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Vesicular Trafficking into the Plant Vacuole

Vezikulární transport do rostlinné vakuoly

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Prohlášení:

Prohlašuji, že jsem tuto práci vypracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatní část nebyla předložena pro získání jiného nebo stejného akademického titulu.

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Abstract

Vacuole is very important plant cell organelle which can occupy 90% of cell volume. It provides wide range of functions. Considering enormous size of the vacuole, vesicle trafficking into the plant vacuole is major vesicle movement inside the cell. Transport pathway into the vacuole is very dynamic field of plant cell biology. This sorting machinery shares similarities within all eukaryotes, but plants also have their own specificities. Soluble cargo is targeted through secretory pathways by vacuolar sorting receptors (VSRs). These proteins due to its transmembrane localization can interact with sorted cargo and take it to the right organelle within the cell. Membrane fusion is facilitated with Rab-GTPase and SNARE protein complexes. A special kind of vesicle traffic is autophagy, the self consuming process that protects the cell from various type of stress or enables apoptosis.

Key words: Vacuole, VSR, SNARE, Rab, Autophagy

Abstrakt (česky)

Vakuola je velmi důležitou organelou v rostlinné buňce, která zajišťuje širokou škálu funkcí. Může zaujímat až 90% objemu buňky. Zvážíme-li obrovský objem vakuoly vůči buňce, váčkový transport do této organely představuje většinu transportu v buňce vůbec. Způsob vybírání a třídění proteinů pro transport je v zásadě podobný mezi všemi eukaryoty, avšak rostliny mají určitá specifika. Solubilní proteiny jsou transportovány pomocí VSR (Vacuolar Sorting Receptors). VSR jsou díky své transmembránové orientaci nejen schopné interagovat se solubilním nákladem, ale také ho směrovat do správné organely v rámci buňky. Fůze membrán je pak umožněna Rab GTPasami a komplexem proteinů SNARE. Specifický transport do vakuoly představuje autofagie, ‘sebekonzumující’ proces, který chrání buňku před různými typy stresu a umožňuje apoptózu.

Klíčová slova: Vakuola, VSR, SNARE, Rab, Autofagie

Abbreviations

PSV-Protein storage vacuole

LV – lytic vacuole

CCV – Clathrin coated vesicles

VSR – vacuolar sorting receptor

PVC – prevacuolar compartment

LPVC – late prevacuolar compartment

RMR - receptor homology domain-transmembrane sequence-RING-H2

TMD – Transmembrane domain

CT – C-terminus

AP – Adaptor protein

TGN – Trans Golgi network

SNX – sortin nexin

MVB – multivesicular body

PAC – precursor accumulating vesicle

BFA – Brefeldin A

TIP – Tonoplast intrinsic protein

PHA – phytohemaglutunin A

NI-mutation - substitution if Asparagine by Isoleucine

CLB – calcineurin like B

ESCRT - Endosomal Sorting Complex Required for Transport

SNARE - soluble N-ethyl-maleimide sensitive factor attachment protein receptor

1 Introduction

The vacuole is an essential compartment in the plant cell which functions are very diverse and play irreplaceable role in the maintenance of cell homeostasis. It can occupy almost the whole volume of the cell and thus influences its physical shape. It provides an environment for metabolic processes and place for storage of nutrients, ions or water, but even for toxic substances. In general, plants must be able to cope with various types of abiotic and biotic stress and vacuoles are necessarily involved in the response to these environmental effects. There are two types of plant vacuoles which diverge in their function and can be distinguished according to the different type of its tonoplast intrinsic proteins (Paris et al., 1996). Protein storage vacuole (PSV) is abundant in tissues with storage function such as seeds or cotyledons. Substances from PSV are mobilized in the case of the need for nutrients; for example, when germination of seeds occurs. Lytic vacuole (LV) is place, where proteins are degraded and is mostly present in vegetative tissues. Proteins determined for degradation in LV can come from PSV or from the cell components in case of autophagy.

Apart from membrane transport, vesicle transport is the main pathway for soluble, as well as membrane cargo into the vacuole. The vesicles can bud from and fuse with various membrane organelles by which they provide communication and movement of particles within the cell and even with the cell surroundings. There are several pathways of vesicle transport into the vacuole. In this thesis I will focus on the mechanism of cargo recruitment provided by vacuolar sorting receptors and on the mechanism of vesicle fusion with the target organelle.

The goal of this thesis is to briefly summarize up-to-date knowledge about key moments in vesicular trafficking into the vacuole.The historical context of process and its methods will also be described.

2 How does vacuolar sorting work?

Knowledge about the vacuolar sorting system is much more dynamic than other topics in this thesis. Many proposed thoughts were afterwards reviewed and refuted. Although many processes are elucidated and confirmed, lot of them are still unknown especially in the plant field.

2.1 Transport of soluble cargo

2.1.1 Vacuolar sorting receptors

Soluble cargo is targeted into the vacuole according to the amino acid sequence either on N- or C- terminus or even inside the protein (Neuhaus et al., 1991, Holwerda et al., 1992). Amino acid targeting sequence is recognized by the vacuole sorting receptor (VSR). This receptor was first found in developing cotyledons of pea (*Pisum sativum)* and labeled as BP-80 due to its molecular weight almost 80kDa (Kirsch et al., 1994). Kirsch et al. isolated it from clathrin-coated vesicles (CCV). These vesicles provide the majority of endocytic processes inside of the eukaryotic cell. Kirsch et al. also postulated the orientation and function of domains of this transmembrane protein. Cargo proteins bind to the N-terminal domain of the BP-80 orientated into the lumen of vesicle whereas the C-terminus of the receptor is exposed to the cytoplasm and provides right vesicle targeting. Properties of this protein was examined by testing its binding ability to already known vacuolar sorting amino acid sequences in protease proaleurain and endopeptidase B in neutral pH. They also showed that when pH was lowered, the receptor and cargo protein dissociates which reflects what likely happens in an acidic environment of further steps of this sorting pathway.

VSRs function was confirmed in a stigma of *Nicotiana alata* (Miller et al., 1999). This experiment was performed using Na-PI precursor protein, which is after departure into the vacuole cleaved into smaller proteins and act as a proteinase inhibitor (PI). PI likely serves as a defense against bacterial, fungal or insect proteases. This process protects plant stigma from the entry of these pathogens (Atkinson et al., 1993). Stigma of tobacco was chosen due to the high accumulation of these inhibitors in the central vacuole. Miller et al. found BP80-Na-PI complex in PVC and thus confirmed that the soluble cargo transport is provided by VSR and also that this pathway leads through PVC. VSRs presence on PVC membrane was proved by VSR signal which merged with PVC membrane marker SNARE protein Pep 12 (Miller et al., 1999).

2.1.2 VSR cargo binding abilities

BP-80 binding to the ligand was closer studied on its soluble form lacking the cytoplasmaic tail and transmembrane domain. This so-called tBP-80 was treated with endoproteinase Asp-N which brought two large fragments, further indigestible by enzyme. According to these results, two main domains of tBP-80, both with an affinity to bind ligand (proalurin in this

case) and the central domain between them were described. The N-terminal domain (molecular weight ~43kDs) has homology to the receptor homology domain-transmembrane sequence-RING-H2 (RMR, see further) and together with the central domain is responsible for ligand binding. C-terminal domain (molecular weight ~38kD) keeps a proper conformation which enables ligand to bind (Cao et al., 2000). The first binding of VSR to lytic vacuolar cargo happens already in ER (Niemes et al., 2010 b). The experiment, which brought the evidence of this idea, was performed by expressing artificial protein construct. It consisted of N-terminal domain from BP80 and than TMD and C-terminal domain from ER resident chaperone calnexin (BP80:CAL). Part of chaperone calnexin was used because it causes retention of bound protein in ER. Similar studies were earlier performed by fusing ligand-binding domain of PV72 with ER targeting motif HDEL in *Arabiopsis* leaves (Watanabe et al, 2004). They showed retention of cargo in ER. However, the localization was not accurate. Proteins tagged with this motive presumably can traffic between ER and the Golgi because the HDEL receptor is placed both on Golgi and ER (Boevink et al., 1998). Calnexin was used as an only-ER marker (Irons et al., 2003). As supposed, upon expression of BP80:CAL, vacuolar cargo was retained in ER and proved VSR-cargo binding in this organelle. These results are also consistent with the fact mentioned below, that VSR-cargo binding occurs in a high concentration of Ca^{2+}, which is sufficient in ER (Niemes et al., 2010 b).

2.1.3 Targeting of cargo is facilitated by C terminus of VSR

Targeting of cargo bound to the receptor, now already inside of the formed vesicle, is facilitated by the C-terminus (CT) of VSR in most cases. This process was examined on *Arabidopsis* homolog of BP-80, AtELP. CT, which is exposed to the cytoplasm contains Tyr motif (YMPL) recognized by clathrin adaptor proteins (APs). This motif is necessary as a single AA mutation causes loss of the binding specificity (Sanderfoot et al., 1998). Another approach used by daSilva et al. in tobacco (*Nicotana bentamiana*). They fused TMD and CT from BP-80 with GFP and formed a chimeric protein that can undergo transport pathway. They also constructed the same chimeric protein but with deleted CT and then compared the distribution of both. GFP-BP80 colocalized with PVC marker Pep12 tagged by YFP, whereas mutant variant lacking CT was partially retained in ER, Golgi and intriguingly even slightly in PVC. These results indicate CT importance in transport. Nevertheless, according to the presence of truncated protein in PVC they also suggested that some cargo can reach the

vacuole without specific targeting sequence only with transmembrane domain of sorting receptor that enables receptor to leave ER (daSilva et al., 2006).

C-terminal domain of the VSR provides right targeting due to its amino acid sequence with conserved Tyr-based motif as mentioned above. The exact mechanism of depletion and recycling, was performed more closely by mutation of the C-terminal VSR motif YxxΦ (Y-tyrosine, x- any AA, Φ-big hydrophobic AA) on different spots. When Y mutated, anterograde transport to PVC was abolished, whereas mutation of Φ led to disruption of retrograde transfer resulting in the situation where VSR-cargo leaked to LPVC (Foresti et al., 2010).

The whole targeting machinery is rather complex as shown in *Arabidopsis* (Song et al., 2006) and apart from VSR and AP it also comprises of the protein EPSIN1 or VTI11 from SNARE family.

VSR-ligand binding happens in high concentration of Ca^{2+}. The experiment was performed by testing PV72, the pumpkin homolog of BP80 (see further) binding ability to pro2S albumin. The significant importance of calcium ions is that they help to hold the bind between the receptor and ligand even though pH is lowered to 4.0, which usually leads to dissociation of the VSR-ligand complex (Kirsch 1994). Mechanism of this stabilization effect includes the conformational change of PV72, due to Ca^{2+} influence on the receptor protein (Watanabe et al., 2002).

2.1.4 VSR recycling, the retromer movement

After targeting and fusion with PVC, it would be very wasteful if the valuable receptors continued to thevacuole and were degraded. Instead of that scenario, receptors are depleted and recycled (daSilva et al., 2005). As the receptors are depleted, PVC matures and transforms into LPVC. Not all receptors are recycled, the pathway also includes renewal of damaged or nonfunctional ones (Foresti et al., 2010). The maturing process is accompanied with membrane changes and the final fusion process is highly dependent on proteins from the Rab family (Rab5, Rab7) (see chapter Rab GTPasis). This model has been taken as the general opinion on recycling of VSR, supported by evidence of VSR in PVC (mentioned above) (Figure 1. A). Recently, another view was postulated considering the retromer vesicle movement (Figure 1 B)

Retrograde pathway is served by vesicles called retromers. Although their importance in vacuolar transport has been pointed out, their accurate localization or binding specificity remains unclear. They were isolated as the yeast homologues from *Arabidopsis thaliana* and also their interaction with VSRs was proposed (Oliviusson et al., 2006).

Closer studies of retromers revealed a new interesting view on the VSR cycling. Niemes et al. discovered that not PVC, but TGN is likely the place where recycling of VSR (BP80) occurs. Evidence of this idea comes from the observation that retromer components, sortin nexins 1 and 2a (SNX1/2a), colocalize with the TGN in both tobacco and *Arabidopsis* cells. When the retromer pathway is abolished, VSR shows accumulation in TGN so presumably this is the place of vesicular return. This happens when sortin nexins 1 and 2a (SNX1/2a) are simultaneously inhibited. This inhibition can be provided either by RNAi knockdown or by mutation of both variant of SNXs. Whole VSR cycling is then from the ER to TGN and back (reviewed in Robinson et al., 2012). The earlier observed VSR signals in PVC are likely receptors, which are determined for degradation. This concept is consistent with the idea of TGN maturing to multivesicular bodies (MVB) (Niemes et al., 2010a; Scheuring et al., 2011).

It was suggested, that even though the retromer recycling pathway is abolished, downstream trafficking (from TGN to vacuole) to the vacuole should continue without any changes. This idea was confirmed by the observation that mutation of above mentioned nexins did not interfere with cargo delivery into the vacuole. These results indicate that VSR (BP80) are not involved in transport any further than to TGN (Niemes et al., 2010a).

Figure 1: Two models of VSR mediated transport into the vacuole (Lousa et al., 2012). (A) Classic VSR transport model: Transport into the PVC is provided by CCV (Kirsch et al., 1994). Receptors are depleted from the PVC by which it maturates into the LPVC (Foresti et al., 2010). (B) Alternative model: TGN maturing into the PVC (Scheuring et al., 2011), receptors are recycled already in the TGN (Niemes et al., 2010a)

VSR recycling was also tested by wortmannin treatment. Wortmannin is a stereoid fungal metabolite, which in small doses specifically inhibits phosphoinositide 3-kinases (PI3K). This causes depletion of the phosphatidylinositol 3-phosphate (PI3P) – an important endosomal membrane lipid, which is recognized and bound by SNX1/2 proteins (Yu et al., 2001). Thus wortmannin inhibits also retromer mediated receptor recycling to the TGN. DaSilva et al. proposed that if wortmanin causes abolishment of the receptor depletion, they are expected to end up in the vacuole. Experiments with BP-80 fused with GFP proved this suggestion as the high amount of fluorescence signal was observed in the lumen of the vacuole, upon

wortmannin treatment. Moreover, overexpression of BP-80 can partially suppress the effect of wortmannin by replenishing receptor amount which would not happened if wortmannin interfered with anterograde transport. All proteins which take part in this process are taken to the vacuole during the wortmannin treatment instead of being recycled such as SNARE or others. Naturally, the suppression of wortmanin effect by overexpression of BP-80 works only until other proteins became limiting. (daSilva et al, 2005).

2.1.5 VSR role in protein storage

VSR are among other roles very important in seed development because they provide the transport of precursors of proteins into the storage vacuole. Receptors providing this transport was identified in developing pumpkin cotyledons (*Curcubita sp.*) and named according to their molecular weight PV72 (72 kDa) and PV82 (82kDa). The binding ability of these putative receptors was investigated using pro2S albumin (the precursor of storage protein 2S albumin) already known to be transported into storage vacuole in pumpkin cotyledons (Harahishimura et al., 1993). Pro2S albumin and PV72 and PV82 coexist in so-called precursor accumulating vesicles (PAC) which brings cargo into the vacuole. These vesicles bud already from ER and interestingly, they reach the vacuole bypassing Golgi. It's assumed that reason of this Golgi independent pathway is that they do not need to be glycosylated (Hara-Nishimura et al., 1998). Nevertheless, PAC vesicles can be enriched by cargo from Golgi, which is recruited by VSR (Hara-Nishimura, 2004). By affinity chromatography analysis using cleaved pro2S albumin into three parts containing C-, N-terminal region or internal sequence, it was found that PV72 and PV82 binds either to the C-terminus or internal sequence of pro2S albumin, but not to the N-terminus which shows where the signaling sequence is placed. The retention ability of C-terminal propeptide in those experiments was weaker than of the internal one, but it was likely caused by bad accessibility of the binding place (Shimada et al., 1997). The storage cargo is transported also by the Golgi dependent pathway. This transport is served by so-called dense vesicles which take cargo to the PSV, as examined in pea cotyledons (Hohl et al., 1996)

In seed development, another interesting phenomenon appears. The total amount of VSR in seeds decreases during the germination from day 1 to day 3. The majority of the VSR is found in peripheral membrane of MBV in the first day of germination. This VSR are involved in recycling pathway. Interestingly, in the third day the majority of them were observed in the lumen of the MVB and determined to be digested in the vacuole. However, it is not yet known, how internalization works (Lam et al., 2007).

2.1.6 VSR in A. thaliana

In *Arabidopsis thaliana,* there are present seven members of the VSR group (Hadlington and Denecke, 2000; Paris and Neuhaus 2002). VSR1, VSR3 and VSR4 share high sequence similarity to each other, as well as to pea BP-80 and pumpkin PV72, which takes part in the transport of storage proteins (Kirsch et al. 1996). VSR found in A. thaliana (AtVSR) was named *A. thaliana* Epidermal growth factor receptor-Like Protein (AtELP) (Ahmed et al., 1997). The evidence of VSR involvement in *Arabidopsis* vacuolar trafficking was performed using the VSR mutant of *AtVSR1/AtELP*. Instead of the vacuole, the cargo for storage was targeted outside of the cell. It was confirmed that AtVSR1 mediates transport of proteins to the PSV. Nevertheless, the effect of mutation did not cause the complete abolishment of the transport. Sorting and transport of proteases into the lytic vacuole was not influenced by *atvsr-1* mutation, which indicates that this type VSR does not mediate pathway into lytic vacuole (Shimada et al., 2003). Latest studies brought evidence that VSR1, VSR3 and VSR4 takes part in sorting of proteins to either lytic or storage vacuole in vegetative cells of *Arabidopsis*. It was observed that mutants of these receptors caused defects in the transport pathway, such as retention of proteins in ER or higher secretion outside of the cell. Defects of the trafficking were restored by expressing of the wild type form of VSR mentioned above (Lee et al., 2013).

2.1.7 RMR

Other vacuolar sorting receptors were discovered in Arabidopsis thaliana. They are called RMR (receptor homology domain-transmembrane sequence-RING-H2). Homology of the first domain is to the N-terminal sequence of VSR. C-terminal domain labeled RING-H2 is longer that in VSR. Unlike VSR, the binding of cargo to RMR is pH independent and the receptors do not recycle, but they are internalized into the vacuole (Park et al. 2007).

Soluble cargo in the above described pathway moves either to the lytic or storage vacuole.On the other hand, membrane cargo is transported into the vacuole differently. Special targeting of protein on the plasmatic membrane (PM) of the cell, but also in the membrane of Golgi is facilitated by the protein ubiquitin (see chapter Vacuoalr transport of membrane attached proteins).

2.2 Vacuolar transport of membrane attached proteins

Membrane proteins undergo different transport pathways into the vacuole than soluble cargo. A simple experiment proves this was based on the different effect of drugs Brefeldin A (BFA) and monesin on either soluble (phytohemaglutinin, PHA) or vacuolar membrane proteins (tonoplast intristic protein, TIP). TIPs are commonly used as tonoplast markers, of both lytic and protein storage vacuole (Jauh et al, 1999). BFA blocks protein transport from ER to Golgi. Monesin affects TGN, where due to the interference with proton gradient on the membrane, causes missorting of proteins in TGN. Both drugs inhibited the transport of PHA, but neither of them influenced the transport of the α-TIP (Gomez and Chrispeels, 1993). These results suggested that transport of soluble and membrane proteins follow different pathways.

2.2.1 Membrane protein transport with respect to the Rab proteins

Recent studies have provided evidence of different transport of soluble and membrane cargo with respect to the function of Rab GTPases in tobacco leaf epidermis cells (Bottanelli et al., 2011). Several proteins from the Rab family facilitate transport of cargo targeted into the vacuole in a sequential manner. Therefore, mutations in different Rab-GTPases cause specific transport defects during the steps of the vacuolar trafficking pathway. Bottanelli et al. used this approach and it yielded interesting results. Asn-to-Ile substitution (NI) in the nucleotide binding domain of the Rha1, Ara6 and Rab7 causes dominant-negative defect, as these GTPases lose their ability to bind GTP (Jones et al., 1995). This affects the transport of α-TIP tagged with YFP. The same effect revealed soluble protein cargo irrespectively of the placement of the sorting signal. For Rab11 mutant no effect was observed. From these results we can conclude that α-TIP-YFP undergoes the same pathway as soluble proteins even though it is inconsistent with earlier found results (Gomez and Chrispeels, 1993). The defective α-TIP-YFP localization was compared to the distribution of protein from Caclineurin B-like family, which is involved in cell response to calcium. CBL6 is the tonoplast marker (Batistič et al, 2010). CBL-RFP transport was not affected by the mutation of Rha1, Ara6 nor Rab7, which suggests that it undergoes different transport during whole pathway than α-TIP-YFP (Bottanelli et al., 2011). Another strong evidence of different trafficking pathway is that CLB6 transport is not served by CCV (Batistič et al., 2010).

2.2.2 Transport of Vam3 protein

Another membrane protein, Vam3, a member of SNARE family, was also found to exploit a different pathway than soluble cargo. Vam3-GFP transport was disrupted by the Rab7 mutant and Vam3 was retained in the post-Golgi structure. As Rab7 is placed on the vacuolar membrane, it likely contributes to the Vam3 transport to the tonoplast (see chapter Rab GTPasis). Therefore by Rab7 mutation, the final part of the transport was abolished. By NI mutants of Rab11 and Rab5, the Vam3 was transport unaffected. This implies, that transport of membrane bound Vam3 undergoes different pathway than soluble proteins and α-TIP-YFP, but likely only until it reaches PVC (Bottanelli et al., 2011).

2.2.3 Ubiquitin transport pathway

A special transport pathway into the vacuole is mediated by the protein ubiquitin. This protein is known to provide internalization of PM proteins. Ubiqitin targeting of PM proteins was examined on two protein constructs: Box-GFP protein, the membrane reporter and RFP-TMD23, the transmembrane reporter (Scheuring et al., 2012). Box-GFP owns its name due to the conserved glycin/cystein motif (denoted as GC-CG box) which is among other AA present in the domain of type II GTPase AtROP10 from *Arabidopsis*. This domain is able to post-translational attach to the PM, when it is fused to C-terminus of GFP (Lavy and Jalovsky, 2006). RFP-TMD23 is protein, which goes through the whole membrane and reaches the PM by vesicle trafficking. Both proteins have been observed to undergo endocytic pathway, after fusion to ubiquitine. Interestingly, they revealed a difference in vacuolar targetting between the transmembrane proteins and proteins, which are only attached to the membrane. The RFP-TMD is transported up to the vacuole, whereas Box-GFP is observed to arrive at the MVB, but not any further. Box-GFP proteins are likely released from the membrane as the interactions are not so strong and end up in thecytosol (Scheuring et al., 2012).

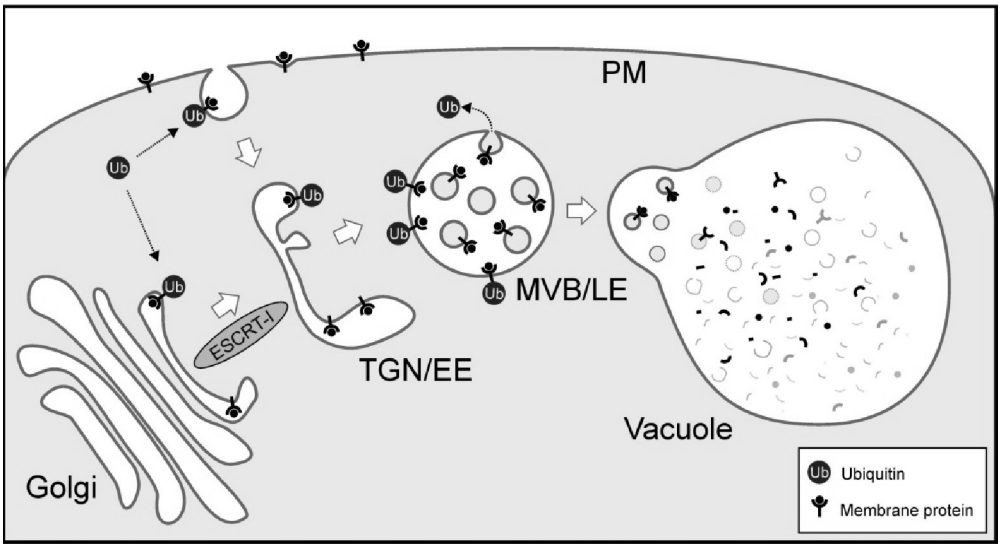

Figure 2: Ubiquitin targeted transport of membrane proteins into the vacuole (Scheuring et al.,2012).

Ubiquitin is also involved in targeting of membrane proteins localized at Golgi. Experiments proving this were performed on protein construct consisting of cytosolic domain of Golgi marker RFP-TMD20 (Brandizzi et al., 2002) fused with either ubiquitin (Ub) or UbΔGG (ubiquitin lacking two terminal glycins), both of which were transported to the lytic vacuole.

It was showed, on example of common vacuolar soluble cargo – aleurain, that proteins fused with ubiquitin undergoes a similar pathway as biosynthesized vacuolar cargo. Transport pathway of protein constructs RFP-TMD23-Ub and RFP-TMD20-Ub, as well as aleurain tagged with GFP was perturbed when one protein associated with ESCRT complex was mutated (Scheuring et al., 2012).

3 Prevacuolar compartment and Late prevacuolar compartment - MVB/PVC and LPVC

PVC is an intermediate cell structure which mediates transport of the proteins from the Golgi apparatus to the vacuole. Due to its structure, it is also called multivesicular bodies (MVB). MVB were firstly taken as a distinct organelle from PVC. Later on, using imunogold labeling on BY-2 cells, it was discovered that these two structures do not differ because VSRs, which were considered as PVC markers, colocalized with MVB membrane (Tse et al., 2004).

3.1 Transport to the vacuole occurs via PVC

The idea of a transient organelle exists between Golgi and the vacuole in plants was proposed when vacuolar sorting receptors were examined. VSR from *Arabidopsis thaliana* colocalized with a membrane organelle which can be distinguished according to its density from ER, Golgi, PM and the vacuole. This intermediate organelle was postulated as a plant counterpart of mammalian or yeast endosome. It was supported by the fact that it fractionates comparable to animal and yeast endosomes (Ahmed et al,. 1997). Another approach to elucidate this, in that time still putative intermediate structure, was used by Paris at al. during examination of the pea (*Pisum sativum*) root tip. In this study, vacuolar sorting receptor BP-80 was shown to localize in the Golgi and the PVC by immunogold labeling (Paris et al. 1997). They also found that the BP-80 cycles between Golgi and prevacuole and this traffic is provided by CCVs (Paris et al., 1997).

Observations using fluorescence microscopy also contributes to knowledge about MVB/PVC. Lam et al. used BP-80 from BY-2 cells lacking the N- terminal domain fused with GFP to characterize these structures (Lam et al, 2007).

Another method to study MVB/PVC dynamic and localization is treatment with drugs such as wortmannin or brefeldin A. These methods can also provide evidence that MVB/PVC differs from Golgi (Tse et al., 2004). Apart from wortmannin interference with the VSR recycling pathway mentioned above (see chapter about VSR), the drug also causes fusion of internal vesicles, as well as swelling of the whole PVC, which was showed on mung bean seeds. It was suggested that the internal vesicles enables PVC enlargement by their membrane fusion. Interestingly, this process has been observed only in plants (Wang et al, 2009). Brefeldin A (BFA) has different effects on MVB/PVC in plant cells than wortmannin. The influence of BFA on the vacuolar transport is dose-dependent. In low concentrations it affects ER and Golgi. In high concentrations it causes MVB/PVC and Golgi aggregation in BY-2 cells. These two distinct origin aggregates stay distinguishable from each other, according to which we can easily and quickly differentiate MVB/PVC from Golgi (Tse et al, 2006).

3.2 Multivesicularity of the PVC

MBV/PVC is fulfilled with the intralumenal vesicles (ILV) which are formed by budding from TGN. There is a direct and sequential connection between TGN and MVB, which is confirmed by experiments showing that abolishment of TGN function leads to inhibition of MVB formation (Scheuring et al., 2011). The mechanism of budding is relatively new found and very interesting. It is provided by Endosomal Sorting Complex Required for Transport (ESCRT) proteins and differs basically from earlier described processes of vesicle budding (CCV) in the cell. The basics of the membrane budding was studied on *Sachcromyces cerevisiae*. The vesicles bud into the MVB to form ILV due to the ESCRT I and II proteins, which assemble at the bud neck (Fig.3.b). ESCRT proteins are placed on the internal side of the rising vesicle. There they induce its forming and stabilize it, but without making any coat around the vesicle as CCV. Scission, which occurs afterwards by ESCRTIII protein also differs from dynamin mediated detachment of CCV, since it is provided from the inside of the neck (Wollert et al., 2009, Wollert and Hurley 2010).

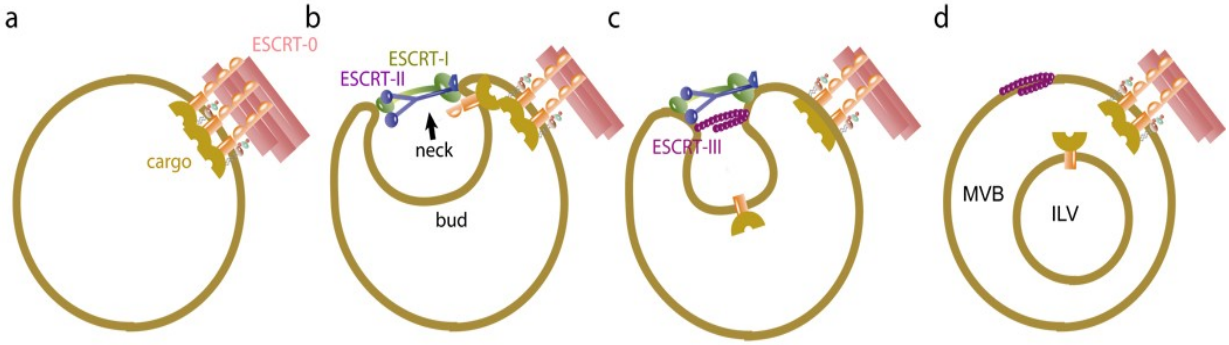

Figure 3: Mechanism of ILV budding (Wollert and Hurley 2010). Plants do not have ESCRT-0 components (Reyes et al., 2011)

In closer examination of the importance of ESCRT proteins in trafficking pathway in plants, the dominant negative mutant of VPS2 protein (part of ESCRT III) was expressed in tobacco protoplasts. Due to this mutation, soluble vacuolar cargo markers amy-spo and spo-GFP were not transported properly. It also caused colocalization of TGN and MVB markers. These results indicate that ESCRT protein plays a role in trafficking of proteins targeted to the vacuole.

When the localization of ESCRT was examined in high-pressure frozen root cells of *Arabidopsis*, it was observed that one component of ESCRTI, the VPS28, colocalizes with Golgi and TGN and not with MVB. On the other hand, experiments in tobacco protoplasts revealed colocalization of other ESCRT components with both TGN and MVB. This inconsistent localization occurs probably because of the different time of release from the membrane. Using the RNAi-mediated knockdown method, it was confirmed the role of another protein which contributes to the TGN to MVB pathway, annexin (ANNAT3). This experiment brought similar effects as when the VPS2-DN mutant was expressed (Scheuring et al, 2011).

3.3 V-ATPase influences the vacuolar transport

The process of the Golgi to MVB/PVC transport is influenced by the proton transporter V-ATPase which is present on TGN membrane. The importance of V-ATPase was examined by using the inhibitor of this enzyme Concanamycin A both on the tobacco (Matsuoka et al., 1997) and *Arabidopsis* cells (Dettmer et al. 2006). Inhibition with ConcA causes retention of the TGN cargo in Golgi, so naturally, the formation of the MVB radically decreases, even in only a short time treatment. MVB itself was not found to content the V-ATPase (Scheuring et al., 2011). As the VSRs dissociate in an acidic enviroment, the absence of V-ATPase in MVB can suggest that VSRs depletion occurs already in TGN, which is consistent with recent findings (Niemes et al., 2010a).

3.4 Pathways to storage and lytic vacuole merge on the MVB/PVC level

Interesting questions rise about the MVB/PVC role in targeting into different types of vacuoles which can exist within one cell. Naturally, the protein storage cargo and the lytic cargo do not leave Golgi in the same vesicles because otherwise storage proteins would be digested already in transport vesicles. Vesicles with both types of cargo fuse afterwards to form MVB (Otegui et al., 2006). In the *Arabidopsis thaliana* embryo, lytic processes such as pro 2Salbumine cleavage to mature 2S albumine start in MVB. This digestion likely starts already in MVB because it offers to process the proteins in a sequential manner. The gradient in organelle acidification from Golgi to vacuole, which enables progressive activation of protein processing enzymes, contributes to this idea (Otegui et al., 2006). Later studies confirmed that both lytic and storage protein cargo reaches the same MVB/PVC using

aleurain-GFP and 2S proalbumin-GFP in Arabidopsis and tobacco BY2 cultured cells (Miao et al., 2008).

3.5 MVB/PVC in seed development

MVB/PVC is very important in seed development and germination. In mung bean seed development VSR are thought to transport proteases to MVB/PVC. In mature seeds MVB/PVC carry proteases and provides physical separation of lytic enzymes from storage proteins and do not fuse with protein storage vacuole (PSV) until the early germination starts. Only then MBV/PVC coalesces with PSV and serves as an intermediate organelle to transport so far stored proteases which afterwards cleave storage proteins and enables germination (Wang et al., 2007). Experiments on germinating mung bean seeds using confocal imunofluorescence and imunogold EM methods showed colocalization of VSR and aleurain to MVB and so proved that their transport to the vacuole happens through MBV (Wang et al., 2007).

3.6 Secretory and endocytic pathway meets on PVC level

It was also postulated that PVC is the place where endocytic and secretory pathway meet. Fuorescent markers of endocyted vesicles, which helped to elucidate the problematic of cell endocytosis was first found in yeast and afterwards successfully applied in plants. These fluorescent styryl dyes, such as FM 4-64, internalize into the plasma membrane and undergo the endocytic pathway up to the vacuole (Vida and Emr, 1995). In plants, it was discovered that FM4-64 colocalize with PVC in tobacco BY-2 cells, which confirmed that endocyted cargo is transported to the vacuole through PVC (Tse et al., 2004).

3.7 LPVC

Late prevacuolar compartment (LPVC) is a further step in transport pathway into the plant vacuole. This intermediate organelle was proved by Foresti et al. in 2010 during examination of VSR. The evidence of LPVC is given by observing its marker Rab5 GTPase Rha1 (Foresti et al, 2010). The biogenesis of LPVC is most presumably from PVC by gradually maturing and depleting of receptors.

4 Rab GTPases

Rab GTPases are necessarily involved in vesicular trafficking. They regulate vesicle budding, tethering and fusion with target membranes during endocytosis and trafficking processes inside the cell (Zerial and McBride 2001). Some of these GTPasis are also linked with cytoskeleton, probably as a molecular motor (for example Rab 6, Echard and others 1998). In general, Rab GTPases are small GTPases from the Ras family. Rabs are present across the whole eukaryotic domain. 70 Rab proteins have been identified in Homo sapiens, 29 in Drosophila melanogaster and 57 in Arabidopsis thaliana. In fact, the Rab protein branch is the largest in the Ras superfamilly (Pereira-Leal and Seabra, 2001).

4.1 Active and inactive state of Rab protein

Rab GTPase can bind either with GDP or GTP. In an inactive Rab-GDP bound state the molecule is in cytosol bounded to the GDP-dissociation inhibitor (GDI). GDI can deliver certain Rabs to membrane compartments (Pfeffer 1995). When the Rab-GDP-GDI complex is delivered to the target membrane, GDI is released. Dissociation of the GDI from the Rab GDP is provided by the membrane-bound protein factor GDF (GDI-displacement factor, DiracSvejstrup, 1997). After fusion is Rab-GDP detached from the target membrane by GDI to start a new cycle (Pfeffer et al., 1995).

The molecule is inactive in the Rab-GDP state, but as soon as specific GEF (Guanin nucleotide exchange factor) exchanges GDP for GTP, the molecule becomes active. Binding of GTP leads to conformational changes, which afterwards activate a function of downstream effector(s) by allowing them to bind at the Rab-GTP binding site. Due to simple regulatory functions and ability to tansfer the upstream signal to downstream effector(s), Rabs are sometimes denoted as molecular switches (Takai et al 2001). Interesting question is what really regulates this mechanism, which signal switches Rab GTPasis on? The termination of the active Rab form is served by GAP (GTPase activating protein) which causes the hydrolysis of GTP to GDP (Trahey and McCormick, 1987).

4.2 Several Rabs participate in vesicle trafficking into the vacuole

In *Arabidopsis thaliana,* there are 57 loci for Rab GTPase proteins. These are divided into eight subfamilies which are marked with capital letters from A to H. This partition was realized according to the homology with mammalian Rabs (Rab1, Rab 2, Rab5, Rab6, Rab7,

Rab8, Rab11 and Rab 18) (Pereira-Leal and Seabra, 2001). There are opinions suggesting further subdividing up to 18 subclasses of the Rab group (Rutherford and Moore, 2002). As this thesis is about the transport to the vacuole, I will focus here on plant homologues of animal Rab 5 and Rab 7, and Rab11. All of them play an important role in vesicle trafficking to the vacuole.

When Rab mutants (with aminoacid substitution in nucleotide binding domain, see chapter 2.2.1) were examined, mutation in Rab 11 showed a decrease of trafficking intermediates of the soluble cargo in a post-Golgi area towards the vacuole; on the other hand, mutation in Rab5 homologues and particularly Rab7 showed an increase. This examination points out that these proteins take part in the transport to the vacuole sequentially (Bottenelli et al., 2011).

4.3 Rha1, Ara6 and Ara7 are homologues of animal Rab5

Animal Rab5 homologues in Arabidopsis thaliana are Rha1, Ara6 and Ara7. Whereas Rha1 and Ara7 share a very similar amino acid sequence (Sohn et al., 2003), as well as C-terminal lipid modification, Ara6 unusually modified on N-terminus by myristolation does not show that high homology as Rha1and Ara7, respectively.

Rha1 protein plays a role in trafficking of soluble cargo to the vacuole (Sohn, et al. 2003). Its contribution to this pathway was examined by the expression of sporamine fused with GFP (Spo:GFP) alone in protoplast in contrast to the expression of Spo:GFP and dominant-negative mutant Rha1 fused with RFP (RFP:Rha1(S24N)) within the cell. In the cells, where were only Spo:GFP after 48 hours, approximately 70% of Spo:GFP was transported through the ER network into the vacuole, whereas in cells transformed also with the mutant RFP:Rha1(S24.N), only 6% of Spo:GFP was present in the vacuole (Sohn, et al.2003). This experiment shows that Rha1 GTPase mutant perturbs the transport of sporamine fused with GFP to the vacuole. Analogical experiment was performed with the mutant of Ara6, another Rab5 homolog which is considered to be involved in endocytosis. However, targeting of Spo:GFP was not interrupted by expressing this mutant, so Ara6 is not necessarily involved in the trafficking of cargo proteins into the vacuole. (Sohn, E. J. et al. 2003).

According to a new investigation, the expression of mutant Rab5 homologues not only causes a disorder of vacuolar transfer, but also an overdose of wild types of these proteins can bring vacuolar transport defects (Bottanelli et al., 2012). Moreover, these defects are not equal within Rab5 plant homologues, Ara6 and Rha1. Ara6 shows a higher sensitivity to even a slight increase of its expression, which causes the fusion of PVC and LPVC. When a stronger

promotor to express Ara6 WT is used, TGN, PVC and LPVC fuse. In contrast, when Rha1 WT is expressed with a strong promotor, fusion of the PVC and LPVC appears.

Further examination shows, that Rha1 colocalize to the prevacuolar compartment (PVC) (Lee et al., 2004), which confirms the role of Rab1 in vacuolar trafficking and also the idea that cargo is transported into the vacuole through the PVC. In the time of these experiments, it was not clear, how the transport from PVC to the vacuole actually works. Later on, Foresti et al. discovered an intermediate compartment between the PVC and Vacuole (Foresti et al., 2010). It is called late prevacuolar compartment (LPVC). They also postulated an opinion that the transport from PVC to LPVC is not provided by vesicles, but by PVC gradually maturating into the LPVC. This process is accompanied with recycling of vacuolar sorting receptors (VSR) back to the beginning of the secretory pathway. Recent studies show, that when expressed from a weak promoter, Rha1 and Ara6 co-localize with the LPVC marker (Botanelli, F. et al. 2012).

4.4 Plant Rab7 colocalizes with vacuolar membrane tonoplast

Another GTPase involved in vacuolar transport is the homolog of animal Rab7. In recent studies, it has been examined and confirmed a Rab7 colocalization with the vacuole membrane, tonoplast (Botanelli, F. et al. 2012). The experiment was performed on tobacco leaf epidermal cells, where CFP-Rab7 was expressed using a strong promotor from Cauliflower Mosaic Virus 35S (CMV 35S) at first. However, this approach did not reach its goal. Overexpressed CFP-Rab7 appeared in cytoplasm on a high level and scientists were not able to detect its exact localization. Accumulation of CFP-Rab7 likely happens because of its incomplete post-translational modification. If the necessary modification, when Rab7 acquires a C-terminus lipid anchor is not done, Rab7 remains in cytoplasm and cannot be localized properly on a subcellular level. However, when expressed with a weaker promotor, Rab7s localization to tonoplast was clearly visible. CFP-Rab7 colocalized with tonoplast marker YFP-Vam3 on the membrane around the vacuole and even on intracellular bulbs and transvacuolar strands. Unlike in Rab5 homologues the overexpression of wild-type Rab7 doses not influence vacuolar sorting, which is only defected when Rab7mutant is expressed (Botanelli, F. et al. 2012). Interestingly, vacuolar sorting defects of this nucleotide-free mutant cannot be suppressed by adding Rab7 wild-type or its supposed GEF Vam6. This negative dominant feature of Rab7 has several explanations. Either Vam6 does not serve as GEF of Rab7 or there is some other strange part of the pathway titrated by the Rab7 dominant negative mutant.

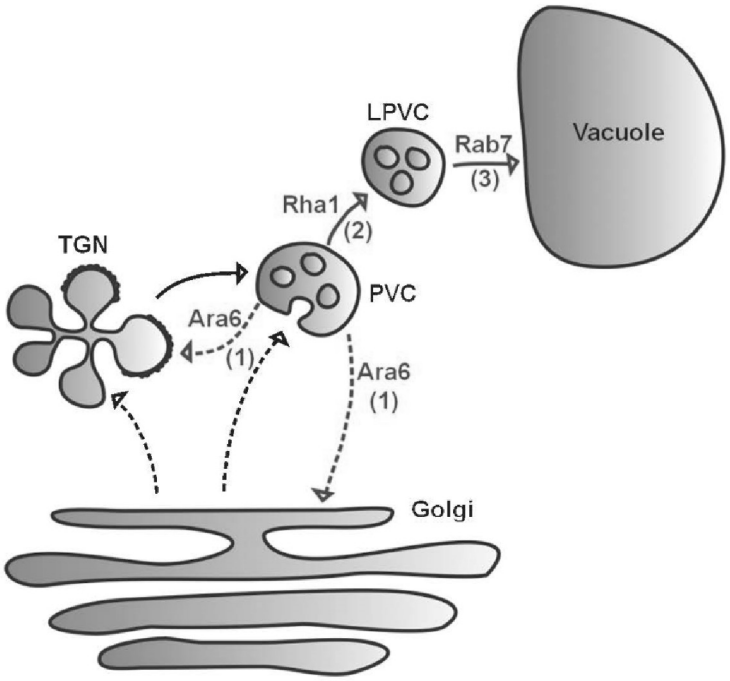

Figure 4: Rab5 and Rab7 role in the secretory pathway. (1) Ara6 is involved in recycling of components. (2) Rha1 likely plays a role in PVC to LPVC maturing. (3) Rab7 provides final fusion with the vacuole (Bottanelli et al., 2012).

4.5 Rab11 might be involved in vacuolar sorting

Plant protein, which shows highest similarity to Rab11, is named RabA. Rab 11 is present in yeast and animal cells; however, plant specific is the great expansion of the genes coding it. In *Arabidopsis thaliana*, 26 out of 57 encoded Rab GTPasis belong to the Rab 11 subfamilly (Pereira-Leal and Seabra, 2001). Rab11 was not speculated as a member of the pathway transporting cargo towards the vacuole until Botanelli et al. proposed its assumed role in vacuolar sorting. Rab11 may influence vesicular trafficking for the Rab11 (NI) mutant causes the reduction of the post Golgi structures (Botanelli et al., 2011).

5 SNARE proteins

SNARE proteins are essential for vesicle trafficking, more precisely vesicle fusion, in every eukaryotic cell. The name SNARE is an abbreviation of soluble N-ethyl-maleimide sensitive factor attachment protein receptors. SNARE proteins include syntaxin (Bennett, et al. 1992),

SNAP 25 (synaptosome-associated protein with molecular weight 25kDa, Oyler et al, 1989) and VAMP (vesicle-associated membrane protein, Trimble et al., 1988). These proteins have two domains, one of which is anchored in membrane (transmembrane domain, TMD) and the other forms coiled-coil SNARE motif. Altogether, they form a fusion complex.

5.1 SNARE structure

The core of the fusion complex consists of a longitudinal bundle of four coiled proteins (Sutton et al, 1998). In the middle of the bundle is an ionic layer denoted as a layer 0, which is highly conserved in its amino acid content. Every helix contributes to this layer with one amino acid, three of them with glutamine (Q) and fourth with arginine (R). According to these amino acids SNARE proteins are distinguished as Q- and R- SNARE (VAMP) (Fasshauer, D et al. 1998). Q-SNAREs are further classified as Qa-SNARE (syntaxin), Qb-SNARE (SNAP N) and Qc-SNARE (SNAP C) (Bock et al, 2001). Complete SNARE complex contents one motif from each subgroup (Qa-, Qb-, Qc and R-SNARE) (Jahn et al. 2003).

SNAREs have been postulated as proteins which constitute minimal machinery for membrane fusion (Weber et al, 1998). The experiment confirmed that SNARE proteins assembly is necessary for fusion. Later on, the mechanism of assembly was proposed. As a first step of assembly of synaptic fusion complex, it has been considered association of the SNAP 25 (consisting from two helixes Sn1 and Sn2) and the syntaxin. Then synaptobrevin binds to this complex. The core of fusion complex is held together with hydrophobic interactions, and the surface is exposed into the solvent then by interactions as salt bridges or hydrogen-bonding. Subsequently, the strains of helix complex provide membrane fusion (Sutton et al. 1998).

5.2 SNARE localization

The subcellular localization of different SNAREs was examined on *Arabidopsis thaliana* protoplasts in suspension cultured cells. The experiment was performed using the CaMV 35S promoter to express SNARE proteins tagged with GFP. It was found that the majority of SNAREs are localized in a single compartment, but some of them are localized in dual or even trial compartments. This indicates that SNARE shuttle between these organelles. The interesting question is what actually determines SNARE localization in different cell organelles. It was speculated that the length of transmembrane domain is a critical factor, but this hypothesis were afterwards found wrong. According to an analysis of TMD lengths it was discivered that SNAREs are mostly conserved within a phylogenetic group and diverge among these groups. Thus it was postulated that subcellular localization of SNARE (found in

Arabidopsis) is not dependent on the length of its TMD. This idea was confirmed by amino acid sequence analysis comparing SNAREs in *Arabidopsis*, *C.elegans*, *Drosophila* and *S. cerevisiae* with one exception – VAMP proteins of Arabidopsis (Uemura et al. 2004).

5.3 SNARE proteins plays key role in transport into the vacuole

The transport pathway of *Arabidopsis* into the vacuole is served by nine SNAREs. Two of them belong to the Qa-SNARE group are labeled SYP21/ AtPep12 and SYP22/AtVam3, another two are from Qb-SNARE group AtVTI11 and AtVTI13, SYP51 and SYP56 from Qc-SNARE group and AtVAMP711, AtVAMP712 and AtVAMP713 representing R-SNAREs. These proteins provide transport between TGN (where overlap), PVC and vacuole by cycling between TGN and PVC or the PVC and vacuolar membrane. Retrograde movement from PVC to TGN is also possible (Uemura et al., 2004).

SNARE mediated transport into a plant vacuole is vitally important. For example when the gene for SYP21 out of syntaxin group was disrupted, male gametophyte lethality occurred (Sanderfoot et al., 2001b). Further examinations discovering the nature of this phenotype were realized by overexpressing this protein. Overexpression of SYP21 interfered with transport of vesicles from the PVC into the vacuole. Instead of the vacuole, soluble cargo was targeted to the cytoplasmic membrane. Explanation of this phenomenon is either that the incomplete SNARE complexes were assembled which led to titration and so proper complexes could not be formed or that the fusogenic complexes do not dissociate (Foresti et al., 2006).

Another example of importance of SNAREs, now from the R-SNARE group, is the VAMP727. It belongs to one subgroup of the R-SNAREs – longins. Longins have 120 to 140 amino acid N-terminal domain (the longin domain) unlike the second R-SNARE subgroup – brevins. VAMP727 is seed plant unique protein (Sanderfoot 2007), which plays an important role as a component of the SNARE complex. It assembles with SYP22, SYP51 and VTI11 to form a fusion complex which mediates the fusion of PVC and the vacuole. Mutants *vamp727* and *syp22* partially defects transport of storage proteins into the vacuole and disrupt its biogenesis and consequently the biogenesis of seeds (Ebine et al., 2008).

The number of genes encoding SNARE proteins in plants expanded during the evolution. Unicellular algae encode 30-35 SNAREs, moss *Physcomitrella* encodes 63 of them and angiosperms encode up to 62-76 SNAREs. All major groups and subgroups of SNAREs in evolutionary most advanced angiosperms are also in moss *Physcomitrella,* but not in the algae. This points to the fact, that the largest leap in SNARE proteins evolutional

development, and likely also the development of secretory system in general, is associated with multicellularity and consequently with the need to serve specialized pathways (Dacks and Dolittle 2002, Sanderfoot et al, 2007).

6 Autophagy

Autophagy is a trafficking process in eukaryotic cells, which can be described as 'self-eating'. It is packing of cell compounds into the membrane vesicle which is further digested either by fusion with vacuole or lysosome (Figure 5). It is very important in dealing with various types of stress such as lack of nutrients, pathogens or toxic substances and also for digestion of organelles, damaged proteins and reuse cellular components before apoptosis. It is also necessary in plant development.

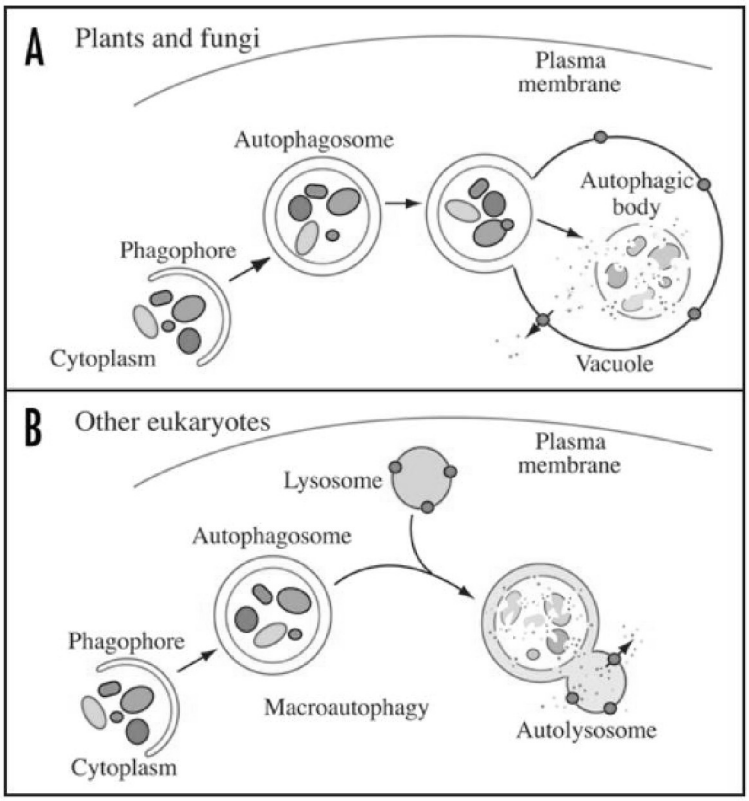

Figure 5: Comparison of autophagy within eukaryots (Klionsky et al., 2008).

Autophagy has been studied on plants since the late 1960's. At the beginning, studies were based on observation of autophagic structures using electron microscope (Van der Wilden et al., 1980). As the genetic methods developed, genes necessary for autophagy (*ATG*) were identified in *Sacharomyces cerevisiae* (Tsukada and Ohsumi, 1993). Subsequently

homologues to some *ATG* yeast genes were identified (Doelling et al., 2002; Hanaoka et al., 2002) as well as in rice (Xia et al., 2011) and maize (Chung et al., 2009). Atg proteins in yeast can be functionally divided into four groups. First is Atg1 protein kinase complex, second is phosphatidylinositol 3-kinase(PI3K) complex specific for autophagy, third is the Atg9 complex and in fourth group are two ubiquitine-like systems causing lipidation (Atg8) and protein conjugation (Atg12) (Yoshimoto 2012). Homologs of thes genes have been found in *Arabidopsis* (Suttangkakul et al., 2011).

6.1 Autophagy regulation

Autophagy is regulated by TOR (target of rapamycine) complex in animal, yeast and also in *Arabidopsis thaliana*. TOR negatively regulates autophagy as the lowered expression of AtTOR caused by RNAi-AtTOR revealed constitutive autophagy response. This occurred without any stress conditions. In yeast and metazoans, Tor reacts with Atg1/Atg13 complex but in plants these kind of interactions have not been discovered (Liu and Bassham, 2010).

6.2 Macroautophagy and micropautophagy

There are two basic autophagic pathways: macroautophagy and micropautophagy, which differs in their origin and amount of taken cell material to digestion. Microautophagy occurs right on the vacuolar membrane. The cytoplasm is than taken inside to vacuole as the membrane invaginates. On the other hand, macroautophagy occurs in the cytoplasm, where is cytoplasm and even parts of organelles encased into vesicles with two membranes called autophagosomes. The outer membrane of this vesicle then fuse with the vacuole and content of vesicle as well as inner membrane ends up in vacuole and yield digestion (Bassham et al., 2006).

Microautophagy process in plants was postulated on the example of mung bean (*Vigna radiata*) cotyledons where are autofagic vesicles invaginated into protein bodies, which becomes lytic compartment and digested invaginated content afterwards (Van der Wilden et al., 1980).

Macroautophagy is also called only autophagy. Autophagosomes are found in plant cells when the plant is under stress conditions. For example, APG7, which activates the APG8/12 protein complex involved in autophagy in elongation and enclosure of formatting autophagosome is not necessary for normal growth and development in nutrients sufficiency. However, when the plant lack nitrogen and carbon, apg7-1 mutant causes premature leaf senescence which suggest the need of APG8/12 complex when autophagy occures (Doelling

et al., 2002). The reason of autophagy is the reuse of necessary nutrients like it has been confirmed by studying nitrogen remobilizatioin *Arabidopsis* (Guiboileau et al., 2012).

6.3 Role of autophagy in vacuole development

Intereistingly, autophagosomes occur also when the biogenesis of vacuole is perturbed in the mutant of gene *VACUOLELESS1 (VCL1)* in Arabiodopsis. VCL1 is a homolog of the yeast VPS16p and vcl1 mutant lacks the central vacuole. Instead, cells are entirely filled up with autophagic bodies (Rojo et al. 2001). This is a crucial observation, revealing the role of autophagy in vacuole biogenesis and its importance in vacuolar trafficking in plants. Vacuole biogenesis usually starts from pre-existing vacuole. This vacuole can be artificially removed by ultracentrifugation from tobacco protoplasts. In such protoplasts (miniprotoplasts), the central vacuole is regenerated within 2-3 days(Wu and Tsai, 1992). If cysteine protease inhibitors are added to the protoplasts, cytoplasmic proteins are observed largely in newly formed vacuole(Yano et al., 2007). This demonstrates, that autophagy has a central role in vacuole biogenesis in plants. However, conventional autophagy suppressing drugs, such as 3-MA (3-methyl adenine) or PI3K (phosphoinositide 3-kinases) inhibitors do not influence this kind of autophagy. Hence, this might be a yet undescribed plant-specific autophagic pathway(Yano et al., 2007). Existence of more than one autophagic pathway is highly expected in plants, as plants must cope with cytoplasm toxicity stress on daily basis (caused by senescent plastids and mitochondrions). Atophagy is therefore also involved in coping with reactive oxygen species (ROS), which cause oxidative stress. Arabidopsis, atg2 and atg5 mutants showed higher accumulation of ROS (Yoshimoto et al., 2009). The cell likely gets rid of the dangerous ROS by mitochondrial autophagy, so-called mitophagy (Minibayeva et al., 2012) and by removal of senescent plastids, which is autophagy (namely ATG5) – dependent (Ishida et al., 2008).

7 Conclusion

Vesicle transport into the vacuole is very dynamic and intensively studied field in plant sciences.

As the problematic of vesicle traffic is very wide, I have mentioned in my opinion crucial proteins involved in vesicle trafficking. I focused on the mechanism, by which are the proteins and their cargos sorted and transferred. I described the development of knowledge

about vacuolar sorting receptors (VSRs). VSRs are very interesting due to its special membrane localization and orientation which can both sort the cargo and also be targeted to the right organelle. Their existence was proved many times (Kirsch et al., 1994; Miller et al., 1999), as well as their importance in vacuolar sorting. However, the theopinion on their transport within the cell, more precisely about the place where are they recycled, has changed lately.

Subsequently, I described essential proteins for vesicle fusion. Rab GTPase proteins, which are involved in membrane fusion, work as molecular switches. They are active or inactive depending on the binding either with GTP or GDP. Next, the proteins I mentioned are SNARE complexes. They provide vesicle fusion.

Finally I mentioned autophagy, as a special vesicular trafficking to the vacuole. It is a necessary process for keeping cell alive under different types of stress, as well as for proper regulated cell death.

I focused on pathways which I find interesting which of course dos not lower the importance of other. Vesicle transport into the vacuole is essential in almost every part of plant life. It begins with accumulation of storage proteins in seeds and their lytic digestion in seed germination. The storage of synthesized substances during plant growth and dealing with stress like salinity, drought, pathogens and finally providing cell death and digestion and reuse of cell compounds are explored.

Although many pathways and proteins in this field have been described, there are still much to reveal in this field.

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