⁷¹ (not identified in Arabidopsis), is required for the destination of ubiquitinated proteins to the lysosome for destruction. Weak binding of ESCRT-0 to the endosome membrane occurs via the interaction of the FYVE domain of HRS with phosphatidylinositol 3-phosphate (PtdIns3p) molecules in endosomes^{72, 73}. Contemporarily, the interaction of HRS with the ESCRT-I subunit named tumor susceptibility gene-101 (TSG101) leads to the recruitment of ESCRT-I to the endosomal membrane. The different ESCRT complexes show subtle differences and functional interactions with each other that determine their role in directing the packaging of ubiquitinated proteins into lysosomes⁶⁶. The ESCRT-I complex consists of four subunits including VPS23/TSG101, VPS28, VPS37, and the MVB sorting factor 12 (MVB12) in mammal, yeast, and plant⁷⁴⁻⁷⁶, with an exception that

no MVB12 been identified in plant⁶⁸. By interacting with ubiquitin, TSG101/VPS23 recognize and sort ubiquitinated proteins to the ILVs engulfed into MVBs. More importantly, the ESCRT-I complex is suggested to help recruiting ESCRT-II (ref^{77, 78}) by the interaction of TSG101 with its core components EAP30/VPS22 and EAP45/VPS36. ESCRT-II consists of EAP30/VPS22, EAP20/VPS25 and EAP45/VPS36(ref^{77, 78}) and functions primarily in membrane deformation. The sub-complexes, comprised of charged multivesicular body proteins (CHMP), including CHMP2/VPS2, CHMP3/VPS24, CHMP4 and CHMP6/VPS20 plus the later described CHMP1, CHMP5/VPS60 and CHMP7 (ref⁷⁹⁻⁸³) further associate to form a functional ESCRT-III complex. Activation of ESCRT-III initiate by the recruitment of CHMP6/VPS20 to endosome through the interaction with the ESCRT-II subunit EAP20/VPS25(ref^{84, 85}).

The disassembly of ESCRT complexes from endosomal membrane and the recycling of individual subunits back to cytoplasm is regulated by a subset of AAA-ATPases termed VPS4⁸⁶⁻⁸⁸ through the interaction with VTA1, a positive regulator for VPS4 activity^{89, 90}.

Deubiquitination by ESCRT-associated deubiquitinating enzymes (DUBs) has been shown for some proteins before release into ILVs. However, deubiquitination seems not essential for the delivery to the lysosomal lumen for degradation⁹¹. The best characterized DUBs are UBPY, likely the homolog of Doa4 (degradation of alpha-4) first identified in yeast⁹², which is AMSH in human⁹³, the JAMM-domain containing DUB. They function in regulating the progress of proteins along the MVB pathway.

This sorting pathway is orchestrated by ESCRT subunits forming complexes that interact with each other and modify, recognize and deliver proteins building a chain of events that result in the degradation of specific proteins in the lysosome.

Table 1. ESCRT components and associated proteins identified in organisms

Human (Hs) Proteins	Yeast(Sc) homolog	Plant(At) homolog	General function in endocytic pathway	Refs
<i>ESCRT-0 components</i>				
HRS	Vps27p	N.I.	Binds to PI(3)P for the recruitment on membrane of EEs; recruiting clathrin to EEs; initial targeting and concentration of the ubiquitinated cargo; interacts with ESCRT-I subunit Vps23p/TSG101 for the recruitment of the ESCRT-I	94-98
STAM	Hse1	N.I.		
<i>ESCRT-0 like</i>				
GGA1, 2, 3	GGA1, 2	N.I.	Recruited to EE membranes by interaction with PI(3)P and clathrin; recognizes ubiquitinated proteins; interacts with ESCRT-I subunit Vps23p/TSG101 by the VHS and GAT domains for the recruitment of the ESCRT-I	99-101 102-106
TOM1, TOM1L1, 2, 3	N.I.	TOL1, 2, 3, 4, 5, 6, 7, 8, 9		
<i>ESCRT-I components</i>				
TSG101	Vps23p	VPS23A, B	Recognizes ubiquitinated cargo via the UEV domain; Interacts with plant specific ESCRT components FREE1/FYVE1 controlling vacuole biogenesis	107-110
VPS28	Vps28p	VPS28-1, 2	Interacts with ESCRT-II subunit EAP45/Vps36p	111
VPS37A, B, C, D	Vps37p	VPS37-1, 2	Endosomal sorting	112, 113
MVB12A, B	MVB12	N.I.	Associates to ESCRT-I complex by binding to VPS37/Vps37p; stabilizes ESCRT-I in an oligomeric, inactive state in the cytosol ; regulates cargo selection	75, 76, 114-117
<i>ESCRT-II components</i>				
EAP20	Vps25p	VPS25	Interacts with ESCRT-III subunit CHMP6/Vps20p, and aids recruitment of CHMP6/Vps20p on endosomal membrane	78, 84, 118-120
EAP30	Vps22p	VPS22	Interacts with RAB7 effector RILP mediating MVBs biogenesis	118, 120, 121
EAP45	Vps36p	VPS36	Binds directly with ESCRT-I subunit VPS28/Vps28p for the recruitment of ESCRT-II to endosomal membrane; recognizes ubiquitinated cargoes; binds to PI(3)P; MVB and vacuole biogenesis	111, 118, 120, 122-124
<i>ESCRT-III components and accessory proteins</i>				
CHMP6	Vps20p	VPS20-1, 2	Interacts with ESCRT-II subunit EAP20/Vps20p; Initiating the assembly of ESCRT-III by recruiting CHMP4/Snf7 on the endosomal membrane	85, 119, 125, 126
CHMP4A, B, C	Snf7/Vps32p	SNF7-1, 2	Binds directly with BRO1/ALIX mediating the ESCRT-III disassembly and membrane scission activity	127-130
CHMP3	Vps24p	VPS24-1, 2	Terminates Snf7/CHMP4 oligomerization; Associates with AMSH/Doa4 for the deubiquitination of endocytosed cargos	131
CHMP2A, B	Vps2p	VPS2-1, 2, 3	Engages Vps4p/VPS4/SKD1 in the dissociation of ESCRT-III	131, 132
CHMP1A, B	Did2/Vps46p	CHMP1A, B	Interacts with VPS4/SKD1 and LIP5/Vat1p	133, 134
CHMP5	Vps60p	VPS60-1, 2	Function as an adaptor protein for the endosomal recruitment of LIP5/Vat1p;	81, 87, 135, 136

Table 1. Continue

Human (Hs) Proteins	Yeast(Sc) homolog	Plant(At) homolog	General function in endocytic pathway	Refs
CHMP7	Chmp7	CHMP7	Associates with CHMP4/Snf7 for the endosomal sorting; nuclear envelope reformation and stability	83, 137, 138
IST1	IST1	ISTL1	Associates with Did2/CHMP1, LIP5/Vat1p and VPS4/SKD1 to aid the recruitment of VPS4/SKD1 to MVB-associated ESCRT-III	139-143
<i>ESCRT disassembly proteins</i>				
VPS4/SKD1	Vps4p	VPS4/SKD1	Binds to ESCRT-III (Dia2/CHMP1, VPS2/CHMP2, and VPS24/CHMP3) by the N-terminal MIT domain; disassembling ESCRT-III polymers from endosomal membrane upon ATP binding and hydrolysis; mediating the recycle of ubiquitin and ESCRT-III	144-150
LIP5	Vat1p	LIP5	Interacts with ESCRT-III component CHMP5/VPS60/Vps60p, promoting the oligomerization and activation of the Vps4p/VPS4/SKD1	81, 145, 151-154
<i>ESCRT machinery associated components</i>				
BRO1	ALIX	BRO1/ALIX	Binds to ESCRT-III components CHMP4/Snf7/SNF7 facilitate cargo sorting and ILV formation; directly interacts with the DUB AMSH/Doa4 stimulating recruitment or stabilization of AMSH/Doa4 on MVBs/LEs controlling vacuole biogenesis in plant	130, 155-158
AMSH	Doa4	AMSH1, 2, 3	Binding to clathrin on EEs; associates with ESCRT-0 (STAM/Hse1), and ESCRT-III (CHMP3/VPS24) components for the deubiquitination of endocytosed cargo; mediating the vacuole biogenesis in plant.	92, 93, 159-163
<i>Unique ESCRT components for plant</i>				
N.I.	N.I.	FREE1/FYVE1	Interacts with ESCRT-I components VPS23, associating to PI(3)P and ubiquitinated proteins to mediate MVB/Vacuole biogenesis	110, 164, 165
N.I.	N.I.	PROS	Interacts with VPS4/SKD1 and Vat1p/LIP5 and boosting the ATPase activity of VPS4/SKD1 in vitro.	166

Hs, Homo sapiens; Sc, Saccharomyces cerevisiae; At, Arabidopsis thaliana; N.I., not identified.

Biogenesis of lysosomes

In spite of the extensive studies on the protein trafficking pathways to lysosome, the formation of lysosomes is still a poorly understood. RAB5 and its effector complex CORVET, RAB7 and its effector HOPS complex, and SNAREs are crucial for the maturation of endosomes and the fusion of MVBs/LEs to lysosomes. Overexpression of GTP-locked RAB7 in HeLa cells induces the formation of enlarged lysosomes by increasing the fusion of lysosomes, whereas the expression of RAB7 dominant-negative mutants leads to the formation of dispersed lysosomes

and a decrease of lysosome luminal acidity¹⁶⁷. Depletion of the HOPS complex-specific subunits, VPS41 or VPS39 in HeLa cells, by contrast, causes no obvious morphological abnormalities of lysosomes, even though the late endosome fusion and the subsequent sorting of endocytosed cargo to endolysosomes (the hybrid organelle originating from the fusion of endosomes and lysosome) are affected⁶³, illustrating the high complexity of the RAB7-HOPS-dependent lysosomal fusion and biogenesis.

Ectopic expression of the type III transmembrane glycoprotein LGP85, also known as LIMPII, also results in enlargement of endosomes and lysosomes, which is suppressed by loss-of-function of RAB5b^{168, 169}. This strongly indicates that RAB5 has a role in the formation of lysosomes. Further analyses confirmed the involvement of RAB5 in the biogenesis and maintenance of late endosomes/lysosomes in coordination with PI(3)K activity¹⁷⁰.

The endosomal lipid kinase PIKfyve is a key player of lysosomal biogenesis, function and morphology. By phosphorylation, PIKfyve converts PtdIns(3)P into phosphatidylinositol-3,5-bisphosphate (PtdIns(3,5)P₂) at endosomes, which inhibits the dephosphorylation and translocation of microphthalmia-associated basic helix-loop-helix (bHLH) leucine zipper transcription factors, including TFEB (transcription factor EB) and TFE3 to the nucleus, the master regulator of lysosome functional association genes. This is shown by the effect of mutations in PIKfyve regulators, like Vac14/ArPIKfyve and Fig4/Sac3, or impairment of PIKfyve activity, which all result in enlarged lysosomes¹⁷¹.

The transcriptional regulation of lysosome biogenesis was elucidated decades ago by studying the effect of nutrient starvation¹⁷², ER stress¹⁷³ and inhibition of PIKfyve. These conditions all trigger the translocation of TFEB, TFE3 and MITF (microphthalmia-associated transcription factor) from the cytoplasm or the lysosome membrane to the nucleus, where they activate genes involved in autophagy and

lysosomal genesis¹⁷⁴⁻¹⁷⁷. In HeLa cells, TEFB binds to coordinated lysosomal expression and regulation (CLEAR) elements to activate transcription of genes encoding lysosomal enzymes (like β -glucosidase, Cathepsin D, and β -glucuronidase,) subunits of CORVET and HOPS complexes (VPS11 and VPS18), the transmembrane proteins of mannose-6-phosphate receptor, M6PR and IGF2R (Insulin-like growth factor 2 receptor)¹⁷⁸. TFE3 also binds to CLEAR elements and induces the expression of genes encoding autophagy-related proteins, several V-ATPase subunits, lysosomal transmembrane proteins, and lysosomal hydrolases, and increases the number of autophagosomes and lysosomes¹⁷⁹. TFE3 and TFE3 can independently promote transcription of genes encoding lysosomal proteins. In TFE3-depleted cells, TFE3 functions normally activating transcription of lysosomal genes and mediating lysosomal biogenesis^{172, 178, 179}. Altogether, the identification of the bHLH-zip transcription factors (TFEB and TFE3) might open a way to the identification of more genes involved in regulating lysosome biogenesis.

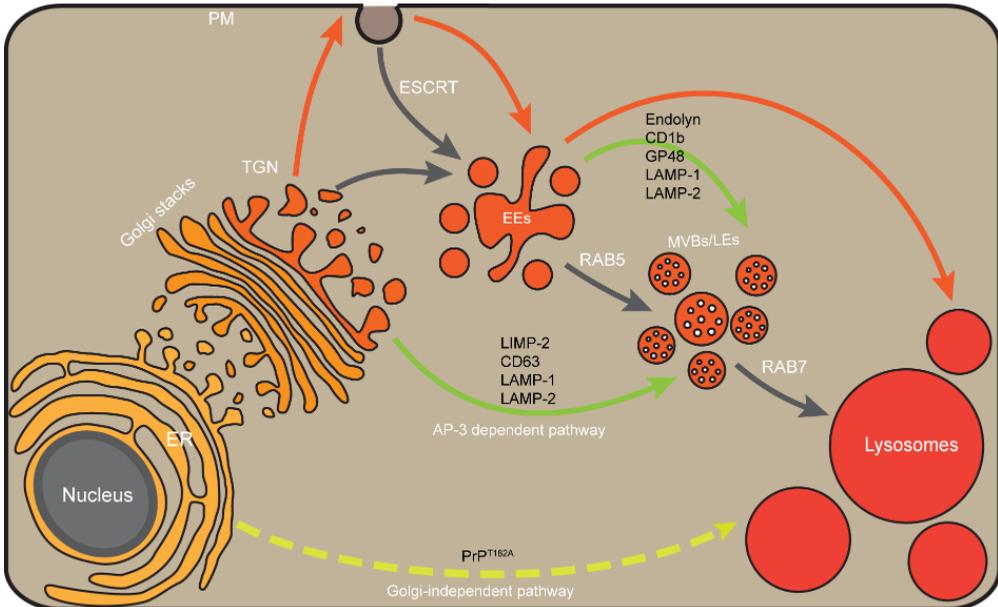


Figure 1. Protein sorting pathway to lysosomes. The M6P pathway, as indicated with grey arrows, plays a crucial role in the delivery most of the newly synthesized lysosomal enzymes to lysosomes in

mammalian cells. The M6P-independent pathway, as shown by orange arrows, has been proved involved in trafficking of multiple protein to lysosomes. For instance, the LIMP-2-mediated transport of β -glucocerebrosidase to lysosome by this pathway, skips MVBs^{49,50}. The AP-3 dependent pathway is an alternative pathway taken by several lysosomal membrane proteins⁶² sorting to lysosome in a direct (TGN-MVBs/LEs-lysosomes) or indirect manner(TGN-PM-EEs- MVBs/LEs-lysosomes) which is show by green arrows. The Golgi-independent pathway to lysosomes, depicted by a yellow dashed arrow, has not been extensively studied yet, however the prion protein (PrP) bearing the disease-associated T182A mutation (Mut-PrP) has been reported to reach lysosomes in a Golgi-independent manner with the involvement of the autophagy-lysosomal pathway¹⁸⁰. Words in white color indicate endomembrane compartments, protein sorting pathways, and key regulators in those pathways; Words in black color indicate proteins delivering to Lysosomes by certain pathway.

Table 2. Proteins involved in Lysosome biogenesis.

Human(Hs) Proteins	Yeast(Sc) homolog	Plant homolog	Function in mammal	Subcellular localization	Refs
<i>Transient Receptor Potential Mucolipin channel</i>					
TRPML1	N.I.	N.I.	Directed movement of nascent lysosomes from late endosome/hybrid organelle in mouse cells	Late endo-lysosome(LEL) compartment	181
<i>Microphthalmia-associated bHLH/Zip transcription factor</i>					
TFEB	N.I.	N.I.	Regulating the biogenesis of autophagosomes and lysosomes in mammalian cells under starvation	Cytoplasm / Lysosome/ Nuclear	172, 182, 183
TFE3	N.I.	N.I.	Mediate cellular adaptation under starvation by simultaneously promoting lysosomal biogenesis and autophagy induction	Cytoplasm / Lysosome/ Nuclear	173, 177, 179
<i>Type III transmembrane glycoprotein</i>					
LGP85	N.I.	N.I.	Overexpression of LGP85 causes an enlargement of early endosomes and late endosomes/lysosomes	Lysosomes / late endosomes	168
<i>Phosphatidylinositol 3-phosphate 5-kinase</i>					
PIKfyve	FAB1	FAB1A/B	Inactivation of PIKfyve induce the nuclear accumulation of TFEB and TFE3, leading to enlargement of lysosomes, while decreasing endo/lysosome number	Early/late endosome	171, 184, 185
<i>RAB small GTPases</i>					
RAB5	YPT5/ VPS21	RAB5a, ARA7/ RHA1	Expression of GTP-locked RAB5 boost the homotypic fusion of early endosomes and gives rise to enlarged hybrid organelles termed endolysosomes	Early endosome	170, 186, 187
RAB7	YPT7	RABG	Overexpressed GTP-locked RAB7 increase the size of lysosome	late endosomes / mainly lysosomes	167, 188-191

Hs, Homo sapiens; Sc, Saccharomyces cerevisiae; N.I., not identified.

Post-Golgi traffic pathway to the plant vacuole (lytic vacuole)

Sorting towards the vacuole is in plants generally assumed to involve post-Golgi traffic. However, in some specific cell types and/or developmental stage of the plant some membrane- as well as soluble proteins traffic directly from the ER to the

vacuole^{192, 193} via a Golgi-independent pathway. Both routes involve vesicles, which identity and destination are defined by proteins in their coat. ER-to-Golgi transport of vesicles is diversified into a pathway dependent on the presence of the coat protein COPII (COPII-dependent), and one that is COPII-independent)¹⁹⁴. More details of the ER-to-Golgi transport can be found in other reviews^{192, 194-196}.

RAB5-to-RAB7 dependent pathway

The post-Golgi vacuolar trafficking includes several distinct pathways. The best studied pathway is a RAB5-to-RAB7 dependent route, which delivers vacuolar proteins from TGN/EEs to vacuoles with the participation of intermediate organelle well-known as pre-vacuolar compartments (PVCs) / multivesicular bodies (MVBs). This pathway involves two RAB small GTPases RAB5/RABF and RAB7/RABG, their activator, the guanine-nucleotide exchange factor (GEF) VPS9a, and the SAND/CCZ1 complex respectively. The set of proteins that traffic along this pathway from PVCs/MVBs to LV include a wide range of vacuolar proteins like aleurain, phaseolin and 12S globulin^{190, 197}. Interestingly, some proteins, like SYP22, a Qa-SNAREs, and VHA-a3, a subunit of the vacuolar proton ATPase, are targeted to the CV depending on the RAB5 machinery only, which suggests the existence of a RAB5-dependent RAB7-independent pathway^{191, 198}.

AP-3 dependent pathway

For certain vacuolar proteins, a shortcut to central vacuole is the AP-3-dependent pathway, which is RAB5 and RAB7 independent. This route, important in plant development and in numerous cellular processes, is mainly contributed by the adaptor protein-3(AP-3)¹⁹⁹. Multiple types of protein have been identified as cargos of the AP-3-dependent pathway. These include the sucrose transporter 4 (SCU4)²⁰⁰, the R-SNARE termed vesicle-associated membrane protein 713 (VAMP713)¹⁹¹ and VAMP711(ref²⁰¹), and protein S-ACYL transferase10 (PAT10)^{201, 202} of

Arabidopsis. A mini review¹⁹⁹ that well summarizes the recent advances on AP-3 dependent pathway in *Arabidopsis* is recommended for further details.

ESCRT-dependent endosomal traffic pathway

The ESCRT machinery plays a crucial role in MVB biogenesis, MVB-mediated protein degradation in CV and autophagy^{68, 203, 204}. Growing evidence improves our understanding of the roles of ESCRTs in multiple non-endosomal sorting processes including cytokinesis²⁰⁵, viral replication²⁰⁶, abscisic acid (ABA) signaling^{109, 165, 207, 208} and chloroplast turnover¹³⁴, which are mediated by both canonical and unique plant ESCRT components⁶⁸. FREE1/FYVE1 and PROS have been characterized as plant-specific ESCRT components, indicating that the ESCRT machinery diverged in plants to adapt it to their unique physiological processes. Impairment of the function of any component of each pathway affects the sorting of specific cargos to their destination organelles, disturbing ubiquitin accumulation and resulting in misregulating of various signaling pathways, leading to very pleiotropic defects in plant growth and development^{197, 209, 210}.

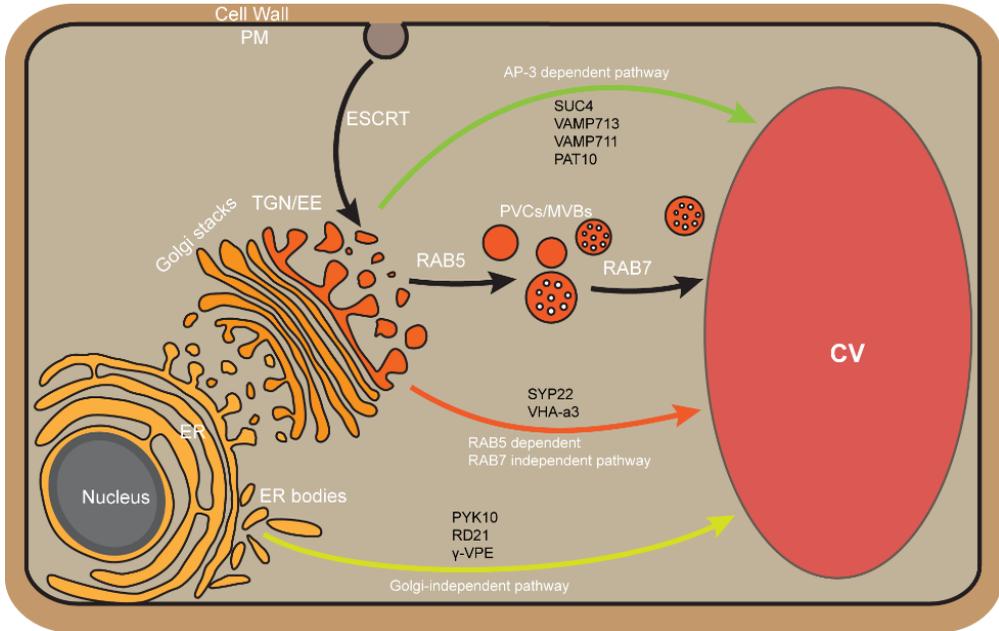


Figure 2. Protein sorting pathway to the vacuole. The canonical protein sorting pathway (RAB5 and RAB7 dependent) is indicated with black arrows. The AP-3 dependent pathway is indicated by green arrows. Sucrose transporter 4 (SUC4)²⁰⁰, the R-SNAREs VAMP713(ref¹⁹¹) and VAMP711(ref²⁰¹) and the protein S-ACYL transferase10 (PAT10)^{201, 202} were shown to reaching the CV by an AP-3-dependent (RAB5- and RAB7-independent) route (green arrow). RAB5-dependent and AP-3-independent pathway is indicated by orange arrows. SYP22, a member of Q_a-SNAREs group of Arabidopsis¹⁹¹, and VHA-a3, a subunit of the V-ATPase¹⁹⁸, traffic to the CV in a RAB5-dependent manner. A Golgi-independent, or ER-bodies²¹¹ mediated Golgi-independent pathway is reported by yellow arrows. PYK10(ref⁵¹), a β-glucosidase with an ER-retention signal (KDEL sequence), and two cysteine proteinases, RD21 and γ-VPE²¹² (under salt treatment) have been proven to accumulated in ER bodies and be targeted to the CV by a direct ER-to-vacuole trafficking route. Words in white color indicate endomembrane compartments, proteins sorting pathways, and key regulators in those pathways are indicated in white font; Proteins tracking to vacuole via specific pathway are indicated in black font.

Mechanisms of biogenesis of vacuoles and lysosomes compared

Biogenesis of the plant Central Vacuole and the mammalian lysosome

Interestingly, most of the core components of the traffic pathways to the lysosome are also major contributors to the biogenesis of vacuoles in plants. No obvious defects in central vacuole morphology were detected in the mutants of *ara7* and *rha1* (ARA7 and RHA1 are plant conventional RAB5, ref²¹³), *vps9a-2* (VPS9a is a

common activator of some plant RAB5, ref²¹⁴), and *rabg* (RABG is also called RAB7, ref¹⁹¹). However, the *Arabidopsis vacuoleless 1 (vcl1)* mutant, impaired in the function of VPS16, a core subunit of the HOPS tether complex, shows defects in vacuole formation accompanied by an increased number of autophagosomes²¹⁵. Similarly, loss of function of the mammalian VPS16 component of CORVET and HOPS abolished the fusion of autophagosomes with lysosomes and impaired the formation of autolysosomes (a component of the autophagy pathway)²¹⁶. Mutation of the *Arabidopsis PAT2* gene, which encodes a putative β -subunit of the AP-3 complex, leads to defects in PVC-dependent biogenesis of the lytic vacuoles and aberrant-shaped vacuoles with numerous multilayered endomembrane enclosures²¹⁷. In mammalian cells, however, AP-3 mutations mainly affect the sorting of proteins from the TGN/endosomal compartment to lysosomes and disturb the biogenesis of several types of LROs²¹⁸. Importantly, subunits of the ESCRT tether system are major contributors to vacuole biogenesis in plants. For example, *Arabidopsis* cells with a mutation in *FREE1*, encoding the plant-specific ESCRT component FYVE1, lack a large central vacuole and contain instead numerous fragmented vacuoles and an increased number of autophagosomes¹⁶⁴. Further analyses revealed that the fragmented vacuoles of *fyve1-1* are interconnected, forming a complex tubular vacuole²¹⁹. Mutations that cripple the ESCRT-III associated deubiquitinating enzyme (DUB) AMSH3 also result in a failure to form the central lytic vacuole, and increases the accumulation of autophagosomes^{161, 162}. Furthermore, *Arabidopsis* mutants for the core component of the ESCRT-II complex VPS36, contain smaller and aberrant vacuoles in root tip cells of seedling¹²³.

Recently, also other genes have been reported to have a role in vacuole formation^{220, 221}. However, none of these appear to be completely essential for the vacuole formation, as mutants that completely lack vacuoles are unknown, possibly because such mutants would be embryo-lethal or even zygotic lethal^{215, 220}, which seriously hampers further characterization of the players in vacuole biogenesis. This points

out the urgency to identify and explore a more versatile model for vacuolar genesis than those explored so far.

Table 3. Proteins involved in plant central vacuole biogenesis.

Plant(<i>At</i>) protein	Yeast(<i>Sc</i>) homolog	Human (<i>Hs</i>) homolog	Mutant phenotype	Subcellular localization	Refs
<i>COVERT components</i>					
VCL1	VPS16p	VPS16	Loss of <i>VCL1</i> function blocks the vacuole formation (defects in docking or fusion of pvc), however triggers the formation of autophagosomes; embryo lethal	PVC/MVB and tonoplast	215, 216, 222, 223
<i>ESCRT-II component</i>					
VPS36	VPS36p	VPS36	<i>vps36-1</i> seeding show smaller and aberrant vacuoles	Mainly PM, rarely EE/TGN or LE/MVB	123
<i>Plant-specific ESCRT components</i>					
FYVE1/FREE1	N.I.	N.I.	Mutation in <i>fyve1/free1</i> lead to defects on vacuolar fusion and central vacuole formation, and resulted in numerous fragmented vacuoles and accumulation of autophagosomes; seedling lethality	Cytoplasm and LE/MVB	164, 219
<i>ESCRT-related proteins</i>					
AMSH3	Doa4	AMSH	<i>amsh3</i> mutant fail to form a central vacuole, accumulate autophagosomes, and mis-sort vacuolar protein to the intercellular space	Cytoplasm/LEs	162
ALIX/BRO1	BRO1	ALIX	<i>alix-4 mutant</i> shown similar vacuole phenotype(tubular interconnected vacuole) as <i>amsh3</i> mutant	Cytoplasm/LEs	156
<i>Adaptor proteins</i>					
PAT2/ AP-3 β	APL6	Ap3B	<i>pat2</i> is specifically defective in the biogenesis, identity, and function of central vacuoles; normal sorting of proteins to storage vacuoles	Cytoplasm / uncharacterized compartment	217
<i>Syntaxin superfamily, calcium-dependent protein kinase 1 adaptor protein 2</i>					
CAP2	N.I.	N.I.	<i>atcap2-1</i> delayed the fusion of PSVs to form central vacuole during seeds germination	PVC/MVB	221

Hs, *Homo sapiens*; *Sc*, *Saccharomyces cerevisiae*; *At*, *Arabidopsis thaliana*; *N.I.*, not identified.

PSV: so far the best characterized model for additional vacuoles in plant cells

Seminal work of Paris et al showed that barely root tip cells can contain multiple vacuoles with distinct content³. One type of vacuoles contain the α -TIP (tonoplast intrinsic protein) and the storage protein lectin, for which they are referred to as protein storage vacuoles (PSVs), while the other vacuoles contained TIP-MA27 (or

γ -TIP) and the cysteine-protease aleurain, for which they were named lytic vacuoles^{3, 224}. These findings in barely root tips are often extended to other tissues, because of which α -TIP (or α -TIP-GFP) positive compartments in these tissues are also considered to be PSVs, and γ -TIP (or γ -TIP-GFP) positive compartments as LVs^{225, 226}. However, it can be debated whether the localization of TIP proteins is a good criterion to discriminate between multiple types of vacuoles in plant cells¹⁰, a.o. because subsequent studies showed that most cells contains both α -TIP and γ -TIP and vacuoles with only γ -TIP are very rare, and because fluorescent protein fusions to α -TIP, γ -TIP, δ -TIP were in petunia cells targeted to unidentified compartments, that were distinct from the central vacuole and vacuolinos⁹. Nevertheless the localization of TIPs is still often (miss) used to identify vacuoles in a wide variety of tissues as either PSVs or LVs.

Several observations in developing embryo support the coexistence of PSVs and LV in one cell, and the transition from one type of vacuole into the other. Coexistence of different types of vacuole, and the transformation of one type of vacuole into the other were observed under particular physiological and developmental constraints, especially during seed germination^{227, 228}. Several studies suggested that PSVs arise by a the *de novo* mechanism in cotyledons of developing pea seedlings (*Pisum sativum*)²²⁹ and embryos of *Medicago truncatula*¹⁰. However, recent findings show instead that PSVs originate from the pre-existing embryonic vacuole (EV) by a remodeling mechanism during *Arabidopsis* seed maturation²³⁰.

While the pathway by which proteins are sorted to lytic vacuoles has been studied in some depth (see above), the pathway to the PSV remains largely unexplored. In addition to the receptor-mediated sorting involving clathrin-coated vesicles (CCVs) and the PVCs/MVBs pathway to the lytic vacuole, certain proteins are targeted to PSVs via plant specific non-coated dense vesicles (DVs)^{231, 232}. Storage proteins are released from the TGN and sorted into DVs²³³⁻²³⁵ by physical aggregation of the

cargo protein mediated by vacuolar sorting receptors (VSRs) and receptor homology transmembrane-RING H2 motif protein (RMR) which have been proposed to act as a co-receptor with VSRs^{236, 237}. PVCs/MVBs would be the intermediate between DVs and PSVs^{233, 238-240}. Although also direct ER-to-PSV trafficking pathway driven by the precursor-accumulating (PAC) vesicles has been identified in maturing pumpkin (*Cucurbita maxima*) seeds²⁴¹, our knowledge on the sorting of proteins to the PSVs remains poor and fragmentary.

Most of the available information was gained from studies on loss of function mutants. For example, mutations in VTI12 (v-SNARE 12)²⁴², VAMP727 (R-SNARE)²⁴³, VPS36 (subunits of ESCRT-II complex)¹²³, VSR1 (vacuolar sorting receptor 1)²³⁶, and NHX5/NHX6 (sodium-proton exchanger)²⁴⁴, have been shown to alter storage protein sorting to PSVs as well as the morphology of these vacuolar compartments. In addition, in rice, mutations affecting OsRAB5A and its activating GEF OsVPS9A, lead to size reduction of PSV, which are named protein body IIs (PBIIs) in rice²⁴⁵. It is not surprising that the core component of the ESCRT-II complex, VPS36, equally contributes to the biogenesis of PSVs and LVs, given the role of ESCRT complexes in the biogenesis of MVBs^{36, 246}, and the general consensus about these being intermediate compartments in the sorting to both LVs and PSVs.

Table 4. Proteins involved in PSV biogenesis

Plant(At) protein	Yeast(Sc) homolog	Human(Hs) homolog	Function (mutant phenotype)	Subcellular localization	Refs
<i>SNAREs</i>					
VTI12	VTI1	VTI1A	<i>vti12</i> mutant mainly alter the transportation of storage proteins to PSVs, lead to the decrease in size of PSV in embryo	TGN/EE	242
VAMP727	N.I.	N.I.	double mutant of <i>vamp727 syp22-1</i> exhibited fragmented and smaller PSVs	PVC/MVB	243
<i>ESCRT-II component</i>					
VPS36	VPS36p	VPS36	maturing seeds of <i>vps36 Arabidopsis</i> mutant showed only numerous small PSVs	Mainly PM, rarely EE/TGN or LE/MVB	123
<i>Sorting receptors</i>					
VSR1	N.I.	N.I.	<i>atvsr1</i> mutant miss-sorts storage proteins to extracellular space of seeds, resulting in a much smaller PSVs than Wild-type	PVC/MVB	247
<i>Na⁽⁺⁾/H⁽⁺⁾ antiporter</i>					
NHX5/ NHX6	NHX1	NHE9	<i>nhx5 nhx6</i> double mutant embryo reduced the size of PSVs, however increased the number of PSVs; thus the overall area of PSV per cell was not significantly different based on the quantification	Golgi/TGN	248, 249
<i>Guanine nucleotide exchange factor (GEF)</i>					
CCZ1	CCZ1	CCZ1	Mutant of <i>ccz1</i> miss-sort vacuolar proteins to apoplast; small and numerous PSV present in these mutant	PVC/MVB	190, 191
<i>Vacuolar processing enzymes</i>					
VPE	N.I.	N.I.	<i>vpe</i> null (a quadruple mutant for α -, β -, γ - and δ VPE) embryos exhibit small and numerous PSVs	N.D.	244

At, Arabidopsis thaliana; Sc, Saccharomyces cerevisiae; Hs, Homo sapiens; N.I., not identified.

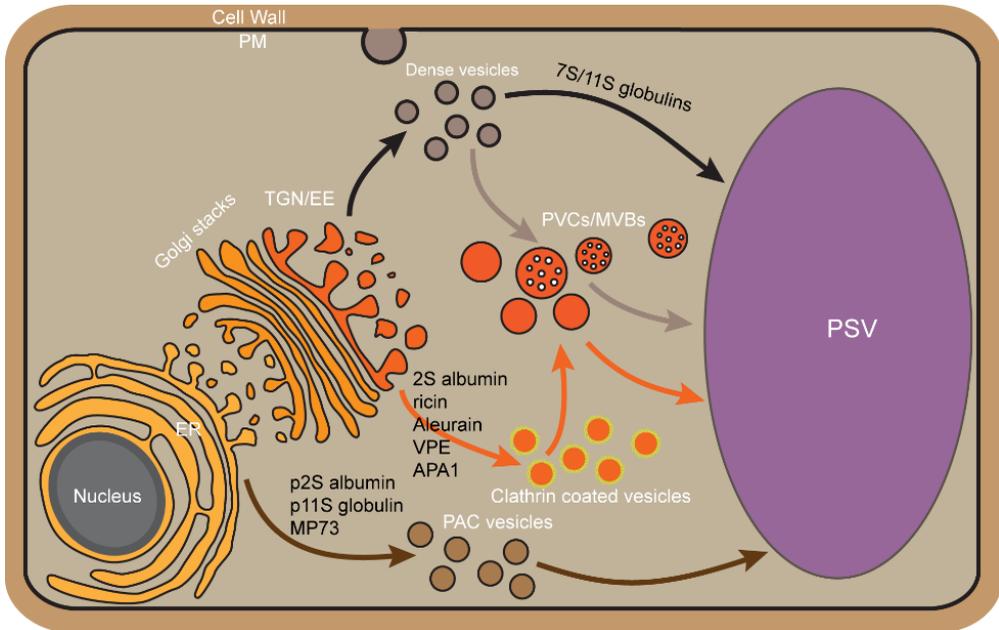


Figure 3. protein sorting pathways to the PSVs. Dense vesicle (DV)-mediated pathway (black and grey arrows) has been identified as the major route for storage proteins to traffic to PSVs in pumpkin seeds²³¹, and other species^{233, 250}. DVs deliver the storage proteins by direct fusion with PSVs, or integration into their PVCs/MVBs mediated protein trafficking pathway to PSVs. The role of the clathrin coated vesicles dependent pathway (depicted by orange arrows) in sorting of proteins to PSV is not well explored and debatable. In castor bean (*Ricinus communis*) endosperm, both 2S albumin and ricin are trafficked to PSVs with the help of the CCVs machinery²⁵¹. In addition, the thiol protease Aleurain, vacuolar processing enzymes (VPEs) and the aspartic protease A1 (APA1) traffic to PSVs via the CCVs machinery in Arabidopsis embryo^{233, 234}. The Golgi-independent pathway (brown arrows) is here depicted as identified in maturing pumpkin (*Cucurbita maxima*) seeds to deliver the precursors of 2S albumin and 11S globulin^{44, 252} and the membrane protein MP73 (ref²⁵³) to the PSVs. This traffic is mediated by the participation of precursor-accumulating (PAC) vesicles (diameters of 0.2 μ m to 0.4 μ m). In rice endosperm cells, PAC-like vesicles have been found to deliver glutelin and α -globulin as well²⁵⁰. Words in white color indicate endomembrane compartments, proteins sorting pathways, and key regulators in those pathways; Words in black color indicate proteins delivering to PSV by certain pathway.

Vacuolino: a novel and suitable model for the study of vacuole biogenesis

Vacuolinos are additional vacuoles first identified in petunia petal epidermal cells⁹. They are widespread in the plant kingdom as their presence has been detected in two species belonging to the two main groups of Eudicots, Petunia (an *Asterid*) and rose (a *Rosid*). Vacuolinos appear to serve as an intermediate station for vacuolar

proteins en route to the central vacuole. After transient transformation of epidermal petal cells, both in the intact tissue and in derived protoplast, with gene constructs expressing FP-fusions of PH1, PH5 (two P-type ATPases required together for hyper-acidification of the CV in petal epidermal cells²⁵⁴), vacuolar SNAREs, Aleu or KCO (ref⁹), these proteins localize after 24 hours in vacuolinos and reach the CV only after 48 hours. This suggests that proteins directed to the CV transit in these cells through vacuolinos which deliver them to the CV, and implies that vacuolinos are distinct from the CV and exchange membranes and proteins with it. The transport of proteins from vacuolinos to the CV requires PH1, the P_{3A}-ATPase, which interacts with vacuolar SNAREs including SYP22 and SYP51 (ref⁹), and a putative vacuolar H⁺/sugar symporter encoded by PH7 (**Chapter 5** in this thesis).

In petunia petals a transcription factor complex, named MBWW, consisting of AN1, AN11, PH4 and PH3, activates genes involved in anthocyanin pigment synthesis, vacuolar hyper-acidification and, surprisingly, also the formation of vacuolinos^{9, 254-257}. In mutants lacking one of the MBWW components (*an1*, *ph3*, *ph4* and *an11*) the formation of vacuolinos is abolished, while the morphology and function of the CV are largely unaffected, as in these mutants proteins can still reach the vacuole along a 'direct' pathway, similar to vacuolar trafficking in other cell types. This supports the independent character of vacuolinos and CV and the presence of multiple vacuolar trafficking pathways, some involving vacuolinos, others not. The presence in plant cells of alternative routes to the CV is in agreement with accumulating evidence suggesting that post-Golgi traffic is diversified into multiple pathways in each organism. In contrast to mutations affecting other vacuolar types, the depletion of vacuolinos has besides changes in the flower color no other dramatic effects on plant development and growth, making the study of mutants for different steps of the biogenesis of this organelle feasible.

The above mentioned features of vacuolinos indicate that they make a nice model system to vacuolar biogenesis and protein trafficking, but also raise questions about the biological function of vacuolinos. Our knowledge on the vacuolino pathway is at the moment still limited, but the recent identification of a true key player in their formation and regulation of maturation, shed some light on it. A RAB GTPase isoform, encoded by the petunia *RAB5a* gene and widely represented in the plant kingdom (with the exception of *Brassicaceae*), is required for the formation of vacuolinos by driving the fusion of a subpopulation of PVCs in petunia (**Chapter 4** in this thesis). The amount of RAB5a protein, regulates the dimension of vacuolinos, by controlling the number of subpopulation of PVCs fusions preceding the vacuolinos fuse to the CV. Expression of *RAB5a* is transcriptionally controlled by the AN1/AN11/PH4/PH3 MBWW complex. The study of *RAB5a* mutant and overexpression lines in petunia, showed that vacuolinos, present in the tip of conical epidermal cells, contribute to the shape of these peculiar cell-type, which was previously reported to affect the visual recognition of flowers by pollinating animals²⁵⁸⁻²⁶⁰. Furthermore, certain vacuolar membrane proteins are in vacuolinos released from membrane and trapped in the lumen of vacuolinos and never reaching the CV, while other proteins are delivered to the CV (**Chapter 4** in this thesis). This suggests that vacuolinos select and degrade/halt certain proteins serving as ‘sorting station’ or ‘gatekeeper’ in the vacuolar trafficking pathway. An example of proteins that are halted in the vacuolinos are the vacuolar sorting receptors BP80 and VSR2, as well as the FADING enzyme involved in destabilization of anthocyanins in the CV (**Chapter 3** in this thesis). More evidence is needed to support such function of vacuolinos and to unravel the mechanism behind it.

The coexistence of vacuolinos (or other types of additional vacuoles in other cell types) with the CV in petal epidermal cells, raises several questions:

- how are vacuolinos and other additional vacuoles generated?

- how are proteins and other molecules sorted to different vacuolar types within one cell?
- how do proteins move from vacuolinos to the CV and how are some (other) upheld in vacuolinos and prohibited from moving on to the CV?

Until now, researchers define identity of endomembrane compartments by the set of specific (marker) proteins residing there. A typical example is the before mentioned definition of lytic vacuole based on the sorting of γ -TIP to their membranes in contrast to α -TIP residing on PSV in seed and leaf cells^{3, 229, 261, 262}. However, in petal epidermal cells, different TIPs do not localize to the CV or the vacuolinos, showing that this type of ‘markers’ for specific compartment types can be dependent on the cell type and/or species and are therefore not of general use. Vacuolinos, by contrast, are defined by the proteins required to make them (like the MBWW complex and target genes, such as *RAB5a*) and to mediate their contact with the CV (like *PH1* and *PH7*). The study of target genes of the MBWW transcription complex and the understanding of their function, will, similarly to *RAB5a*, likely provide some answers to the biogenesis and functions of vacuolino.

The proteins *PH1* and *PH5*, which are essential for the hyperacidification of the CV, pass via vacuolinos on their way to the CV, it is likely that hyperacidification starts already in vacuolinos. If and to what extent the lumen of vacuolinos is (hyper) acidified has not been measured so far. This parameter is crucial for the activity of degrading enzymes, like proteases, and can therefore provide information on the mechanism by which some proteins are halted in the vacuolino and prevented from reaching the CV or recycle back to ER. The isolation of vacuolinos and proteomics study will identify both membrane and soluble proteins in these organelles. An acidic lumen and abundance of soluble hydrolases are known as the essential characteristics of degradative compartments^{48, 263}. In addition, comparative analysis on the composition of lipids in PVCs/MVBs, vacuolinos and LV membranes will help defining the identity of vacuolino and distinguishing them from other

endomembrane organelles. This will set the first steps towards the understanding of how different vacuoles are generated and kept distinct within a single cell, and possibly give the first clues about the function of vacuolinos.

Table 5. Proteins involved in vacuolino biogenesis and physiology

Plant(Ph) protein	Yeast(Sc) homolog	Human(Hs) homolog	mutant phenotype	Subcellular localization	Refs
WRKY transcription factor					
PH3	N.I.	N.I.	<i>ph3</i> mutant abolished the formation of vacuolinos and affected the acidity of CV.	Nucleus	9
MYB transcription factor					
PH4	N.I.	N.I.	<i>ph4</i> mutant abolished the formation of vacuolinos and affected the acidity of CV	Nucleus	9
P_{3B}-ATPase					
PH1	N.I.	N.I.	Impairment in PH1 induced formation of enlarged vacuolinos and blocked the fusion of vacuolinos with CV	Vacuolinos and tonoplast	9
RAB small GTPases					
RAB5a	VPS21	RAB5A	Mutation in RAB5a blocked the fusion of PVCs/MVBs to form vacuolinos. By contrast, ectopic expression of RAB5a induced enlarged vacuolinos.	Cytoplasm, PVC/MVB, vacuolinos	Chapter 4 in this thesis

Ph, *Petunia hybrida*; *Hs*, *Homo sapiens*; *Sc*, *Saccharomyces cerevisiae*; *N.I.*, not identified.

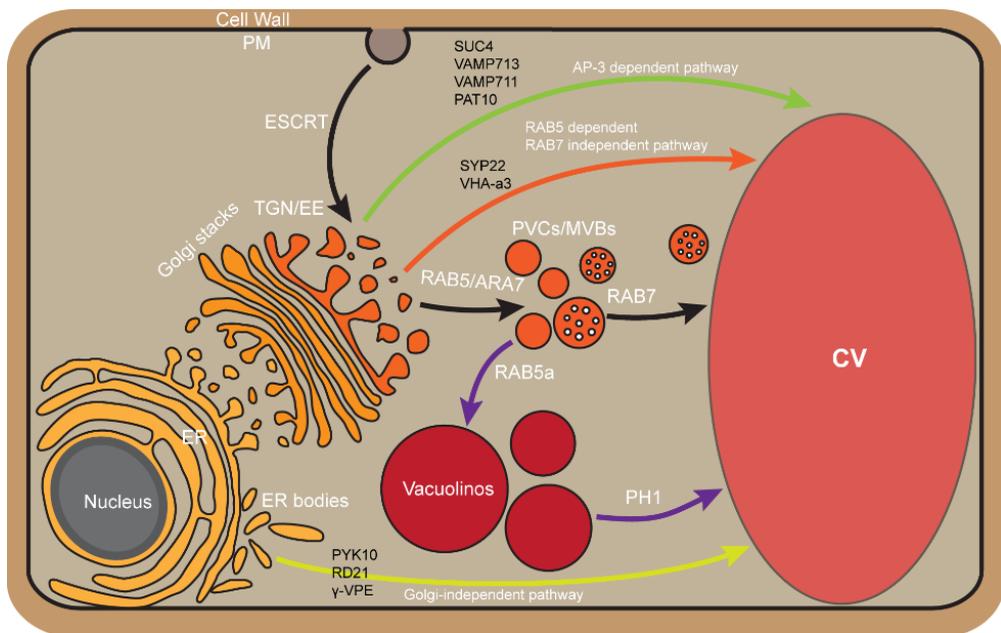


Figure 4. Vacuolar proteins sorting to CV by a vacuolino-dependent pathway. Golgi-dependent and Golgi-independent pathway, described in this review (see legend of Fig. 2), and the vacuolino pathway identified so far in the petal epidermal cells of *Petunia* and *Rose*⁹. An ancient RAB5a, distinct from the ARA7/RHA1 group of plant RAB5 based on phylogenetic analysis, mediates the fusion of subpopulation of PVCs/MVBs and leads to vacuolino formation (**Chapter 4** in this thesis); PH1, a P_{3B}-

ATPase, controls the fusion of vacuolinos with CV⁹ probably by interacting with SYP22 and SYP51. Multiple vacuolar proteins have been characterized as cargo of the vacuolino pathway including the vacuolar luminal protein Aleurain, P_{3A}-ATPase type of proton pump PH5, SNAREs SYP51 and SYP22, and the potassium channel opener protein KCO⁹, (some of this proteins are not in the diagraph due space limitation. Words in white color indicate endomembrane compartments, proteins sorting pathways, and key regulators in those pathways; Words in black color indicate proteins delivering to vacuole by certain pathway.

Evolution of core components of the lysosomal/vacuolar traffic pathway

To better investigate the conservation and diversification of cellular processes such as endomembrane trafficking system during evolution, a hypothetical ancestral state, named last eukaryotic common ancestor (LECA), has been phylogenetically inferred and reconstructed^{264, 265}. LECA is deduced to possess all the canonical endomembrane organelle/compartments, as well as the core regulatory components for driving the endomembrane system function properly, including vesicle coat complexes, fusion machinery, tethering complex, RAB GTPases and other regulators²⁶⁴. Although the core components of the lysosomal/vacuolar traffic are evolutionarily conserved in all modern organisms, new set of factors were acquired after separation of the different eukaryotic lineages, mostly via duplication, neofunctionalization and secondary loss of genes encoding different players of this pathway. This allowed to meet the unique and specific demands of the single type of organisms for specialized endomembrane traffic and physiological functions. Among all components, SNAREs complexes²⁶⁶, RAB GTPases^{267, 268}, clathrin coat proteins²⁶⁹, and ESCRT complexes²⁷⁰ have been selected and extensively studied by comparative genomic and phylogenetic analyses in order to understand the evolution of the endomembrane system in eukaryotes^{264, 271}. A plethora of RAB GTPase isoforms residing in different intracellular compartments, function as core components for membrane fusion and the remodeling of actin in concert with motor proteins, tether complexes and trafficking cargos. Their involvement in the

physiology of many types of compartments, makes them well-accepted markers of organelle identity^{267, 272, 273}.

The diversity of RAB proteins, present in the endomembrane system, where they fulfill several different function, is described as a driving force of endomembrane evolution²⁷⁴. By systematic phylogenetic reconstructions it was first proposed that up to 14 ancient RAB paralogs were presents in the LECA^{275, 276}. However, the high resolution phylogenetic strategy termed ScrollSaw, predicts that LECA had instead as many as 23 RAB GTPases²⁶⁷. This would be similar to the numbers of RABs, ranging from 10 to 20 that most modern unicellular eukaryotes, with few exceptions²⁷⁶⁻²⁷⁹. Multicellular organisms have more RAB GTPases, with an average of 60 or more²⁷⁵. RAB5, a subfamily of RAB GTPases, provides a good model to understand the lineage-specific evolution of endomembrane trafficking pathways by the expansion of the number of paralogous genes. Next to duplications, resulting in new members of the family and acquisition of new functions by some of them, secondary losses of RAB5 paralogs in specific species or groups have been reported as well. A good example is offered by the *Arabidopsis* RAB5 subfamily. ARA7 and RHA1, which are two closely related conventional RAB5 homologs, localize on MVBs and function as a major player in endocytic and vacuolar trafficking pathways²⁸⁰. *Petunia* (and many other higher plant species) contains besides RAB5a2, a homolog of ARA7 and RHA1 from *Arabidopsis*, two additional RAB5s named RAB5a and RAB5a1, which define two different phylogenetic RAB5 clades. Among which, RAB5a has been characterized as ancient RAB5 widely spread in the whole plant kingdom, mediating the fusion of PVCs/MVBs to form vacuolinos in *petunia* petal epidermal cells, which was missing in *Arabidopsis* (and other *Brassicaceae*) probably by secondary loss during evolution (**Chapter 4** in this thesis). Higher plants possess a plant-specific RAB protein, known as ARA6 in *Arabidopsis*, which is often referred to as a plant specific RAB5, even when it seems in phylogenetic analysis more similar to members of other RAB subfamilies. ARA6

marks in *Arabidopsis* distinct subpopulation of MVB, shows considerable co-localization with the conventional RAB5s of *Arabidopsis* and regulates a distinct endosomal trafficking pathway from the MVB to the PM^{181, 213, 281}. However, plants also lost the homolog of RAB4 and RAB9 subgroups governing endocytosis in mammals²⁷⁵.

Taken together, the RAB5 proteins in plants well depict how lineage-specific expansion, neofunctionalization and secondary loss of paralogs belonging to specific clades has driven the appearance of new endomembrane compartments and/or trafficking pathways to meet newly emerging functions, adapt to novel environmental conditions and meet different survival strategies. An example is given by the association to specific pollinators for reproduction that is associated with the cell shape in the epidermis of petals.

Conclusion

Although lysosomal and vacuolar trafficking pathways share conserved core components in their traffic machinery and mechanisms of membrane fusion as well as remodeling, the pathways controlling the biogenesis and physiology of the two types of organelles present clear differences. This strongly suggests a common evolutionary origin of lysosomes and vacuoles accompanied by independent evolution of the pathways leading to these organelles to meet unique functions in different organisms and distinct tissues. The discovery of vacuolinos provided new evidence of the presence of multiple vacuoles in specialized cell-types and produced novel information on the regulation of vacuolar biogenesis opening the way to a deep analysis of the genetics and physiology of this phenomenon which is of vital importance for the cell life and the differentiation of distinct cell-types.

The evidence till now available shows that vacuolinos originate from compartments sharing markers (like BP80) with PVCs/MVBs without solving all questions about

the genesis of these vacuolar type. However, the fact that mutants where vacuolinos are abolished are viable, allows to design experimental approaches to address these questions. Next to shed light on the origin and function(s) of vacuolinos, these studies will possibly provide insights into lytic vacuole formation and development.

Thesis outline

In most plant cells, the vacuole is by far the largest organelle and may take up to 90% of the cell's volume. Vacuoles are multifunctional endomembrane compartments, surrounded by a single membrane called tonoplast, and serve a plethora cellular processes. The large central vacuole in plant cell is involved in the generation of cell turgor, cell expansion, the detoxification of xenobiotics, the accumulation of a multitude of other organic or inorganic compounds and ions and degradation of proteins and glycosides. Unlike other organelles, such as chloroplasts and mitochondria, vacuoles can “differentiate” (i.e. assume distinct functions) depending on the differentiation status of the cells, which makes vacuoles also one of the most elusive of all organelles. Adding to the complexity, it was found some two decades ago that a single plant cell may harbor multiple vacuoles with distinct protein content and distinct functions. The presence of multiple vacuoles contributes to the flexibility of plant cells to execute distinct (conflicting) functions at different developmental stages and/or to adapt to different environmental conditions.

The pathways by which proteins are delivered to vacuoles after their synthesis in ribosomes, and the regulation of a multitude of transport process into or out of the vacuole have been studied for decades. Most of these studies were based on biochemical, electrophysiological or microscopy approaches. Genetic approaches, exploiting mutants that are impaired in vacuolar functions, or protein trafficking pathways to the vacuole, have the advantage they can uncover factors involved in these process that could not be predicted (or identified) otherwise. Such genetic

approaches proved very powerful in yeast, leading to a Nobel Prize for Randy Schekman in 2013, but had little success in higher eukaryotes as in those multicellular species such mutations proved to be very pleiotropic and often lethal.

Despite the pivotal role of vacuoles in the growth and development of plant, the mechanisms underlying the evolution, biogenesis, and function of both vacuoles and additional vacuoles remain largely unknown. This thesis addresses several key aspects of vacuole biology:

- **Chapter 1** reviews the protein endocytic and sorting pathways and the machinery leading to the biogenesis of different types of lysosome/vacuole;
- **Chapter 2** identifies in juice cells of *Citrus* fruits the very same mechanism of vacuolar lumen hyper-acidification (and might suggest the presence of the same type of additional vacuoles) as the one discovered in petals of *Petunia* flowers;
- **Chapter 3** reports the identification of the FADING gene, which triggers the degradation of anthocyanins and fading of the petal color, after opening of the flower bud. This chapter shows that vacuolinos act as a “gatekeeper” of sorting station that prevents specific proteins from reaching the central vacuole
- **Chapter 4** investigates the role of a small GTPase, RAB5a, of petunia. This chapter shows that plant RAB5 proteins are more diverse than previously known, and consist of at least three distinct phylogenetic clades of ancient evolutionary origin. The analysis of loss and gain of function mutants revealed that RAB5a is specifically involved in the formation of vacuolinos.

- **Chapter 5** describes the isolation and first characterization of the *PH7* gene, showing that *PH7* encodes a monosaccharide sugar transporter that is required for the formation of vacuolinos and subsequent traffic from vacuolinos to the central vacuole
- **Chapter 6** summarizes and globally discusses the major results of the thesis and highlights new issues raised from the finding in each chapter. These pin points novelty and contributions to general knowledge and indicates potential directions for future research.

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