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# Evaluation of a yeast system for studying the function of plant Vacuolar Sorting Receptors (VSRs)

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# Evaluation of a yeast system for studying plant Vacuolar Sorting Receptors (VSRs)

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## Abbreviations

| | |
|---|---|
| CCV | clathrin-coated vesicle |
| ct-VSD | C-terminal vacuolar sorting determinant |
| CT | cytosolic tail domain |
| DV | dense vesicle |
| ER | endoplasmic reticulum |
| EDTA | ethylenediaminetetraacetic acid |
| EtOH | ethanol |
| GFP | green fluorescent protein |
| LV | lytic vacuole |
| MES | 2-(-N-morpholino)-ethanesulfonic acid |
| M | mutant strain |
| NT | luminal domain |
| O.N. | over night |
| PVC | prevacuolar/endosomal compartment |
| PSV | protein storage vacuole |
| PM | plasma membrane |
| PCR | polymerase chain reaction |
| pps | protoplasts |
| PEG | polyethylene-glycol |
| PAGE | polyacrylamide gel electrophoresis |
| RT° | room temperature |
| SDS | sodium dodecyl sulphate |
| ss-VSD | sequence-specific vacuolar sorting determinant |
| TGN | *trans*-Golgi network |
| TIP | tonoplast intrinsic protein |
| TMD | transmembrane domain |
| TCA | trichloroacetic acid |
| VSR | vacuolar sorting receptor |
| VSD | vacuolar sorting determinant |
| WT | wild type |
| HA | influenza hemagglutinin epitope tag |
| AC | genbank accession number |

Abbreviations used only in single subchapters are explained in the text.

# Chapter 1. General introduction

The secretory pathway of eukaryotic cells is the site of synthesis, modification, and sorting of proteins that are secreted, accumulate in vacuoles, or are retained in various other compartments of the endomembrane system. Despite a number of unique features with regard to the plant secretory pathway, there is a remarkable conservation of general features with the yeast and mammalian secretory pathways. Therefore, the sorting of proteins in the plant secretory system has also been studied by analogy with the yeast and animal systems.

The yeast *Saccharomyces cerevisiae* has been extensively used for studies on vacuolar transport. In contrast to plants and mammalian cells, yeast has a shorter generation time and can be much more easily genetically manipulated. In addition, the existence of many convenient markers to monitor protein delivery to the vacuole, along with the availability of hundreds of well-characterized mutants involved in vacuolar protein sorting, makes this unicellular eukaryote an attractive model system for the plant biologists. Many classical tools of the cell and molecular biology as well as biochemical approaches have already been well established in yeast. However, many publications already illustrated the need to carefully interpret the results obtained when expressing plant proteins in yeast.

This thesis evaluates the merits of *S.cerevisiae* to study plant vacuolar sorting interactions. This first chapter will present an overview of the yeast and plant secretory pathways, focusing on each compartment and step and in particular on the Golgi-to-vacuole pathways. I will then compare the vacuolar targeting between the two systems, and the possible problems encountered when using yeast as a heterologous system.

## The secretory pathway

All eukaryotic cells are characterized by the existence of many specialized compartments defined by complex membrane systems. The targeting of different kinds of molecules to these compartments requires many complex systems. Among these systems, the secretory pathway is considered as a biochemical pathway because it is responsible for the synthesis and transport of important subsets of proteins, lipids and carbohydrates. It comprises the endoplasmic reticulum (ER), the Golgi complex (GA), the endosomal/prevacuolar compartments (PVC), the vacuoles, and the plasma membrane (PM). Trafficking between these organelles is thought to be primarily mediated by a system of transport vesicles. Many molecular components involved in the vacuolar transport have been first identified in yeast and mammalian cells, and related components have been then identified in plants. However, a number of other components can be found in only one of these systems.

Apart from its crucial roles during cell division (Lukowitz et al., 1996), cell polarization (Morita et al., 2002), protein metabolism, and biogenesis, the plant secretory pathway is also involved in specialized processes such as stress physiology and plant-pathogen interactions (Jelitto-Van Dooren et al., 1999).

## The endoplasmic reticulum

The ER is the site of synthesis and maturation of proteins destined to secretion, the PM, and the secretory and endocytic compartments. Additionally, it plays a crucial role in safeguarding the correct folding and assembly of proteins through “quality control” (QC) mechanisms. The ER is also involved in the synthesis of many lipids.

The ER of plant cells possesses some additional functions not found in mammalian and yeast cells. The plant ER is involved in cell to cell communication via the plasmodesmata, and in specialized cells, it serves as a storage site for proteins. Its also has enzymes and structural proteins that are involved in the process of oil body biogenesis and lipid storage (Galili et al., 1998).

### Protein translocation into the ER

Proteins that enter the secretory pathway are first sorted from those destined to reside in the cytosol or those destined to be translocated into specific organelles such as mitochondria, chloroplasts, peroxisomes because they have a specific signal peptide (soluble proteins) or one or more permanent hydrophobic membrane-spanning domains (membrane proteins) (Vitale and Raikhel, 1999). Signals peptides of eukaryotic cells are about 13 - 30 amino acid residues long, they have a basic amino-terminal region, an uninterrupted stretch of 7 or 8 apolar, largely hydrophobic residues, and a more polar carboxyl terminal region that defines the cleavage site (von Heijne, 1986). These signal peptides are first recognized and bound by a ribonucleoprotein complex, the signal recognition particle (SRP). Binding of SRP to the signal peptide inhibits translation (High and Dobberstein, 1991). The SRP-preprotein-ribosome complex is then recognized by a membrane receptor, the docking protein (DP). The nascent protein will cross the ER membrane through a translocation complex the translocon (Gorlich and Rapoport, 1993). This complex is formed by Sec61p which constitutes the translocation pore, the signal peptidase (SP), the oligosaccharyltransferase (OST) and the TRAM protein. It is suggested that a conformational change of Sec61p and TRAM opens the translocation channel. The Sec61p complex is found in mammals, yeast and plants (Deshaies and Schekman, 1987; Stirling et al., 1992; Hartmann et al., 1994; Rapoport, 1996). The signal

peptide is co-translationally cleaved off at a specific site by the membrane-bound signal peptidase (SP) (Shelness and Blobel, 1990).

Membrane proteins can be inserted into the ER membrane via either SRP-dependent or SRP-independent mechanisms. Certain Type I membrane proteins have a cleavable signal peptide that is similar to the signal peptide of soluble proteins. Other Type I and II membrane proteins do not have a cleavable signal at their N-terminus and their insertion into the ER membrane is mediated by “signal anchor” sequences, i.e. internal uncleaved signal sequences that also function as transmembrane domains. The orientation of these proteins depends on the preferential translocation of the C-terminal or N-terminal end of the signal anchor sequence. Although many other factors could also determine this translocation, the distribution of charged residues flanking the transmembrane segment appears to be most important (Hartmann et al., 1989; Wahlberg and Spiess, 1997).

During and after translocation, the polypeptides undergo additional modifications to achieve their proper final conformation. The ER is the only place within the secretory pathway where proteins change their topology.

## Maturation of secretory proteins within the ER

### *Glycosylation and early maturation of the glycans*

Proteins of the secretory pathway are often glycosylated, i.e. modified by the addition of sugar residues. The glycan moiety can be linked to the polypeptide backbone via either N- or O- glycosidic bonds, giving rise to N-glycosylated or O- glycosylated proteins, respectively. In most cases, the N-glycosylation occurs co-translationally while addition of O-linked glycans occurs after the proteins have left the ER (Moore et al., 1991; Matsuoka et al., 1995a).

For N-glycosylation, glycans with the structure $Glc_3Man_9GlcNac_2$ are pre-assembled on dolichol lipid carriers and then transferred to specific asparagine residues on nascent polypeptides (within the tripeptide Asn-X-Ser/Thr where X is any amino acid except proline or aspartic acid) (Kornfeld and Kornfeld, 1985). The preformed glycan is transferred to the nascent protein by the oligosaccharyl transferase (OST) which is part of the translocon complex. This enzyme glycosylates the nascent protein unless folding of the growing polypeptide chain masks the potential glycosylation site. Therefore, polypeptide folding can determine the efficiency of potential glycosylation sites. The N-glycans within glycoproteins are thought to favour glycoprotein folding and transport (Helenius, 1994).

The three terminal glucose residues are removed by two ER-resident enzymes, the glucosidases I and II, yielding $Man_9GlcNac_2$ structures (Sturm, 1987).

### *Folding and assembly of the polypeptide*

Once glycosylated, proteins are correctly folded and assembled with the help of various ER resident proteins, the molecular chaperones (Chrispeels, 1991; Gething and Sambrook, 1992). Unfolded and/or unassembled proteins tend to form aggregates in the ER. Such aggregates may be unable to enter the transport vesicles. The function of molecular chaperones is to prevent aggregation or the formation of incorrect three-dimensional structures by assisting the folding of nascent proteins. Chaperones dissociate from the nascent protein when maturation is completed. The best characterized ER-localized molecular chaperone is the HSP70 cognate called binding protein (BiP). This highly conserved ER-resident protein transiently binds newly synthesized secretory proteins and promotes their correct folding (Denecke, 1996).

Two ER-resident lectins specifically bind glycoproteins in the ER: the soluble calreticulin (CRT) and the membrane-bound calnexin (CNX). In association with other proteins, they mediate retention and promote proper folding of glycoprotein substrates (Helenius et al., 1997).

### *Formation of Disulfide Bonds*

Enzymes also play a role in the structural maturation of proteins in the ER. The ER-resident protein disulfide isomerase (PDI) binds to unfolded proteins and catalyzes the formation and exchange of the disulfide bonds necessary for proper folding (Bednarek and Raikhel, 1992). In addition, PDI seems to possess other functions. It is likely to serve as a subunit of prolyl hydroxylase, another ER-resident which functions in the hydroxylation of proline residues in a number of proteins (Denecke, 1996; Galili et al., 1998).

### *Protein oligomerization*

Oligomerization is essential for the function of many proteins. Transport of most oligomeric proteins from the ER is dependent on the assembly of the correct oligomeric structure (Bednarek and Raikhel, 1992). BiP also plays a role in oligomer assembly. Binding of properly folded but unassembled proteins to BiP is responsible for retention of unassembled subunits of oligomeric proteins such as immunoglobulin heavy chains, although other unassembled proteins are retained in the ER by other mechanisms (Hurtley and Helenius, 1989; Gething and Sambrook, 1992).

**Retention and recycling of ER-resident soluble proteins**

ER-resident proteins generally require specific signals for retention or retrieval which are often short amino acid sequences at their C-terminal end, and are highly conserved in eukaryotes.

Many soluble proteins of the ER lumen have the consensus C-terminal tetrapeptide H/KDEL. KDEL is more usual in mammals whereas HDEL is more usual in yeast. In plants, both tetrapeptides have been identified (Denecke et al., 1992; Gomord and Faye, 1996; Gomord et al., 1997). For example, the alfalfa protein disulphide isomerase harbors a KDEL sequence, although most plant ER soluble resident proteins carry an HDEL signal (Vitale et al., 1993). Specifically, HDEL proteins show a characteristic ER distribution whereas KDEL proteins are immunodetected on a discrete part of the ER network (Napier et al., 1992).

Many studies have shown that the addition of KDEL to the C-terminus of various secreted proteins leads to ER-retention of these proteins in animal cells. Similarly, reporter proteins fused to H/KDEL tetrapeptides are at least partly retained in the ER of plant cells (Denecke et al., 1992; Boevink, 1996; Gomord et al., 1997).

Soluble ER-resident proteins are diverted and retrieved from the default pathway or 'bulk flow' by membrane receptors that recognize the H/KDEL C-terminal extensions. In yeast, two HDEL-specific receptors have been identified: ERD1 and ERD2 (Hardwick et al., 1990; Semenza et al., 1990). The sequence of ERD2 is 50% similar to its human equivalent and 52% similar to the *A. thaliana* ERD2 (Lewis and Pelham, 1992; Lee et al., 1993). These ER-resident receptors do not appear to retain ER-resident proteins but are responsible for their transport back to the ER when they escaped from this compartment. In mammals, the KDEL-receptor concentrates mainly in a post-ER intermediate compartment and in the GA, but with a concentration that decreases towards the *trans*-side. Accordingly, it has been shown that reporter glycoproteins fused to the KDEL motif undergo N-glycans modifications that are believed to happen in the GA. In plants, a direct retention in the ER of proteins bearing the HDEL signal has been described (Gomord et al., 1997) and other ERD2-related proteins exist (they are termed ERPs) (Hadlington and Denecke, 2000).

***Retention and recycling of ER integral membrane proteins***

While the concept of default pathway (a constitutive secretory pathway that automatically delivers material via GA to the PM, if no sorting signals are present) is not generally applicable to membrane proteins, specific signals and mechanisms are found to be necessary to retain proteins within the ER membrane. For instance, a cytosolic di-lysine motif (i.e. KKXX or KXKXX) at the C-terminal end has been identified in many type I integral membrane proteins

of the ER of yeast, animal and plant cells. This sequence is both necessary and sufficient for ER retention. As for H/KDEL containing soluble ER proteins, the dilysine motif mediates retrieval of type I membrane proteins from the GA back to the ER in mammalian (Jackson et al., 1993) and yeast cells (Gaynor et al., 1994) by interacting with the COPI coatomer.

For type II membrane proteins a di-arginine motif at the cytoplasmically orientated N-terminus has also been identified as retention and retrieval signal in the ER (Schutze et al., 1994).

Retention and retrieval mechanisms also appear to co-exist for ER resident membrane proteins. For example, ER retention of the yeast Sec12p (type II transmembrane glycoprotein) is mediated by its cytoplasmic tail domain (CT) while its retrieval is only dependent on the transmembrane domain (TMD) (Sato et al., 1996).

Many ER type I membrane proteins have been identified from different plant species (Huang et al., 1993; Hasenfratz et al., 1997) and similar motifs in the CT could also be involved in their ER-retention.

Plant and mammalian CNXs harbor a di-arginine motif, a retention/retrieval motif for type II membrane ER proteins (Schutze et al., 1994). When fused with GFP or phosphinothricin acetyl transferase (PAT) as reporter proteins the TMD and CT of tobacco and castor bean calnexins are sufficient for ER-retention in tobacco protoplasts. In addition, when a part of the CT including the di-arginine motif of these two proteins is deleted from the fusion proteins, they are in part exported from the ER, similarly to results obtained in mammalian cells (Phillipson and Denecke, 1997).

## The quality control system

Incorrectly folded proteins are common side products of protein synthesis in the ER (Ellgaard et al., 1999). It is well known that when N-glycosylation is prevented by inhibitors or mutations, proteins tend to misfold and aggregate in the ER lumen and membrane. Both soluble and membrane proteins are subject to quality control in the ER. This system has several strategies: the retention of incompletely or incorrectly folded proteins, the degradation of irreversibly misfolded proteins by the ER-associated degradation system (ERAD), retrieval to the ER from downstream organelles (i.e. Golgi complex), and rerouting from the Golgi complex to the lysosomes or vacuoles (Ellgaard and Helenius, 2003; Trombetta and Parodi, 2003) (Figure 1.1). Therefore, the QC system (1) increases the efficiency of correct structural maturation by retaining polypeptides in a favorable environment which is rich in folding factors (2) avoids delivery of immature or defective secretory proteins to locations where they could negatively interfere with the cell metabolism, and (3) recovers amino acids and maintains

homeostasis of the endomembrane system by disposing of defective proteins (Vitale and Denecke, 1999).

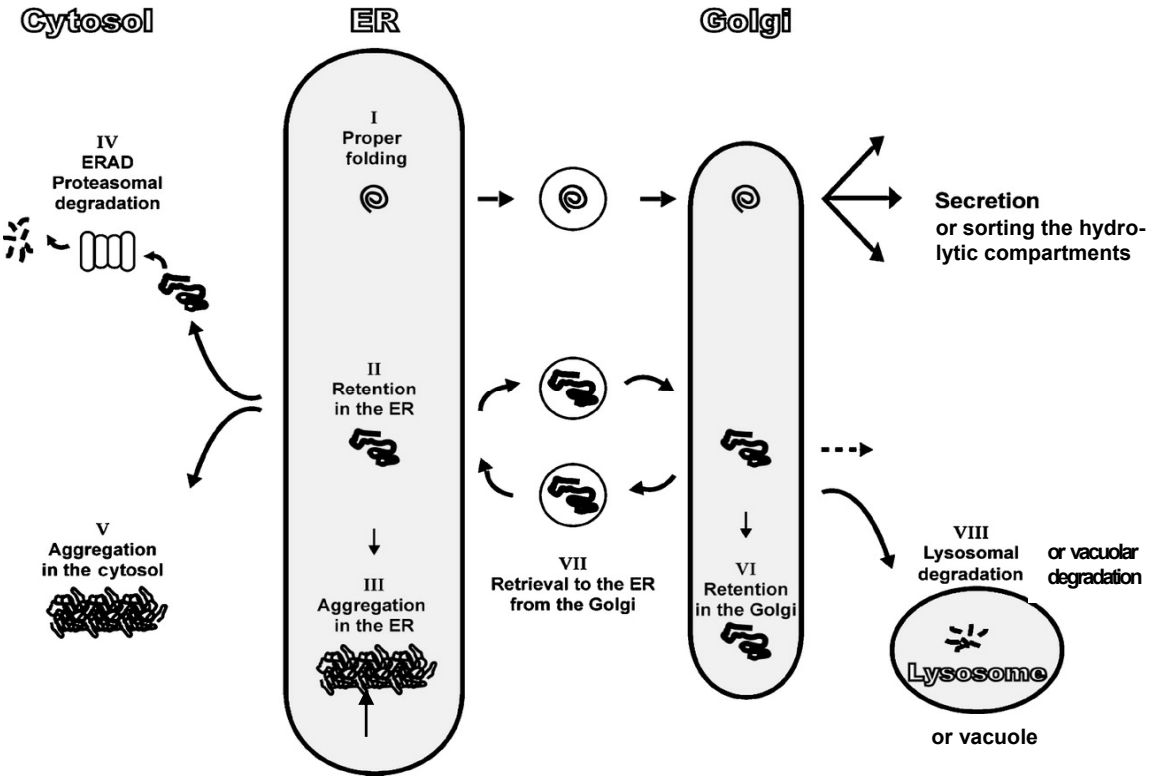



**Figure 1.1. Summary of quality control scenarios observed along the secretory pathway.** Proteins that fold properly are transported out of the ER (I). Proteins that fail to fold correctly are generally retained in the ER and exposed to ER-resident chaperones to attempt refolding (II). They may transiently or permanently aggregate (III), or they may be retro-translocated to the cytosol resulting in proteasomal degradation (ERAD) (IV) or aggregation (V). Some incompletely folded proteins reach the GA, where they may be retained (VI), or from where they can be either retrieved to the ER (VII), or diverted to lysosomes/vacuoles for degradation (VIII). This figure is modified from Trombetta and Parodi (2003).

Multiple problems cause proteins to fail the QC. Among these, truncations or mutations interfere with proper folding, orphan subunits of hetero-oligomeric complexes or homo-oligomers cannot reach their proper quaternary structure. Incomplete co-and post-translational modifications (signal peptide cleavage, disulfide bond formation or N-glycosylation) or the exposure of polar amino acids within the TMDs can also interfere with correct folding.

## Some basic principles of the QC system

### *Association with ER chaperones*

The first QC mechanism involves the association of newly synthesized proteins with the ER chaperones and folding enzymes such as BiP, CRT, CNX and PDI. These factors are not only responsible for assisting the folding and assembly process, but also serve as retention anchors for immature proteins, preventing premature degradation by either vacuolar proteases or cytosolic proteasomes (Lord, 1996; Brodsky and McCracken, 1997; Gomord et al., 1997). For instance, BiP is a retention factor for unassembled IgG heavy chains in the ER (Bole et al., 1986) while CRT and CNX bind to incompletely folded glycoproteins. In plants, the HDEL tetrapeptide also seems to participate in targeting misfolded protein complexes that escape the ER to the plant lytic vacuole for degradation (Gomord et al., 1997).

### *ER-associated degradation (ERAD)*

When the folding machinery in the ER is not sufficient to promote a native conformation proteins are generally degraded by the ERAD (Bonifacino and Weissman, 1998; Plemper and Wolf, 1999; Lord et al., 2000). This process is mainly carried out by the *26S* proteasome in the cytosol (Hiller et al., 1996; Brodsky and McCracken, 1999; McCracken and Brodsky, 2003). This process occurs in several steps: terminally misfolded or unassembled proteins are recognized by the ER chaperones. They are then retranslocated into the cytosol through the Sec61p channel with the help of BiP, deglycosylated, polyubiquitinated, and finally degraded by the proteasomes (Wiertz et al., 1996; Plemper et al., 1997). In yeast as well as in mammalian cells, many defective proteins have been shown to be degraded in the cytosol as described (Richter-Ruoff et al., 1994; Hiller et al., 1996; Wiertz et al., 1996; Galan et al., 1998). Studies on castor bean cells (Frigerio et al., 1998) strongly suggest that ER-to-cytosol dislocation can also occur in plant cells. Mannose-trimmed oligosaccharides have also been proposed to target glycoproteins to ERAD (Trombetta and Parodi, 2003).

As in yeast cells (Holkeri and Makarow, 1998), some defective proteins in plant cells may be also degraded in an early compartment of the secretory pathway, maybe in the ER itself (Pedrazzini et al., 1997).

## Quality control beyond the endoplasmic reticulum

Some defective secretory proteins have been shown to escape ER retention, but are diverted from the GA to the lysosome/vacuole for degradation (Lippincott-Schwartz et al., 1988; Holkeri and Makarow, 1998). In other cases, after normal proteins leave the ER, having successfully passed QC, modifications occurring in Golgi seem to induce non-native

structures, leading also the protein to the lysosome/vacuole for degradation. In yeast, Vps10p acts not only as a cargo receptor for vacuolar residents (see below), but it has also been suggested to bind non-native structures in the Golgi and to deliver them to the vacuole (Hong et al., 1996). Selective ubiquitination of transmembrane proteins also addresses them to the vacuole for degradation (Reggiori and Pelham, 2002).

In plants, studies on maize zeins (Coleman et al., 1996) and the vacuolar storage glycoprotein phaseolin (Pueyo et al., 1995) suggest that vacuoles could also be a site for degradation by the QC.

Recent studies demonstrated that some ERAD substrates require trafficking between the ER and the GA. In yeast, many soluble misfolded glycoproteins showed a retarded proteasomal degradation in mutants defective in exit from the ER, in vesicle delivery to the Golgi or in retrograde transport from the Golgi to the ER. However, this apparently did not affect misfolded membrane-spanning domains (Caldwell et al., 2001; Sato et al., 2001; Vashist et al., 2001). In mammalian cells, lysosomal targeting may depend on aggregation in the *trans*-Golgi network (TGN) (Wolins et al., 1997).

## Transport from the endoplasmic reticulum to the Golgi apparatus

After passing the quality controls, most proteins are ready to be directed to their specific compartment of residence within the secretory pathway, depending on the information contained in their polypeptide chain.

Protein transport from the ER to the GA is mediated by carrier vesicles that are formed on the ER membrane and selectively fuse with the *cis*-Golgi membrane. Two types of coat proteins define the vesicles that shuttle between the ER and the GA, COP-I and COP-II. It is generally accepted that COP-II mediates the transport of material from the ER to the Golgi (Barlowe et al., 1994; Schekman et al., 1995; Paccaud et al., 1996; Schekman and Orci, 1996) whereas COP-I plays an important role in Golgi-to-ER retrieval and in maintenance and function of the *cis*-Golgi (Cosson and Letourneur, 1994; Letourneur et al., 1994; Gaynor and Emr, 1997; Gaynor et al., 1998). Additionally, the transport from early to late endosomes also depends on COPI vesicles (Aniento et al., 1996; Gu and Gruenberg, 1999).

A general mechanism of vesicle budding and fusion has been proposed (Kuehn and Schekman, 1997; Aridor et al., 1999; Bonifacino and Glick, 2004). Vesicles bud from a donor compartment by a process (“vesicle budding”) that allows selective incorporation of cargo into the forming vesicles while retaining resident proteins in the donor compartment (“protein sorting”). The vesicles are subsequently targeted to a specific “acceptor” compartment (“vesicle targeting”), into which they unload their cargo upon fusion of their limiting membranes (“vesicle fusion”) (Figure 1.2).

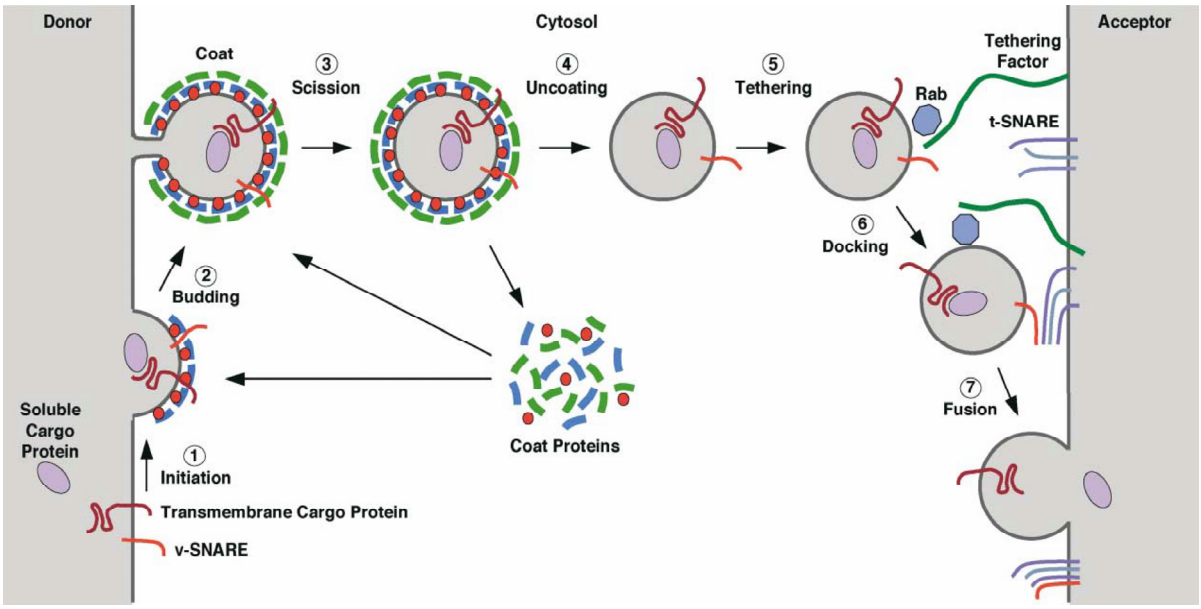



**Figure 1.2. Steps of vesicle budding and fusion.** (1) Initiation of coat assembly. The membrane-proximal coat components (blue) are recruited to the donor compartment by binding to a membrane-associated GTPase (red) and/or to a specific phosphoinositide. Transmembrane proteins and v-SNAREs begin to gather at the assembling coat. (2) Budding. The membrane-distal coat components (green) are added and polymerize into a mesh-like structure. Cargo becomes concentrated and membrane curvature increases. (3) Scission. The neck between the vesicle and the donor compartment is severed either by direct action of the coat or by accessory proteins. (4) Uncoating. The vesicle loses its coat due to various events including inactivation of the small GTPase, phosphoinositide hydrolysis, and the action of uncoating enzymes. Cytosolic coat proteins are then recycled for additional rounds of vesicles budding. (5) Tethering. The “naked” vesicle moves to the acceptor compartment, possibly guided by the cytoskeleton, and becomes tethered to the acceptor compartment by a combination of a GTP bound RabYTP protein and a tethering factor. (6) Docking. The v-and t-SNAREs assemble into a four-helix bundle. (7) This “trans-SNARE complex” promotes fusion of the vesicle and acceptor lipid bilayers. Cargo is transferred to the acceptor compartment, and the SNAREs are recycled. This figure was taken from Bonifacino and Glick (2004).

The mechanism by which cargo molecules are packaged into COP-II vesicles have been described in details (Kuehn and Schekman, 1997; Bonifacino and Glick, 2004). Briefly, the COPII coat assembles by the gradual deposition of Sar1p•GTP, Sec23p•Sec24p, and Sec13p•Sec31p onto sites where newly synthesized proteins exit from the ER. The cytosolic Sar1p•GDP is converted to membrane bound Sar1p•GTP by the transmembrane protein Sec12p, its GDP-GTP exchange factor (GEF). Sar1p•GTP recruits the Sec23p•Sec24p subcomplex by binding to Sec23p, forming the “pre-budding complex”. Then, transmembrane

cargo proteins can gather at the assembling coat by binding to Sec24p. The Sec13p•Sec31p subcomplex polymerizes onto Sec23p•Sec24p and crosslinks the pre-budding complexes. Cargo proteins are further concentrated. The dissociation of the coat requires GTP hydrolysis by Sar1p which is stimulated by Sec23p (a GTPase-activating protein (GAP)) destabilizing the coat (Goldberg, 1998).

In the case of COP-I, the coat formation begins by the recruitment of a preassembled coatomer (Orci et al., 1993). In this case, the coatomer directly interacts with the membrane-bound ARF•GTP via its β- and γ-COP subunits. COPI dissociation also requires GTP hydrolysis by ARF1, stimulated by a cytosolic ARF-specific GAP (Goldberg, 1998).

In plants, homologues to the yeast COP coatomer (Pimpl et al., 2000), ARF1 (Regad et al., 1993); Sar1p, Sec12p (d´Enfert et al., 1992); Sec21p and Sec23p (Movafeghi et al., 1999) have been identified.

### Vesicle fusion with the target membrane

SNAREs are cytosolic-facing membrane proteins that reside on vesicular carrier (v-SNAREs) and target organelles (syntaxins or t-SNAREs). According to the SNARE hypothesis, specific vesicle fusion between compartments requires that v-SNARE proteins recognize cognate t-SNAREs in the target membrane (Schekman and Orci, 1996; Hay and Scheller, 1997; Pfeffer, 1999). As shown in figure 1.2, the SNARE proteins also have an important role at the final fusion step. Different v-/t-SNARE complexes form at different steps of intracellular transport. SNAREs cannot be the only specific determinants for membrane fusion because each v-SNARE recycles and is thus present in both anterograde and retrograde vesicles. Additional specificity is provided by tethering proteins that link the opposing membranes prior to the SNARE complex formation. These various tethers assemble with the aid of Rab family GTPases (known as Ypt proteins in yeast) to promote initial specific association of two membranes. Linked to SNAREs, multiple Rab proteins operate at different steps of transport. Rabs, tethers, and SNAREs collaborate to ensure that membranes fuse at the correct time and place (Bonifacino and Glick, 2004). Although many aspects have been clarified about the role of these factors before or after docking and in the SNARE complex formation, much remains to be determined (Sanderfoot and Raikhel, 1999).

Almost every fusion step in the membrane trafficking is carried out by a distinct set of SNARE pairs. In the yeast, *S.cerevisiae*, the complete set of SNAREs has been described (Hay and Scheller, 1997; Holthuis et al., 1998; Pelham, 1999; Burri and Lithgow, 2004).

Several plant SNARE proteins have also been found (Sanderfoot and Raikhel, 1999). In Arabidopsis, 24 genes encode members of the syntaxin family, grouped in 10 subfamilies that each contain one to five members (Sanderfoot et al., 2000). These genes have been recently

reclassified under the name of “syntaxin of plants” or SYP (Sanderfoot et al., 2000). Some plant syntaxins have homologues in yeast, although it is not always clear whether they have the same localization and/or function (see below, page 56). In addition, one can often find multiple homologues to a single yeast gene.

*Arabidopsis* AtSYP21 (AtPEP12p), a syntaxin isolated by functional complementation of a yeast *pep12* mutant was localized to a post-Golgi compartment (Bassham et al., 1995; da Silva Conceicao et al., 1997). In yeast, Pep 12p is the specific syntaxin for the PVC (Becherer et al., 1996). Another Arabidopsis syntaxin that may be involved in vacuolar assembly, AtSYP22 (AtVAM3), has also been identified via complementation of the yeast *vam3* mutant (Sato et al., 1997). In yeast, Vam3p is the vacuole-specific syntaxin (Darsow et al., 1997).

## The Golgi apparatus

Over the past decades, the GA has received the attention of many researchers because of the different morphologies and activities depending on the host cell. In contrast to animals cells in which the stacks are interconnected by tubular elements and clustered around the nucleus (Mellman and Simons, 1992), plant cells contain up to several hundred individual Golgi stacks (or dictyosomes) dispersed in the cytoplasm (Harris, 1986). The GA is composed of a number of flattened cisternae. The *cis*-Golgi, the *medial*-Golgi, and the *trans*-Golgi network (TGN) can often be distinguished, both microscopically and functionally.

In higher plants, each Golgi stack consists of 3 to 10 cisternae displaying a morphological polarity from the *cis* to the *trans* face. Morphological parameters such as width of the cisternae, the spacing between the cistenae, the staining of cisternal membranes and contents, as well as the location of intercisternal elements and of the TGN, are used to define the Golgi cisternae (Staehelin and Moore, 1995). The plant GA does not disassemble during mitosis as in animal cells, but continues to synthesize cell wall and membrane components.

The GA functions as an important biosynthetic compartment that modifies proteins and synthesizes lipids and polysaccharides. The central biosynthetic function of the Golgi is to be the factory for complex glycans. The Golgi is the site of modification of N-linked high-mannose glycans on glycoproteins that were synthesized in the ER, of O-glycosylation of hydroxyproline-rich glycoproteins (HRGPs) and arabinogalactan proteins (AGPs) (Nebenführ and Staehelin, 2001). These modifications occur in a series of sequential reactions, while the glycoprotein is transported across the stacks, by numerous Golgi enzymes such as glycosidases and glycosyltransferases. In addition, the plant GA is engaged in the synthesis of complex cell wall polysaccharides such as hemicelluloses and pectins, but not of cellulose.

The GA, a multifunctional organelle, is also the major sorting point in the secretory pathway since it packages its macromolecular products in membrane-bound vesicles, which are

targeted from the *trans*-face to different destinations within the cell. As a consequence, the GA plays a key role in regulating the membrane economy of the cell.

A major controversy about the GA concerns the progression of secretory material and membrane from the ER to the *cis*-face of the stack, then across the stack to the *trans*-face, and the final exit. Two basic models have been proposed (Hawes and Satiat-Jeunemaitre, 1996) although they may be not mutually exclusive: the vesicle shuttle model suggests that there is a sequential vesicle-based transport of cargo through a static stack. The cisternal maturation/progression model suggests that the functional gradient of the cisternae is generated by a progressive maturation from the *cis*-cisternae into *trans*-cisternae.

**Vesicle shuttle model**

This model proposes that the anterograde transfer of material not only from the ER to the *cis*-Golgi cisternae but also between the cisternae occurs by means of small vesicles. The presence of such a vesicle-based pathway for protein transport from the ER to the GA was supported by experiments showing that the fungal metabolite brefeldin A, which inhibits the formation of COPI vesicles in mammals and yeast, blocks the transport of sporamin (a sweet potato storage protein) from the ER-to-GA in transgenic tobacco cells (Holwerda et al., 1992; Satiat-Jeunemaitre et al., 1996). As in mammalian cells and in yeast, it was assumed that the transport of products between the cisternae and the retrieval of 'escaped' enzymes are mediated by vesicles (COPI for retrograde and, possibly, also anterograde transport (Staehelin and Moore, 1995) or, according to a variant of this model ('tubular intercisternal model') by fusogenic tubules, the buds of which are stabilized by COP coats (Staehelin and Moore, 1995). Small coated vesicles were seen in many electron micrographs of plant GA (Andreeva et al., 1998). This model suggests that each cisterna would be a permanent structure with a potentially unique complement of processing enzymes. Each cisternal compartment would receive and deliver cargo molecules via transport vesicles.

**Cisternal maturation model**

This model supposes the *de novo* synthesis of cisternae on the *cis*-face and the decay by vesiculation of old cisternae on the *trans*-face. The whole cisternae with their contents move as units from the *cis* to the *trans* face through the stack by progressive maturation without need for anterograde vesicular transport. As new cisternae are assembled at the *cis*-face and old cisternae are disassembled at the *trans*-face, a single cisterna will appear to progress through the Golgi stack (Mironov et al., 1997). During this process, retrograde vesicles must retrieve Golgi-specific proteins. The strongest support for this model has come from ultrastructural

studies on scale-forming unicellular protists, which have Golgi stacks composed of numerous cisternae (Becker et al., 1995). Scales can be detected in *cis*-cisternae of the stack, but were never observed in the small coated vesicles budding from the periphery of the cisternae. Therefore, coated vesicles cannot be involved in anterograde progression of the scales through the GA. In animal cells, procollagen type I was also shown to be secreted after moving through the Golgi stacks without even leaving the lumen of the cisternae (Bannykh and Balch, 1997; Bonfanti et al., 1998). However, this model may also involve additional intra-Golgi traffic mechanism, such as anterograde carrier vesicles. Indeed, anterograde cargo proteins have been immunolocalized within COPI vesicles near the GA in animal cells (Orci et al., 1997).

So far, the maturation models predicted that the vesicular tubular cluster (an intermediate compartment formed by the fusion of ER-vesicles) membrane should not mix with pre-existing cisternae, but simply become organized into new cisternae at the *cis*-Golgi face. However, Trucco and coworkers (2004) recently showed that cargo-containing intermediate compartment membranes fuse with the *cis* cisternae and are absorbed into the stack, resulting in the formation of one to two new *cis* cisternae and the expansion of the pre-existing cisternae. Additionally, a traffic wave entering a Golgi stack triggers the formation of intercisternal connections which could play an important role in the formation of new *cis* cisternae and would allow the anterograde movement of cargo through the stack. Connections between cisternae at different levels in the GA have also been identified in glucose-stimulated mouse islet ß cells (Marsh et al., 2004). It has been suggested that these connections may help the retrieval of Golgi-resident membrane proteins such as the glycosylation/ processing of enzymes to earlier cisternae in the stacks.

**Targeting of integral membrane proteins to the Golgi apparatus**

The targeting and localization of integral membrane proteins to the GA depends on targeting determinants in the cytoplasmic tail and/or in the transmembrane domain. Three yeast processing proteases, Kex2p, Kex1p and DPAP-P, are localized in the late Golgi compartment, the equivalent to the TGN of higher eukaryotic cells. For Kex2p, a tyrosine-containing signal in the CT was found to be required for Golgi retention. Indeed, mutation of the tyrosine residue of Kexp2 CT mistargeted the protein to the vacuole. A Phe-X-Phe-X-Asp motif is responsible for DPAP-P retention (Wilcox et al., 1992; Nothwehr et al., 1993).
Resident proteins of the mammalian TGN such as TGN 38 and furin are also targeted by tyrosine-containing signals (Humphrey et al., 1993). Furin is concentrated in the TGN not only by a tyrosine motif, which is also believed to act as a retrieval signal for ‘escaped’ proteins from the PM, but also by an acidic residue (Trowbridge et al., 1993).

The localization of GA membrane proteins is also mediated by their TMDs. The length and sequence within and adjacent to the TMD have been shown to play an important role. It has been determined that Golgi membrane proteins generally have shorter TMDs than plasma membrane proteins, and sorting has been postulated to be a result of the difference in thickness of the lipid bilayer between the GA and the PM. In mammalian cells, for example, proteins with 14-16 or 17-18 amino acids tend to remain in the ER or in the Golgi complex respectively (Munro, 1995a, b; Pedrazzini et al., 1996). However, yeast differs from animal cells in that proteins with relatively short TMDs are not restricted to the GA but also can reach the PVC (Rayner and Pelham, 1997).

In mammals, the length of the TMD is a major factor for Golgi retention. Becker et al. (2000) found that the TMDs of murine α1,2-mannosidase IB and α1,2- mannosidase were the major targeting determinant in Golgi localization in COS7 cells (Becker et al., 2000). A series of TMD replacements and mutations increased β1, 4 GalT secretion in COS7 cells. In this case, the cysteine 29 and histidine 32 that are located in the TMD played a crucial role (Aoki et al., 1992; Masibay et al., 1993). Although the TMD has often a dominant role in Golgi retention, some studies have also demonstrated that occasionally the stem region (a region found between the luminal and transmembrane domains) also determines the localization of certain proteins. In plants, the CT and TMD of Arabidopsis β1,2-xylosyltransferase were necessary and sufficient to target GFP chimeras to the GA in *N.benthamiana* leaf cells (Dirnberger et al., 2002; Pagny et al., 2003).

The TMD and CT of type I plant membrane proteins are also essential for their localization. Paris et al. (1997) showed that a truncated form of the vacuolar sorting receptor, $VSR_{PS-1}$ (see below) lacking the TMD and the CT was secreted when expressed in tobacco suspension cell protoplasts (Paris et al., 1997). This suggested that when the luminal domain alone was not anchored in the membrane, it was transported out of the cell by the default route for soluble proteins. More recently, Brandizzi and coworkers (2002) showed that a chimeric GFP fused to the 23-amino acid TMD of the human lysosomal protein LAMP1 was retained in the ER, while the 17-amino acid TMD containing chimera was accumulated in the plasma membrane (PM) and a TMD of 20 amino acids targeted GFP to the GA. These results clearly confirmed that the destination of membrane proteins depends on the length of the TMD.

### Models for Golgi integral membrane proteins retention

#### *The oligomerization/kin recognition model*

This model proposes that some Golgi enzymes form (homo-/ or hetero-) oligomers within this compartment. These oligomers would be too large to enter anterograde vesicle trafficking between the Golgi cisternae or to the PM (Munro, 1998). This is thought to happen by interaction of their TMD and the neighboring stems. A 'kin recognition' theory was proposed for hetero-oligomers based on evidence obtained from the two medial Golgi enzymes N-acetylglucosaminyl transferase I (NGAT I) and mannosidase II (Mann II) (Nilsson et al., 1994). A chimeric protein containing the luminal domain region of NAGT I and the ER localization signal-containing cytoplasmic tail of a reporter protein (P33) was targeted to the ER. Man II concommitently also relocalized to the ER, suggesting the existence of a hetero-oligomer.

#### *Lipid bilayer features*

The essential role of the TMD in Golgi retention could be due to thickness and composition differences of the lipid bilayers within the secretory pathway (Munro, 1995b). The bilayer thickness model suggests that the concentration gradient of cholesterol and glycolipids from the ER to the PM results in different membrane thickness. The Golgi membrane is thinner than the PM. Golgi proteins would be unable to progress across or beyond the Golgi stack due to their relatively short TMDs.

TMD regions of Golgi proteins are on average five residues shorter than those of plasma membrane proteins (Munro, 1995b). The hydrophobicity of the first fifteen residues of Golgi proteins is slightly higher due to a higher content of phenylalanine compared to plasma membrane proteins. In Golgi proteins, the distribution of tyrosine and tryptophane residues, which are localized around the ends of the TMD at the water/lipid interface, peaks about five residues closer to the start of the TMD than in PM proteins (Gomord et al., 1999). Therefore, it is believed that localization of proteins could result from an optimal interaction between their signal anchor region and the lipid membrane. This suggests that it is more favorable for Golgi proteins with shorter and more hydrophobic TMDs to remain in the Golgi compartments than to be transported further along the secretory pathway.

## The *trans*-Golgi network: the late secretory sorting station

The complexity of sorting mechanisms at the TGN is believed to be a general feature in all eukaryotic cells. The TGN plays a pivotal role in directing soluble and membrane proteins in the secretory pathway to the appropriate cellular destination. One route, the constitutive or default pathway, delivers proteins to the cell surface (Denecke et al., 1990; Graham and Emr,

1991) while other selective pathways sort proteins into the intracellular endosomal membrane system (Bryant and Stevens, 1998; Conibear and Stevens, 1998; Neuhaus and Rogers, 1998; Jiang and Rogers, 1999; Vitale and Raikhel, 1999).

Each pathway leaving the TGN is mediated by distinct carrier vesicles. In yeast, for example, at least five distinct types of vesicles bud from the TGN: two separate populations of vesicles carry secretory proteins to the cell surface (Harsay and Bretscher, 1995), one mediates the retrieval of Golgi enzymes to earlier Golgi compartments (Harris and Waters, 1996), and two different types of vesicles follow separate pathways to the vacuole (Cowles et al., 1997a; Piper et al., 1997).

## Clathrin-coated vesicles, adaptors, and adaptor-related proteins

### Clathrin

Clathrin triskelions form the outer cytoplasmic coat of the clathrin-coated vesicles (CCVs). Triskelions are hexameric proteins, with three heavy-chain (CHC) and three light-chain (CLC) polypeptides (Pishvaee and Payne, 1998; Robinson et al., 1998a). Triskelions from plant and yeast are morphologically identical to those from mammalian CCV, but have a higher molecular mass. CHC polypeptides are highly conserved and are encoded by a single gene in all eukaryotes. In the case of CLC, mammalian cells have two distinct genes ($CLC_a$ and $CLC_b$) while yeast has only one (Robinson et al., 1998a). Several candidates for CLC have also been found in plants, but their function still remains unclear.

In mammalian cells, CCVs have been shown to bud from three different membranes: the PM, where they function to internalize cell surface receptors-bound ligands (Robinson, 1996); the TGN, where they are responsible for the selective transport of lysosomal acid hydrolases out of the GA (Braulke, 1996); and the late endosomes from where they recycle receptors back to the cell surface (Stoorvogel et al., 1996). In yeast, CCVs participate in the sorting of vacuolar proteins in the GA (Seeger and Payne, 1992a; Deloche et al., 2001). However, in yeast clathrin also appears to be involved in the retention of resident Golgi membrane proteins, thereby preventing their transport to the cell surface (Seeger and Payne, 1992b).

In plant, CCV also seem to be formed at the PM and the TGN, but unlike in mammalian and yeast cells, there is no clear proof for receptor-mediated endocytosis, although the uptake of unspecific electron dense tracers via CCV has been demonstrated on numerous occasions (Robinson et al., 1998a).

**Adaptors and adaptor-related proteins**

The adaptor complexes (AP) are believed to provide membrane binding sites for vesicles coat components such as clathrin and also to interact with membrane proteins to recruit specific cargo into transport vesicles They constitute the linkers between the cytosolic tails of receptor proteins and the triskelions. In animal cells, AP-1 and AP3 are both located at the TGN (Le Borgne et al., 1996; Cowles et al., 1997b; Dell'Angelica et al., 1997; Stepp et al., 1997) while AP2 is located at the PM (Pearse and Robinson, 1990), and AP4 is located at a perinuclear compartment (Hirst et al., 1999). Each of the AP complexes consists of two large subunits: a β subunit (β1- β4) and a more divergent subunit (α, γ, δ, ε), a medium subunit (μ1- μ4), and a small subunit (σ1- σ 4). The carboxyl-terminal domains of the two large subunits from 'ears' connected to the head of the complex by flexible hinges (Robinson and Bonifacino, 2001).

The different subunits of the AP complexes perform different functions. β1 and β2 , for example, function as clathrin-binding partners (Shih et al., 1995; Owen et al., 2000) while μ1- μ3 (Bremnes et al., 1998; Heilker et al., 1999) are main partners for the CTs of various membrane proteins. AP1 and AP2 are the only APs associated with clathrin triskelions, while there is no evidence for inclusion of AP3 or AP4 in CCVs.

Two other families of monomeric proteins with adaptors-related domains have been identified. The GGAs (Golgi-localizing γ-adaptin ear homology domain ADP-ribosylation factor binding protein) were described in mammalian and yeast (Dell'Angelica et al., 2000; Hirst et al., 2000; Costaguta et al., 2001) but have apparently no equivalents in plants. A further family of adaptor proteins is the stoned B family, which acts at the PM level.

**CCV formation**

***Receptor-coat protein interactions***

The first step in CCV formation is the attachment of cytosolic APs onto their target membrane, a process regulated by ARF (ADP ribosylation factor), a small GTPase, and by phospholipids (Le Borgne and Hoflack, 1998). Clathrin APs concentrate transmembrane proteins by interacting with their tyrosine or dileucine-based sorting signals, which are exposed to the cytosol (Kirchhausen et al., 1997; Bonifacino and Dell'Angelica, 1999; Bonifacino and Traub, 2003). They also interact with clathrin triskelions which organize into cages and constitute the outer layer of the coat. The assembly mechanism is similar to the mechanism described above for the COP vesicles.

*In mammalian cells*: The PM, TGN, and endosomes are the three main sites for receptor-mediated clathrin sorting events in animal cells (Robinson et al., 1998a). Some of the receptors at the PM of mammalian cells are always found concentrated in coated pits (region of PM coated with clathrin on its cytosolic face), and are internalized via CCVs. For example, an interaction of the cation-independent mannose-6-phosphate receptor (ci-M6PR) with the AP2 has been well established (Glickman et al., 1989). At the TGN newly synthesized acid hydrolases are specifically diverted from the pathway to the PM by M6PRs. The CTs of these receptors interact with AP1 (Mauxion et al., 1996) via both a tyrosine motif and a dileucine motif (Robinson et al., 1998a). Then, the ligand-receptor complex is transported via CCVs to the prelysosomal, endosomal compartment from which the receptors are recycled to the TGN, again via CCVs. The binding of the tyrosine signal occurs via the μ1 and μ2 adaptins (Bremnes et al., 1998). The acidic cluster dileucine signal from the MPR is recognized by GGA adaptors. The AP3 complex recognizes the tyrosine motif YQRL in the cytoplasmic tail of TGN38 (Dell'Angelica et al., 1997).

Although the isolated APs interact with the sorting signals in the cytosolic tails of transmembrane receptors and are essential components of the adaptor docking site, they also require additional docking proteins. For instance, in the TGN-derived CCV, the putative AP-1 docking proteins p75, p80 and p60 could be found specifically cross-linked to γ-, β1, μ1-adaptins, respectively (Seaman et al., 1996).

*In yeast*: Several yeast integral membrane proteins also possess a tyrosine motif in their CT which is also believed to be important for the interactions with the clathrin adaptors. Kex2p, a protein of the TGN, Vps10p, the receptor for vacuolar carboxypeptidase Y and the membrane–bound proform of alkaline phosphatase (ALP) also contain tyrosine motifs (Wilcox et al., 1992; Stack et al., 1995; Cowles et al., 1997a). Vps10p interacts with AP1 (Yeung et al., 1999; Deloche et al., 2001), while ALP interacts with AP3 (Cowles et al., 1997b).s

*In plants*: All members of the Vacuolar Sorting Receptor (VSR, see below) family contain a form of the tyrosine motif (YXXø, see Appendix 10) (Paris et al., 1997). $VSR_{PS-1}$ was found to be enriched in CCVs (Kirsch et al., 1994; Robinson et al., 1998a; Hinz et al., 1999; Hillmer et al., 2001), which is also true for some of its *Arabidopsis* homologues, the AtVSRs (Ahmed et al., 1997)., It was recently demonstrated that a *Arabidodpsis* μA-adaptin (component of the CCV coat) binds the $VSR_{PS-1}$'s tyrosine motif and the tyrosine residue was also confirmed to be crucial for this binding (Happel et al., 2004). In plant cells, there are no informations yet about the mechanisms underlaying the recruitment of the AP and of clathrin triskelions, but both ARF (Memon et al., 1993) and dynamin (Park et al., 1997) homologues have been described.

### Other types of vesicles in plant

Apart from the CCVs, two other types of vesicles have also been described in plants: the dense vesicles (DVs) (Hohl et al., 1996; Robinson and Hinz, 1997), and the precursor accumulating (PAC) vesicles (Hara-Nishimura et al., 1998). They appear to participate in the same vesicular sorting pathway since both transport storage proteins to vacuoles. However, they are generated in different organelles within the cell. It is believed that most of the storage proteins are transported to the vacuole via the GA by the DVs while some other storage proteins are directly transported from the ER by PACs, bypassing the GA. The mechanism responsible for such Golgi-independent transport has not been yet characterized.

In contrast to lysosomal and vacuolar acid hydrolases, which are sorted at the TGN, at least certain storage proteins are already sorted, by an unknown mechanism in the *cis*-cisternae of the GA, before entering DVs (Hillmer et al., 2001). Hohl et al. (1996) demonstrated in maturing pea cotyledons that electron-dense material, as apparent within cis-, medial-, and trans-cisternae of Golgi and contained storage proteins (Hohl et al., 1996). In contrast, in pumpkin cotyledons and castor bean endosperm, proglobulin and pro2S albumin (two storage proteins) were transported from the ER to the storage vacuole via the PACs (Hara-Nishimura et al., 1998). A receptor from the VSR family was isolated from these vesicles (Shimada et al., 1997). Unlike CCV, which are enriched in VSR, DVs are free of VSR but carry the typical storage vacuole aquaporin, α-TIP (Robinson et al., 1998b; Hinz et al., 1999).

## Sorting to vacuoles

Once the vacuolar proteins are packed into vesicles, they can travel further toward their final destination, the vacuole. Most proteins first transit via a PVC before they reach the vacuole (Vida et al., 1993; Jiang and Rogers, 1998; Robinson et al., 1998b; Miller et al., 1999; Tse et al., 2004).

In the next part of the introduction, I will first briefly refer to the sorting pathway to the lysosome in the mammalian system and then I will mainly focus on the different sorting pathways to the yeast and plant vacuole (s), an the intermediate compartments, as well as on the molecular aspects of vacuolar protein sorting in these two organisms.

## The lysosomal protein sorting in mammalian cells

The best characterized lysosomal targeting pathway for soluble hydrolases is the mannose-6-phosphate pathway (Man6P). First, an N-acetylglucosamine phosphate residue is added to the carbon atom 6 of a mannose of an N-linked oligosaccharide by a transferase. Subsequently, the N-acetylglucosamine is removed by a phosphodiesterase, leaving the

mannose 6-phosphate residue. The Man6P-tagged lysosomal proteins are then recognized and bound by M6PRs at the TGN and the ligand-receptor complexes are transported by CCVs to an acidic endosomal compartment where the ligands dissociate from the receptors in a pH-dependent manner. The soluble ligands continue to the lysosome, while the M6PRs recycle back to the GA for additional rounds of transport.

There are two M6PRs (MPR46 and MPR 300) in mammalian cells, and the cytoplasmic tail of each contains signals for delivery from the Golgi to the endosome and for endocytic internalization from the cell surface. Deletion and mutagenesis studies have identified a short sequence at the C-terminus of each of the MPRs consisting of a di-leucine motif that is required for the sorting of proteins from the lysosome (Johnson and Kornfeld, 1992). Upon transport of M 6-P-containing ligands to the endosome by CCVs, the MPRs either recycle to the Golgi or are delivered to the cell surface. Endocytic internalization of MPRs is again mediated by CCV. In this process plasma membrane-adaptins proteins link clathrin to the MPR CTs by binding to tyrosine-containing signals (Kornfeld, 1992). It is known that AP2 adaptins are associated with tyrosine-containing motifs in the MPRs to facilitate clathrin-dependent endocytosis, whereas Golgi adaptins (AP-1) associate with other determinants in the CT, including di-leucine motifs, to direct the transport from the GA to the endosome also in a clathrin-dependent process.

The delivery of membrane proteins to the lysosome appears to be independent of the mannose-6-phosphate recognition system, since these proteins do not contain mannose-6-phosphate modifications. (For further information, see the reviews: Hille-Rehfeld, 1995; Stack et al., 1995; Ghosh et al., 2003).

## Vacuolar Protein Sorting in yeast

The yeast vacuole, like the mammalian lysosomes and the plant lytic vacuoles, is an acidic organelle that is responsible for the degradation of macromolecules. It is also essential for pH and osmoregulation and it serves as an important storage reservoir of amino acids, small ions and polyphosphates. To carry out all these functions, it is important that the yeast vacuole contains its full complement of proteins (Klionsky et al., 1990).

The yeast vacuole is a final compartment station into which different transport pathways converge (Bryant and Stevens, 1998):

i) A two Biosynthetic pathways, following transit through the early stages of the secretory pathway diverging from the pathway to the cell surface (Figure 1.3). The CPY pathway is similar to the Man-6P pathway in animal cells and to the plant sorting pathway that uses

sequence-specific Vacuolar Sorting Determinants (ss-VSD) (see below). The ALP pathway transports proteins directly from the Golgi to the vacuole, bypassing the PVC.

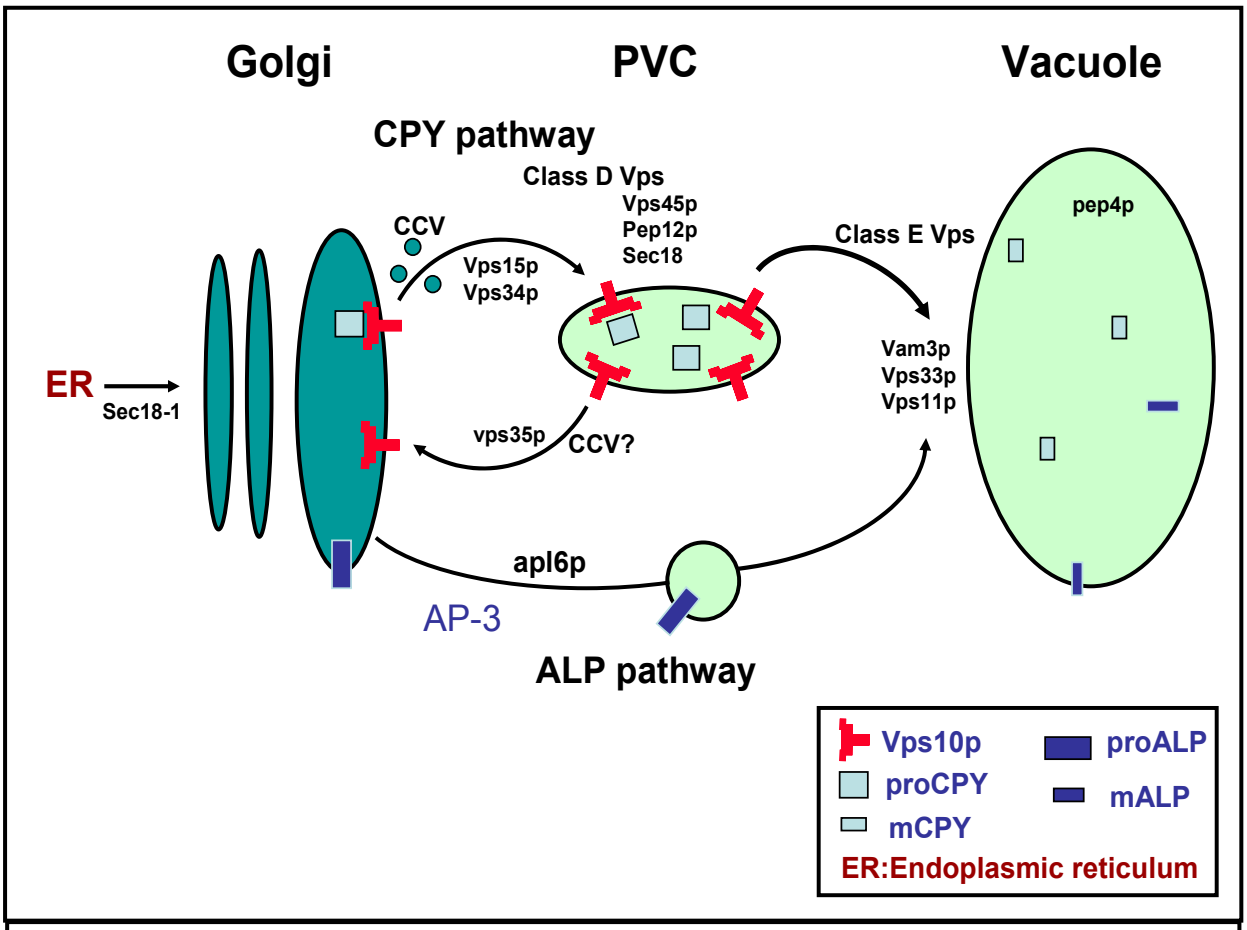



**Figure 1.3. Transport pathways from the yeast TGN to the vacuole.** Newly synthesized proteins can be transported to the vacuole by two different pathways. Proteins of the CPY pathway enter CCV at the TGN and reach the vacuole by way of an PVC that also receives material from the endocytic pathway. Proteins that enter the ALP pathway are sorted into a distinct class of vesicles at the level of the GA. Although they reach the vacuole without passing through the PVC, it is not known whether they transit through an alternative intermediate compartment. CPY and ALP pathways converge at the vacuole, which also receives material from autophagy. A large collection of genes are implicated in the budding of vesicles carrying vacuolar cargo at the different steps of the secretory pathway. The *Sec18-1*(yeast NSF) protein is required for the docking and/or fusion of transport vesicles derived from different organelles with their appropriate target membrane (in particular, in the anterograde ER-to-Golgi transport). Whereas class D Vps proteins (e.g. *VPS45* and *PEP12*) act before vacuolar proteins gain entry into the PVC, class E Vps proteins (e.g. *VAM3*, *VPS33*, and *VPS11*) control traffic out of the PVC. *VPS15* and *VPS34* are examples of class D *VPS* genes that appear to act at the level of vesicle formation from the TGN. *VPS35* is required for the recycling of Vps10 from the PVC back to the GA. The *PEP4* gene is required for the activation of numerous vacuolar hydrolases. This figure is modified from Bryant and Stevens (1998).

ii) Endocytosis from the PM. Two mechanisms coexist: constitutive (ligand-independent) and ligand-dependent (Roth and Davis, 1996). These pathways require phosphorylation and ubiquitination of the molecules, prior to their internalization (Hicke and Riezman, 1996; Roth and Davis, 1996). The endocytic machinery implicates many components such as *END* genes, Rab proteins and CCVs as well as the actin cytoskeleton (Bryant and Stevens, 1998). Piper and coworkers (1995) demonstrated that the endocytic and the Vps pathways converge at the PVC (Piper et al., 1995).

iii) Cytoplasm-to-vacuole targeting pathways, which do not pass though the early stages of the secretory pathway. Autophagy is the best characterized mechanism of cytoplasm-to-vacuole targeting (Klionsky, 1997), and is a response to nutrient deprivation in which cells degrade large amounts of intracellular proteins. Two different types of autophagy exist: non-specific/bulk and specific. The latter mechanism is used to deliver hydrolases such as the aminopeptidase I (API) to the vacuole. ER-to-Golgi transport is not required (Klionsky, 1998).

iv) Inheritance of vacuolar material by daughter cells during cell division. Cells do not synthesize organelles de novo but, instead, build upon material inherited from their mother cells. Yeast cells inherit vacuoles from their mothers through a mechanism that involves the formation of vesicles and tubules, known as segregation structures, from the mother-cell vacuole, and their transport into the growing daughter cell (Weisman and Wickner, 1988; Raymond et al., 1990).

Although these four routes are different, as genetic screens have revealed, there is a significant overlap. Therefore, all four pathways are intimately related.

It is noteworthy that vacuolar proteases are often synthesized as inactive zymogens whose activation requires proteolytic cleavage to remove a propeptide. This processing occurs in the vacuole and requires proteinase A (PrA), encoded by *PEP4*, to initiate a vacuolar protease activation cascade (Moehle et al., 1989; Nebes and Jones, 1991). The fact that the *PEP4*-dependent cleavage of many vacuolar proteins results in the formation of a lower-molecular weight form of these proteins, provides a convenient signal to monitor the kinetics of vacuolar delivery.

**Sorting of soluble vacuolar proteins**

In yeast, soluble (luminal) vacuolar proteins such as carboxypeptidase Y (CPY), proteinase A (PrA), and proteinase B (PrB) transit through the early stages of the secretory pathway and are sorted away from proteins destined for the cell surface (secreted proteins) in the TGN. Soluble hydrolases require a sorting signal to mediate their vacuolar delivery from the TGN, and in the absence of such information, these proteins are secreted by the default pathway.

Entry into and progression through the secretory pathway are accompanied by compartment-specific modifications of vacuolar proteins. The posttranslational modifications of CPY serve as good indicators of its localization in the pathway to the vacuole. CPY is synthesized as a prepro-enzyme and translocated into the lumen of the ER, where its signal sequence is cleaved off to form proCPY (Stevens et al., 1982; Johnson et al., 1987). proCPY receives N-linked core glycosylation in the ER, which results in a 67-kDa form (p1CPY). Once p1CPY arrives at the GA, further oligosaccharide modifications produce the 69-kDa form (p2CPY). p2CPY is sorted away from proteins destined for the cell surface in the TGN through a receptor-mediated process that leads to its delivery to the vacuole, where it is cleaved by vacuolar proteases into its active, mature, 61-kDa form (mCPY).

Unlike mammalian lysosomal hydrolases, delivery of yeast proteins to the lysosome-like vacuole does not involve a glycan-based sorting signal. Instead, the sorting signals of yeast vacuolar hydrolases reside in their amino acid sequences. In the case of CPY, the sequence QRLP (residues 24 to 27) in the propeptide region of pro-CPY forms the core of the targeting signal required to divert p2CPY away from the secretory pathway, into the vacuolar biogenesis pathway. Alteration of this signal results in secretion from the cell (Johnson et al., 1987; Valls et al., 1987; Valls et al., 1990).

**Sorting of vacuolar membrane proteins**

In addition to soluble hydrolases, the yeast vacuole contains a variety of membrane proteins that function in macromolecular degradation and maintenance of an acidic environment (Klionsky et al., 1990). Membrane proteins such as alkaline phosphatase (ALP), the vacuolar ATPase (Vph1p), and the two dipeptidylaminopeptidases A (localized in the GA) and B (localized in the vacuole) (DPAP A and B respectively) also travel through the early stages of the secretory pathway.

In contrast to soluble hydrolases, integral membrane proteins lacking sorting information are not delivered to the cell surface. Instead, they are transported to the vacuole (Roberts et al., 1992; Wilcox et al., 1992). Yeast Golgi membrane proteins such as DPAP A, Kex2p, Kex1p achieve their localization through an aromatic type of signal within their CTs (i.e. motifs

containing tyrosine and phenylalanine residues) which prevents their exit from the TGN (Wilsbach and Payne, 1993; Nothwehr and Stevens, 1994). Removal of these signals or overproduction of the wild type proteins result in their delivery to the vacuole where they are subject to vacuolar protease-dependent (PEP4) cleavage (Jones et al., 1982; Cooper and Bussey, 1992; Roberts et al., 1992; Wilcox et al., 1992; Nothwehr et al., 1993; Nothwehr and Stevens, 1994). Mutations of the localization signals of resident ER membrane proteins also result in their transport to the vacuole (Gaynor et al., 1994). These results led to postulate that delivery of membrane proteins to the vacuole does not require specific sorting information, and that the vacuole rather than the PM is the default destination for membrane proteins in yeast (Nothwehr and Stevens, 1994).

The mechanisms of Golgi retention and vacuolar delivery are rather complex in yeast. For example, loss of clathrin function results in delivery of Kex2p to the cell surface rather than to the vacuole (Payne and Schekman, 1989).

## Receptor-Mediated Sorting of Soluble Vacuolar Proteins: The CPY sorting receptor Vps10p

Like the M6PR system in mammalian cells, delivery of soluble proteins to the yeast vacuole is mediated by transmembrane receptors. Early studies revealed that overproduction of CPY (Stevens et al., 1986; Johnson et al., 1987) and PrA (Rothman et al., 1986) results in missorting and secretion of a substantial portion of these proteins. This saturation effect could be due to a limiting component such as a receptor molecule that mediates their recognition and sorting to the vacuole. A few years later Marcusson and coworkers (1994) isolated and characterized the sorting receptor for CPY by a complementation screen in a mutant strain that secretes a CPY-invertase fusion (Marcusson et al., 1994). Cells lacking Vps10p missorted more than 90% of their CPY, and Vps10p could be cross-linked to pro-CPY but not to a sorting defective form of pro-CPY. Vps10p also cofractionated with Golgi membranes containing Kex2p and A-ALP (Nothwehr et al., 1993) as well as with lighter membrane fractions corresponding to a prevacuolar, endosomal compartment (Marcusson et al., 1994; Cooper and Stevens, 1996). This localized Vps10p at the expected site in the secretory pathway.

Vps10p is a 178k-Da type I transmembrane protein of 1579 residues with a signal sequence for ER import at its amino terminus (amino acids 1-21), a large luminal domain of 1,380 amino acids, a 17-amino acid TMD (amino acids 1392-1413) and a CT of 164 amino acids. Vps10p is synthesized at a rate 20-fold lower than that of its ligand CPY, which in light of the 1:1 binding stoichiometry, suggested that Vps10p must repeatedly cycle between the TGN and the PVC.

Vps10p was later shown to be also involved in the sorting of another soluble vacuolar hydrolase, PrA. As well as secreting CPY, cells lacking Vps10p secreted >60% of PrA (Cooper

and Stevens, 1996). The PrA propeptide does not contain the QRPL sequence identified in CPY as sorting determinant.

A model of the pathway taken by CPY (as well as by other vacuolar hydrolases) from the TGN to the vacuole was proposed based on the delivery of proteins to the lysosome in mammalian cells by the M6PRs (Marcusson et al., 1994; Cereghino et al., 1995; Cooper and Stevens, 1996): (Figure 1.3) (i) Vps10p binds p2CPY in the TGN, (ii) the receptor-ligand complex travels to the PVC in Golgi-derived transport vesicles, (iii) CPY dissociates from its receptor in the PVC and is transported to the vacuole, while Vps10p is recycled back to the GA for another round of protein sorting. This cycle requires many cytosolic components of the vesicular machinery and additional VPS gene products.

Vps10p also targets to the vacuole certain misfolded proteins that exit from the ER. For instance, while a fusion protein containing the $NH_2$-terminal domain of the λ repressor and invertase was secreted, hybrid proteins with amino acid substitutions that cause this domain to be thermodynamically unstable (Inv- λ* hydrids) were targeted to the vacuole in a Vps10p-dependent manner (Hong et al., 1996).

The Vps10p luminal domain was predicted to interact with the soluble ligands. Therefore, in an effort to determine the domain structure and ligand binding of Vps10p, Jørgensen and coworkers deleted parts of this luminal domain (Jorgensen et al., 1999). The luminal region of Vps10p has two domains (known as domains 1 and 2) with some sequence homology to each other. Domain 2 has two different binding sites, one for CPY, PrA and the aminopeptidase Y (APY) and another for CPY-invertase and PrA-invertase. The latter interaction seems not to be sequence-specific, and it was suggested that an unfolded structure in these ligands is recognize by Vps10p.

In summary, Vps10p is believed to mediate two types of ligand interactions: (a) binding to native vacuolar proteins via a sequence-specific interaction, and (b) binding to misfolded regions of proteins via a more general structure-dependent interaction.

**The Cytosolic domain of Vps10p is essential for its localization and for CPY sorting**

The CT of Vps10p is essential for the proper function and stability of this receptor. Cells expressing a mutant form of Vps10p lacking the CT (Vps10pΔCT) secreted 90-95% of CPY, and in addition Vps10pΔCT itself was missorted to the vacuole, where it was rapidly degraded in a *PEP4*-dependent manner (Cereghino et al., 1995; Cooper and Stevens, 1996).

Cooper and Stevens (1996) also determined whether the CPY secretion resulted from mistargeting of Vps10pΔCT or from its inability to bind CPY. Overexpressed Vps10pΔCT was able to correctly sort 34% of CPY to the vacuole compared with 6% in cells expressing wild-type levels of the truncated protein (Cooper and Stevens, 1996). This confirmed that the

efficient sorting of CPY normally requires recycling of Vps10. It was also proposed that since Vps10p must recycle rapidly to sort all the newly synthesized CPY, Vps10p may be localized to the TGN exclusively by the retrieval mechanism.

The CT of Vps10p contains the tyrosine-based signal, $Y_{77}SSL_{80}$ (the numbers indicate the position within the CT), similar to late Golgi proteins and to the M6PRs. This signal is essential for the retrieval of the receptor to the TGN (Cereghino et al., 1995; Cooper and Stevens, 1996), and mutations within this signal resulted in 35-45% CPY secretion.

A dileucine motif and several aromatic residues also contribute to the function and stability of Vps10p (Cereghino et al., 1995; Cooper and Stevens, 1996). For example, the deletion of the sequence containing both the tyrosine motif and the dileucine motif, $Y_{77}$SSLLDDL resulted in 56% missorting of CPY. A single phenyalanine ($F_{106}$) is also required for receptor recycling. Its substitution by alanine (A) resulted in 20% missorting of CPY. Finally, a cluster of aromatic residues ($F_{41}YVF_{44}$) which is a retention signal for late Golgi proteins, only plays a structural role in Vps10p.

Similarly to Vps10p$\Delta$CT, the series of deletions or mutations within the Vps10p CT mentioned above did not only lead to CPY missorting but also to the rapid degradation in the vacuole of the mutant receptor (Cereghino et al., 1995; Cooper and Stevens, 1996). Additionally, as for Golgi membrane proteins (see above), overproduction of Vps10p also lead to its delivery and subsequent degradation in the vacuole (Cereghino et al., 1995).

### Vacuolar Delivery via a Prevacuolar/Endosomal compartment

Subcellular fractionation studies revealed that CPY transits through another compartment on its journey from the TGN to the vacuole (Vida et al., 1993). This compartment is the PVC (Horazdovsky et al., 1995; Stack et al., 1995).

### Mutants in Vacuolar Protein Sorting

Alteration of the vacuolar sorting signal in wild type CPY or in CPY-invertase fusion proteins results in their localization at the cell surface. This mislocalization of vacuolar proteins is the basis of several genetic selection screens that resulted in the isolation of a large number of yeast mutants specifically defective in the delivery of proteins to the vacuole (Bankaitis et al., 1986; Rothman and Stevens, 1986). Complementation analyses of the vacuolar protein sorting (vps) mutants determined that they have extensive genetic overlaps and define more than 40 complementation groups (Rothman et al., 1989). This large number of genes indicates that protein sorting to the vacuole is a complex process. Possible molecules involved in vacuolar protein sorting include (a) receptor(s) for vacuolar protein precursors; (b) proteins that direct

the segregation and packaging of receptor-ligand complexes into vesicles carriers; (c) factors responsible for vesicle formation, (d) membrane fusion; (e) vesicle coat proteins; (f) proteins involved in the targeting and recognition of vesicles carriers with their target organelles; (g) receptor recycling mechanisms; and (h) proteins involved in the maintenance of vacuolar structure and function (Stack et al., 1995).

***vps mutants***

The large collection of vps mutant was divided into six classes (A to F) on the basis of a number of criteria including vacuolar morphology (Banta et al., 1988; Raymond et al., 1992). It appears that mutants that fall into the same class are blocked in the same stage of vacuolar protein sorting (Stack et al., 1995). The class A of mutants (*vps10*, *vps13*, *vps44*, *vps46*) contain 1-3 large vacuoles that are morphologically indistinguishable from those in the parental strain, suggesting that only a subset of the proteins destined for delivery to this compartment is mislocalized (Raymond et al., 1992). Mutants from the class E (Figure 1.3), for example, accumulate an exaggerated form of the PVC containing CPY and also endocytose proteins such as the mating pheromone receptor Ste3p, as well as vacuolar membrane proteins, such as Vph1p. This indicates that the PVC represents a point of overlap between the endocytic pathway and the vacuolar biogenesis pathway (Piper et al., 1995; Rieder et al., 1996; Babst et al., 1997).

The mutants from the class E are unaffected in anterograde traffic between the Golgi and the PVC and are competent for endocytosis as far as the PVC (Piper et al., 1995; Rieder et al., 1996; Babst et al., 1997). The accumulation in the PVC results from a block in membrane traffic out of the PVC both to the vacuole and back to the Golgi apparatus. Therefore, these gene products seem to control the exit from the PVC. Using a v*ps27* mutant, the role of the aromatic amino acid localization signal in the retrieval of Golgi membrane proteins from the PVC was elucidated (Piper et al., 1995).

The classes D Vps proteins act before vacuolar proteins gain access to the PVC (Figure 1.3). *VPS15* and *VPS34* are examples of class D *VPS* genes that appear to act at the level of vesicle formation from the late Golgi, and thus a loss of function of these genes blocks traffic between the late Golgi and the PVC (Stack et al., 1995). Other class D mutations appear to prevent the formation of Golgi-derived transport vesicles (i.e. Vps1p, a homolog of the mammalian protein dynamin (Conibear and Stevens, 1998). Clathrin is also implicated in the formation of these vesicles. Other mutations prevent the fusion of such vesicles with their acceptor membrane, the PVC (i.e. Vps45p, a Sec1p-like protein (Piper et al., 1994); Vps21p, a rab GTPase (Horazdovsky et al., 1994); Pep12p, a t-SNARE of the PVC (Becherer et al., 1996)). It has been shown that they are all parts of the same SNARE complex which is

regulated by *SEC17* (yeast α-SNAP) and *SEC18* (yeast NSF). In this class of mutants the endocytosis is not affected but CPY is secreted.

Even though the mechanism for signal recognition and recycling of membrane proteins from the PVC back to Golgi is not well understood, a number of *VPS* genes have been implicated in this step (i.e. Vps29p, Vps35p, Vps26p, Vps26p, Vps5p and Vps17p). These gene products form the retromer complex in which Vps35p, Vps29, Vps26 are believed to select cargo for retrieval, and Vps5p and Vps17p to assemble on the membrane to promote vesicle formation (Seaman et al., 1997; Seaman et al., 1998). Vps26p promotes the interactions between the cargo-selective component Vps35p and Vps5p/Vps17p (Reddy and Seaman, 2001). Vps35p is the subunit required for the retrieval of both Vps10p and A-ALP (Nothwehr et al., 2000).

Trafficking from the PVC to the vacuole is dependent on genes represented by the class B and C vps mutants. The vacuolar t-SNARE (Darsow et al., 1997; Wada et al., 1997) Vam3p, seems to play a key role for the delivery of all vacuolar or tonoplast proteins even if they take different pathways to the vacuole (CPY; tonoplast alkaline phosphatase, ALP (see below); aminopeptidase I (API), and the Vamp3 itself). The four known class C *VPS* gene products (Vps11p, Vps16p, Vps18p and Vps33p) have been localized in the vacuolar membrane and have been shown to interact with each other physically and genetically (Rieder and Emr, 1997).

There are two competing theories for the PVC-to-vacuole transport, the vesicular transport and the maturation theories. In contrast to mammalian cells in which the maturation theory has been supported by several experiments (Futter et al., 1996), protein traffic from the PVC to the yeast vacuole seems to be mediated by vesicles, since several SNAREs encoded by class B and C *VPS* genes mediate this step (Bryant and Stevens, 1998).

In summary, the PVC seems to be the point at which the Vps and endocytic pathways merge (Piper et al., 1995). Many gene products are required to enter into and to exit from this compartment such as the components of the machineries for the formation, docking and fusion of vesicles at the TGN-PVC level.

***pep mutants***

Yeast strains defective in CPY enzyme activity, were designated as *pep* mutants (Jones, 1977). *Pep* mutants define 17 complementation groups. These mutants show pleiotropic defects in several vacuolar proteolytic activities including CPY, PrA, and PrB. Two subclasses of pep mutants have been described so far. One class is represented by the *pep4* mutant, affected in the structural gene for PrA (Jones et al., 1982; Ammerer et al., 1986). PrA is involved in the proteolytic maturation of several other hydrolases, including CPY and PrB, which accounts for the pleiotropic nature of the *pep4* mutants. Other pep mutants resemble

*vps* mutants and secrete p2CPY and proPrA. A substantial genetic overlap between the *pep* and *vps* mutants has been revealed (Rothman et al., 1989). Therefore, it is believed that many *pep* mutants are defective in vacuolar hydrolase activity as the result of missorting and secretion of vacuolar protein precursors (Stack et al., 1995).

## The Alkaline Phosphatase (ALP) pathway: ALP bypasses the PVC

Early studies demonstrated that the membrane protein ALP is correctly localized to the vacuole even in class E or D vps mutants (Raymond et al., 1992; Horazdovsky et al., 1996; Cowles et al., 1997a; Piper et al., 1997). Careful analysis of ALP trafficking in mutants with conditional defects in the trafficking into or out of the PVC demonstrated that ALP follows an alternative transport pathway to the vacuole that completely bypasses the PVC, the 'ALP pathway' (Cowles et al., 1997a; Piper et al., 1997) (Figure 1.3). The vacuolar t-SNARE Vam3p is the another protein which has been identified as cargo for the ALP pathway. The delivery of both proteins to the vacuole requires neither prior transport to the cell surface nor endocytosis.

Because ALP and Vam3p are not trapped in the Golgi-derived vesicles that accumulate in *vps45* mutants, the existence of a different class of transport vesicles that bud from Golgi was predicted. Vps1p is involved in the formation of both classes of vesicles from the TGN, but clathrin is only implicated in the formation of those bound for the PVC. This model is based on observations that ALP as well as proteins that utilize the Vps pathway, are diverted to the plasma membrane in *vps1* mutant cells (Nothwehr et al., 1995), while ALP is not significantly mislocalized to the cell surface of *chc1-ts* clathrin-defective cells under restrictive conditions (Seeger and Payne, 1992b). The ALP pathway is saturable, and overproduction of ALP leads to transport of the excess ALP to the vacuole in a Pep12p-dependent manner (Cowles et al., 1997a).

The 33-residue N-terminal cytosolic tail of ALP is required for the entry into the 'ALP pathway'. Addition of this sequence diverts membrane proteins from the Vps pathway to the ALP pathway while deletion of this sequence redirects ALP to the Vps pathway (Cowles et al., 1997a; Piper et al., 1997).

### Machinery required for sorting in the ALP pathway

'ALP vesicles' require the same machinery for fusion with the vacuole as vesicles derived from the Vps pathway or vesicles derived from the cytoplasm-to-vacuole pathway. Vam3p and Vam7p act at a convergence point for these three pathways (Wada et al., 1992; Wichmann et al., 1992; Darsow et al., 1997). Similarly, *YPT7* is required for the delivery of ALP to the vacuole and also for traffic from the PVC to the vacuole (Wichmann et al., 1992; Schimmoller

and Riezman, 1993). However, another class B *VPS* gene, *VPS41* is only involved in the ALP pathway: *vps41* mutants are blocked in ALP processing but process CPY and carboxypeptidase S (CPS) (Cowles et al., 1997a).

The AP-3 adaptor protein complex has also been shown to be involved in the trafficking of ALP. Disruption of genes encoding subunits of the AP-3 complex resulted in accumulation of pro-ALP (Cowles et al., 1997b).

A model for the function of the AP-3 complex has been proposed. The AP3 complex recognizes a sorting signal in the cytosolic tail of ALP at the TGN, and recruits it into a distinct class of non-clathrin coated vesicles. When ALP fails to be sorted into this alternative pathway it enters the CPY pathway by default (Conibear and Stevens, 1998).

Little is known about the interactions between the ALP CT, the AP-3 complex, and the vesicle coats involved in this pathway. The ALP CT does not contain aromatic residues, but a potential dileucine motif is present.

Clathrin is important for sorting into the CPY pathway, but not the ALP pathway. Indeed, the yeast AP-3 complex was not found to be associated with this type of vesicles. However, the dynamin-like GTPase Vps1p appears to be required for the formation of both types of Golgi vesicles.

In summary, two pathways are known to transport proteins from the TGN to the yeast vacuole. The CPY pathway requires the PVC as intermediate compartment, clathrin-coated vesicles and the associated machinery, and is mediated by the Vps10p receptor, while the ALP pathway does not require an intermediate compartment, and targets proteins such as the ALP directly to the vacuole with the help of the AP-3 complex and so far unidentified coat components.

## Transport of proteins to the plant vacuoles

### Plants contain different types of vacuoles

Plant cell vacuoles are multifunctional organelles that serve essential physical and metabolic functions for plant life. They share some basic properties with the yeast vacuole and the lysosomes of animal cells. They are lytic compartments, function as reservoirs for ions and metabolites, including pigments, and are crucial for processes of detoxification and general homeostasis. They are also involved in cellular responses to environmental and biotic stress factors. In the vegetative organs of the plant, they act in combination with the cell wall to generate turgor. In seeds and specialized storage tissues, they serve as storage sites for proteins and soluble carbohydrates (Boller and Wiemken, 1986; Wink, 1991; Okita and

Rogers, 1996; Marty, 1999). The plant vacuoles are also the final compartment in which the intracellular biosynthetic and the endocytic pathways converge.

In contrast to yeast cells, which have a single vacuole, plant cells could have up to three different vacuoles with different functions: the lytic vacuole (LV), the storage vacuole (PSV) (Hoh et al., 1995; Paris et al., 1996; Di Sansebastiano et al., 2001) and the neutral vacuole, which could also serve as a storage vacuole (Di Sansebastiano et al., 1998).

The LV is an acidic compartment that contains enzymes analogous to the lysosomal enzymes of animal cells. The membrane, or tonoplast, of such vacuoles contains the aquaporin γ-TIP (for tonoplast intrinsic protein, Höfte and Chrispeels, 1992; Marty-Mazars et al., 1995; Paris et al., 1996; Barrieu et al., 1998). In some cell types (e.g. leaf epidermis) defense or signal compounds are also stored in the vacuole, and such specialized vacuoles contain specific ATP-binding cassette (ABC) transporters (Rea et al., 1998).

The PSV is found in cells from storage tissues of seeds and fruits, where its major function is the storage of proteins (Okita and Rogers, 1996; Muntz, 1998; Herman and Larkins, 1999). The tonoplast of these neutral vacuoles contains another distinct aquaporin, called α-TIP (Höfte and Chrispeels, 1992; Paris et al., 1996; Swanson et al., 1998).

In some cases, storage proteins are also accumulated in specialized vegetative cells in response to wounding and to developmental switches. The membrane of these vegetative storage vacuoles contains the aquaporin, δ-TIP (Jauh et al., 1998; Neuhaus and Rogers, 1998).

Two separate vacuolar compartments were well defined by α-TIP and γ-TIP in the root tip cells of pea and barley root cells, pea cotyledons and mature tobacco plants (Hoh et al., 1995; Paris et al., 1996). For example, barley lectin, in root tips, was exclusively contained within the α-TIP-positive vacuoles but not in γ-TIP-positive vacuoles, whereas the acidic cysteine protease aleurain was specifically contained within γTIP-positive vacuoles but was absent from α-TIP-positive vacuoles. However, in some cells a colocalization of the γ-TIP and α-TIP marker membrane antigens was observed which indicated that the lytic and storage compartments could have merged. Thus, a model was proposed in which γ TIP defines an acid compartment (LV) whereas the α-TIP defines a separate PSV in which proteins are protected against degradative enzymes (Neuhaus and Rogers, 1998; Jiang and Rogers, 1999). In addition, δ-TIP seemed to determine the transformation of a degradative vacuole into a storage vacuole in vegetative tissues (Jauh et al., 1998).

However, the classification of vacuoles types based on their complement of TIP proteins is even more complicated because vacuoles could be identified with two different TIPs (Jauh et al., 1999). A fourth TIP-isoform called DIP (for dark induced protein) was also identified. This isoform did not immunologically cross-react with others TIPs and was found in rare root tip

cells and developing seeds (Culianez-Macia and Martin, 1993). In seed storage vacuoles, DIP is associated with the crystalloid membranes.

In mature plant tissues, barley lectin and sporamin were found together in aggregates in the central vacuole of transgenic tobacco (Schroeder et al., 1993), indicating that the sorting pathways must have converged to the same central vacuole (Paris et al., 1996).

Due to the diversity of plant vacuoles, vacuolar sorting in plants could be more complex than in the mammalian and yeast systems, since plants must send the correct proteins to each vacuolar type.

## Sorting of soluble proteins to vacuoles

### The vacuolar sorting determinants (VSDs)

As in yeast, the sorting of the soluble plant vacuolar proteins is mediated by direct recognition of a short peptide sequence or the structure of the polypeptides. A large number of signals mediating vacuolar transport have been described in plants (Neuhaus and Rogers, 1998; Matsuoka and Neuhaus, 1999). Originally, the position of the signal within the protein was used to classify them. However, it was later suggested that the sequence of some signals is more important than their position (Matsuoka, 2000). Three main groups of vacuolar sorting determinants (VSDs) have been described: (1) the 'sequence-specific' VSDs (ss-VSD) with an NPIR tetrapeptide or equivalent, which can function when exposed elsewhere in the protein (2) c-terminal VSDs (ct-VSD) which must be exposed at the C-terminus of the protein and (3) the 'internal or physical structural' VSDs (ps-VSD) which require the tridimensional structure of the protein and probably contributes to aggregation.

The two first groups of signals are part of propeptides that are cleaved from the mature vacuolar proteins. In the next paragraphs I will describe examples of such vacuolar signals.

#### *The sequence-specific vacuolar sorting determinants (ss-VSD)*

The best studied examples of ss-VSDs are in the N-terminal propeptides of sweet potato prosporamin and of barley proaleurain. Sporamin is an abundant storage protein in sweet potato tubers that does not form protein bodies unlike most seed storage proteins (Muntz, 1998). After cleavage of the signal peptide, prosporamin carries a 16 amino acid N-terminal propeptide that is later processed and thus absent in the mature sporamin tubers (Matsuoka et al., 1990). When expressed in tobacco suspension culture cells, sporamin is sorted to the vacuole (Nakamura et al., 1993). Expression of a mutant lacking the 16 amino acid propeptide resulted in secretion of sporamin, demonstrating that the propeptide contains the essential VSD (Matsuoka and Nakamura, 1991). Additionally, Nakamura and coworkers (1993) showed

that the amino acid sequence SRFNPIRL from the propeptide was essential for vacuolar targeting. The point mutation Asn-26 to Gly caused about 40% secretion, while the mutation of Ile-28 to Gly abolished vacuolar sorting. When the prosporamin propeptide was attached to the N-terminus of barley lectin lacking its C-terminal propeptide, it acted as an efficient VSD (Matsuoka et al., 1995b).

Barley aleurain was the second protein identified to contain a ss-VSD (Holwerda and Rogers, 1993). It is synthesized as a proenzyme and transported to an acidic compartment where it is processed to the mature form. In barley aleurone cells, aleurain was localized by immunoelectron microscopy in a vacuole that is morphologically and physically distinct from the PSV (Holwerda et al., 1990). This is the reason why aleurain is used as a marker to define LV (Paris et al., 1996).

Results from different substitution and deletion experiments on the aleurain propeptide revealed that an SNPIR motif was sufficient to redirect a secreted protease to the vacuole of tobacco cells (Holwerda et al., 1992), although the efficiency of targeting depended on the presence of other neighbouring determinants (SSSSFADSNPIRPVTDRAAST).

Although, signals of both sporamin and aleurain are within N-terminal propeptide, this position appears less important than their specific sequence, since the sporamin vacuolar propeptide is still functional when transferred to the C-terminus of the protein (Koide et al., 1997). The fact that the NPIR (AsnProIleArg) motif is conserved between the prosporamin and the proaleurain propeptides indicates that this sequence may be recognized by a receptor that would be responsible for directing the proteins to the proper pathway. A detailed mutation analysis revealed that the requirement for the sporamin signal ($X_1$-$X_2$-I/L-$X_3$-X4) is that the large and hydrophobic alkyl chain (I/L) is essential; X1 is preferentially an Asn residue, but at least should not have a small hydrophobic side chain, $X_2$ should not be an acidic residue; $X_3$ can be any amino acid and $X_4$ has to be a residue with a large hydrophobic side chain (Matsuoka and Nakamura, 1999; Matsuoka and Neuhaus, 1999; Matsuoka, 2000). Based on this analysis, the aleurain's NPIRP does not appear optimal for vacuolar sorting but this seems to be compensated by flanking sequences that also interact with the receptor.

Two additional peptides also apparently belong to the category of ss-VSDs: the C-terminal sequences of *Bertholletia excelsis* 2S albumin (2Sa) and of *Nicotiana alata* proteinase inhibitor (NAPI). In the case of the 2Salbumin, the last 20 C-terminal amino acids PSRCNLSPMRCPMGGSIAGF were sufficient to target the invertase to the yeast vacuole (Saalbach et al., 1996). Most of these 20 amino acids are in fact part of the mature protein. The propeptide IAGF was found to be a weak vacuolar sorting signal by itself, but was important for binding to a receptor protein (i.e. necessary but not sufficient). Additionally, the

motif NLSP appeared to be involved in the vacuolar targeting of the protein (Kirsch et al., 1996).

NA-proPI is a 40.3 kDa precursor protein found in the stigma of the ornamental *Nicotiana alata*. It is processed to a series of five 6kDa proteinase inhibitors (one is chymotrypsin- and four are trypsin-specific, Atkinson et al., 1993) which are thought to play an important role in the plant defense reactions. The precursor forms an unusual loop with a disulfide bridge linking the N- and C-terminal regions which together form a sixth inhibitor domain (Lee et al., 1999). The C-terminal propeptide, EYASKVDEYVGEVENDLQKSKVAVS, appears necessary for the vacuolar targeting. The sufficiency of the propeptide for vacuolar targeting remains to be shown and the sequence specificity has not been addressed. As detected by antibodies against the propeptide portion, the precursor-accumulating fractions partially overlapped with those containing PEP12 (a marker for a PVC) in a sucrose gradient (da Silva Conceicao et al., 1997; Sanderfoot et al., 1998). Additionally, the PEP12 fractions partially overlapped with fractions containing homologues of BP-80. A possible interaction of a VSR-like protein with this NAPI propeptide was reported (Miller et al., 1999). However, the fact that this propeptide does not contain a NPIRL-like sorting signal led to postulate the existence of another signal within the C-terminus.

Castor bean ricin was the first example of an ss-VSD located within an internal propeptide. This toxin is accumulated in the PSV, apparently via PACs that are filled with storage-type proteins (Hara-Nishimura et al., 1998). It was found to have an internal linker SLLIRPVVPNFN which was required for vacuolar targeting (Frigerio et al., 2001). This linker connects the A-to the B-chain in the precursor and is cleaved off to give the mature protein with the two domains still linked by disulfide bridges (Lord, 1985). The pentapeptide LLIRP fits the sequence requirements described above. The mutation of the ILe to Gly led to secretion of the preproricin (Frigerio et al., 2001). The linker was also sufficient for vacuolar targeting since its was able to redirect the reporter GFP to the vacuole when attached to its C-terminus. It is worth noting that in this context, the ILe was no longer important for targeting. The authors suggested that in this context the linker probably behaved as a C-terminal type of VSD.

***The C-terminal vacuolar sorting determinants (ct-VSDs)***

Several C-terminal propeptides have been identified: in barley lectin (Dombrowski et al., 1993), tobacco chitinase A (Neuhaus et al., 1991) and glucanase (Sticher et al., 1992), and in phaseolin (Frigerio et al., 1998). Deletion of these propeptides from the precursor proteins resulted in a secreted form of the protein. In the case of tobacco chitinase A, it was shown that the C-terminal propeptide (GLLVDTM) was both necessary and sufficient for vacuolar targeting (Neuhaus et al., 1991; Di Sansebastiano et al., 1998). Deletion and mutation analysis revealed

little sequence requirements for the VSD of chitinase A (Neuhaus et al., 1994). In contrast to ss-VSD, no essential motif was found. Apart from the terminal Met, which was dispensable, all partial deletions strongly reduced the percentage of intracellular chitinase. Single and multiple replacements affected sorting to varying degrees, but no general rule could be deduced. The single most effective replacement was unexpected: while deletion of the terminal Met or its replacement by Phe or Lys had little effect, its replacement by Gly reduced the sorting efficiency by more than 50%.

In barley lectin the propeptide VFAEAIAANSTLVAE was found to be necessary and sufficient for vacuolar targeting (Dombrowski et al., 1993). Either the first four or the last four residues were sufficient for targeting. Even a minimal length of three amino acids beyond the cleavage site was sufficient for a significant vacuolar targeting. While mutation of an internal *N*-glycosylation site had no effect on vacuolar targeting, its displacement to the very C-terminus resulted in secretion of the protein (Wilkins et al., 1990). Addition of two glycines to the end of the C-terminal propeptide also caused secretion, even more efficiently than complete deletion of the propeptide. These results indicated that the accessibility of the C-terminus is crucial for the function of this type of VSD and that the terminal amino acid cannot be a glycine, reminiscent of the ER retention signals (-K/HDEL), which can also be inactivated by addition of amino acids. A functional ct-VSD thus tolerates tremendous sequence changes.

Phaseolin, the 7S storage protein of common bean is a third example of ct-VSD (Frigerio et al., 1998). Deletion of the last four amino acids AFVY of the propeptide caused the complete secretion of the protein in transgenic tobacco cells. This vacuolar sorting was found to be saturable, resulting in Golgi-mediated secretion of the protein.

It was suggested that sequence comparison of natural sequences (e.g. in cereal lectins and chitinases proteins) may be more informative than analysis of mutants (Matsuoka and Neuhaus, 1999) in systems without a conserved motif. This comparison for ct-VSD indicated a preference for Met at the last position, along with Leu, Ile and Val. Amino acids with a long aliphatic side-chain and a terminal charge, as Glu or Lys, are also often found at the last position. The next two amino acids are generally hydrophilic or negatively charged. Finally, there was a slight preference for hydrophobic side-chains at the more distal positions. From these comparisons, the putative receptor was suggested to bind the propeptide at its terminal carboxyl group by interactions involving the backbone of the peptide rather than the side chains. At the C-terminus there must be a hydrophobic patch requiring at least one methyl group but preferably one or several methylene groups, while the end group of the side chain would not interact with the putative receptor (Neuhaus and Rogers, 1998; Matsuoka and Neuhaus, 1999).

The low sequence specificity of the ct-VSD could be one reason why several attempts to identify a receptor for the ct-VSD have failed, although the possibility that this sorting could also be mediated by aggregation (as the ps-VSD, see below) without requiring a receptor has so far not been ruled out.

Neuhaus and Rogers (1998) also discussed another mechanism by which a ct-VSD might function. The final step in protein folding may involve binding of the C-terminus to the surface of the folded core (Fedorov and Baldwin, 1997). If the three-dimensional structure of proteins is the determinant for sorting them into vesicles in the pathway leading the PSV, removal of C-terminal propeptide could then indirectly affect sorting. However, this kind of mechanism is difficult to reconcile with very short ct-VSD such as those of chitinase A and with the successful fusion of the VSD to reporter proteins.

***The physical structure vacuolar sorting determinant (ps-VSD)***

This third group of VSD involves heterogeneous proteins which do not have propeptides such as those described for ss-VSD and ct-VSD. If propeptides are present, they have been shown not to be required for vacuolar sorting. The sorting determinant must somehow be carried within the mature polypeptide.

This type of VSD has been described for the phytohemagglutinin of common bean and for legumin-like proteins. A targeting study with legumin indicated the spread of sorting information over several sequence elements, suggesting an important role for higher structures (Saalbach et al., 1991). Vitale and Chrispeels (1992) proposed aggregation as a possible mechanism of sorting (Vitale and Chrispeels, 1992). Sorting by aggregation is known in animal cells, where it may be induced by lowered a pH (Castle et al., 1997). A form of aggregation is also presented by cereal prolamins which aggregate within the ER to form protein bodies (Okita and Rogers, 1996). Deposition of other storage proteins into protein bodies may occur in PSV after transit through the Golgi. Determinants for aggregation are often associated with hydrophobic regions on the surface of the molecule formed by folding of the three-dimensional structure. It is interesting that when pea prolegumin is isolated from the ER and Golgi vesicles, it is more hydrophobic and binds more tightly to membranes than the mature protein (Hinz et al., 1997). The sorting of phaseolin may be similarly linked to a transient membrane association mediated by its hydrophobic C-terminal propeptide (AFVY, Castelli and Vitale, 2005).

## Plant Vacuolar Sorting Receptors (VSRs)

### $VSR_{PS-1}$ (from pea (*Pisum sativum*), formarly called, BP-80), the first potential vacuolar receptor for ss-VSD

Kirsch et al. (1994) using an affinity column approach identified a single 80 kDa protein in membranes of pea CCV, by its ability to specifically bind to the N-terminal propeptide of barley aleurain in a pH-dependent manner. This protein was named BP-80, but later renamed as $VSR_{PS-1}$ (VSR for vacuolar sorting receptor, PS for *Pisum sativum*).

Subsequent competition assays on affinity columns established the ability of other VSD-containing propeptides to bind $VSR_{PS1}$. The prosporamin VSD (SRFNPIRLPT) competed weakly with the proaleurain peptide, while a peptide with the essential Ile mutated to Gly (SRFNPGRLPT) did not compete. A peptide representing the C-terminal propeptide of barley lectin did not compete either (Kirsch et al., 1994). Kirsch et al. (1996) also observed that $VSR_{PS-1}$ binds the C-terminus of *B. excelsis* 2S albumin. Efficient binding to $VSR_{PS-1}$ required the C-terminal propeptide (IAGF) and the adjacent five amino acids of the mature protein, PMGGS.

In 1997, an Arabidopsis EST (Z38123), homologous to $VSR_{PS-1}$ was identified by homology to peptide sequences from the N-terminus and from internal peptides from $VSR_{PS-1}$ (Paris et al., 1997). This clone was used as a probe to isolate the cDNA from *Arabidopsis* and the cDNA for $VSR_{PS-1,}$ and three homologues from developing pea. Shimada and coworkers (1997) identified two pumpkin homologues (PV72 and PV82) in preparations of ER-derived PACs. Homologues from rice and maize were also found in EST databases (Paris et al., 1996; Paris et al., 1997). When the *Arabidopsis* genome became available in 2000, the entire VSR family in *Arabidopsis* could be identified.

$VSR_{PS-1}$, the prototype of the VSR proteins, is a type I membrane protein with a large luminal domain, two hydrophobic regions, the first corresponding to a signal peptide of 22 amino acids and the second to a TMD of 23 amino acids, and finishing with a cytosolic tail domain (CT) of 37 amino acids. The luminal domain contains a PA domain, followed by three Cys-rich EGF (epidermal growth factor) repeats, one of which is predicted to coordinate calcium ions. Finally, a short Ser- and Thr-rich sequence precedes the TMD. The CT contains a putative tyrosine sorting motif (YMPL), which had been demonstrated to mediate incorporation into CCV in mammalian and yeast systems (see above).

Using antibodies raised against a synthetic peptide representing the N-terminal 20 amino-acids of $VSR_{PS-1}$ and a monoclonal antibody raised against the purified VSR, its localization was analysed by confocal microscopy (Paris et al., 1997). $VSR_{PS-1}$ was found in small punctate structures in pea root tip cells. By comparison with labeling patterns obtained with anti α-TIP or

anti γ-TIP antibodies it was demonstrated that neither type of tonoplast was labeled with the anti-$VSR_{PS-1}$ antibodies, but a network of small punctate organelles positive for $VSR_{PS-1}$ appeared to surround LVs labeled with anti γ-TIP antibodies, which could specifically contain aleurain (Paris et al., 1996). Immuno-electron microscopy revealed $VSR_{PS-1}$ at the *trans*-face of the Golgi and in prevacuoles of root tip and cotyledon cells of pea (Paris et al., 1997). In subcellular fractionation, $VSR_{PS-1}$ co-fractionated partially with Golgi membranes from pea cells (Hinz et al., 1999). A further analysis of the distribution of $VSR_{PS-1}$ within the Golgi stacks revealed that it is concentrated in the TGN with very low labeling in the cis-cisternae (Hillmer et al., 2001). In addition, $VSR_{PS-1}$ was highly enriched in pea CCV, while pea DV are enriched in storage proteins but have no detectable $VSR_{PS-1}$ (Robinson et al., 1998a; Hinz et al., 1999).

Cao and coworkers (2000) carried out a detailed study to elucidate the binding of $VSR_{PS-1}$ using a combination of protease digestion and binding assays to synthetic peptides. The luminal domain of $VSR_{PS-1}$ could be divided in three regions (1) an N-terminal part containing a PA domain (also found in the RMR-proteins, see below) (2) a central domain and (3) the EGF repeats. *In vitro* binding assays demonstrated that the EGF repeats did not bind to the aleurain ss-VSD but could ensure an optimal conformation for binding since their deletion decreased markedly the affinity of $VSR_{PS-1}$ for the aleurain and also for the sporamin VSDs. The aleurain ss-VSD was also divided into the NPIR motif and the flanking motifs, i.e., SSSFADS and VTDRAAST (Holwerda et al., 1992). NPIR binding was proposed to require both the N-terminal (1) and the central (2) domains of $VSR_{PS-1}$, while the luminal domain of $VSR_{PS-1}$ alone recognizes the non-NPIR motifs. In the case of the aleurain ss-VSD, the binding is strong since two vacuolar sorting motifs could be bound simultaneously. The sporamin propeptide was found to be a poor competitor, and the authors proposed that it competes only for one of the two binding sites.

The CT of the AtVSRs (34-40 amino acids) contain a highly conserved sequence of 27 amino acids (also conserved in $VSR_{PS-1}$), but then sequences diverge. They all contain the tyrosine motif (YMPL). This motif is predicted to have the same function as in mammalian and yeast systems, where it mediates the incorporation into CCVs. Consistently, Happel et al. (2004) demonstrated that the *Arabidodpsis* μA-adaptin (a subunit of the AP complex necessary for the assembly of the clathrin coat) binds the tyrosine motif of $VSR_{PS-1}$. Furthermore, the tyrosine residue was confirmed to be crucial in this binding.

These membrane-bound receptors (membrane proteins) were proposed to mediate the vacuolar sorting of soluble proteins to the plant LV as do the M6PRs in mammals and Vps10p in yeast. This sorting involves four steps: (1) recognition of the vacuolar sorting signal by the receptor at the TGN, diverting the soluble protein from secretion, (2) incorporation of the ligand-receptor complex in a CCV, (3) fusion of the vesicle to a PVC where the more acidic pH

causes release of the ligand and (4) recycling of the free receptor to the GA (Paris and Neuhaus, 2002).

### The *A. thaliana* Vacuolar Sorting Receptor family

The *A. thaliana* VSR family comprises 7 very similar members (Laval et al., 1999; reviewed in Hadlington and Denecke, 2000). They are referred as AtVSR1, 2, 2',3, 4, 5 and 6 (Paris and Neuhaus, 2002). AtVSR2 and 2' are a particular pair of variant that derived from a recent local duplication. The first two were identified along with the pea receptor $VSR_{PS1}$ as EST clones, Z38123 (AtVSR1) and MJ447 (AtVSR2) (Paris et al., 1997). The group of Natasha Raikhel also identified AtVSR1 (AtELP), based on the presence of EGF repeats (Ahmed et al., 1997). The same protein was also identified by the group of Rafael Pont-Lezica who looked for β-integrin-like proteins in plants (AtELP1, Laval et al., 1999). Using *N-terminal* sequencing and mass spectrometric (MS) analysis in a proteomics program, the group of Paul Dupree identified SPOT3 from *Arabidopsis* callus, which also matched AtVSR1 (Prime et al., 2000).

The *A.thaliana* isoforms have the same structure as $VSR_{PS-1}$. Sequence homology is very high. For instance, AtVSR1, 2 and 4 share about 80% homology, whereas AtVSR3 and AtVSR5 showed about 60% of homology with AtVSR1 (Laval et al., 1999). The amino acid identity between $VSR_{PS-1}$ and AtVSR2 is 86%, whereas the similarity between $VSR_{PS-1}$ and AtVSR1 is 70% (Hadlington and Denecke, 2000).

Because of the sequence conservation, it has been proposed that they share the same function (Cao et al., 2000; Hadlington and Denecke, 2000). On the other hand, the existence of several homologues in *Arabidopsis* also opens the possibility that they have different ligand specificities, and therefore that they are involved either in pathways to the different plant vacuoles or function at different stages of the plant development. Indeed, analysis of the AtVSRs expression during plant development indicated that they are differentially expressed. Therefore, it has been proposed that some AtVSRs may have a role in trafficking between the ER and the PM, in early germination, in mobilization of storage proteins and in the deposition of cell wall material (Laval et al., 2003). Several teams are now investigating the function of these individual *Arabidopsis* isoforms in terms of ligand binding affinity, subcellular localization and tissue-specific expression.

Ahmed et al. (1997) reported that AtVSR1 binds *in vitro* to the propeptides of *Arabidopsis* and barley aleurains as well as to the propeptide of sweet potato sporamin, but not to the propeptide from barley lectin. These interactions were dependent on the NPIR motifs present in the peptides.

At the subcellular level, AtVSR were detected in a membrane fraction enriched in CCVs (Ahmed et al., 1997). Immunogold EM also localized AtVSRs in the TGN as well as in the PVC

(Sanderfoot et al., 1998). Furthermore, in both subcellular fractionation and double-labelling immunogold EM experiments, AtVSRs partially colocalized with AtSYP21 (AtPEP12p), an *Arabidopsis* homologue of a yeast t-SNARE that resides on a PVC in Arabidopsis roots (Bassham et al., 1995; da Silva Conceicao et al., 1997; Sanderfoot et al., 1998). The trafficking of AtVSRs also appears to be mediated by vesicles since the cytosolic domain of AtVSR1 can interact *in vitro* with the mammalian TGN-specific AP-1 clathrin adaptor complex (Sanderfoot et al., 1998). AtVSRs also colocalized with sporamin at the Golgi complex (Ahmed et al., 2000). Later, Li et al. (2002) showed that 80% of the structures labeled with anti AtVSR antibodies were also labeled by anti-AtSYP21 antibodies, suggesting that the AtVSRs are preferentially concentrated in the PVC.

Protein structure, binding properties and subcellular colocalization indicated that AtVSRs could function as a sorting receptor for NPIR-containing proteins to the LV. Further experiments suggested that AtVSR1 may specifically function as a vacuolar sorting receptor. Using a reverse-genetic approach, Shimada et al. (2003) found that *atvsr1* mutant plants missorted a large amount of seed storage proteins, which were secreted out of the cells, resulting in an enlarged and electron-dense extracellular space in seeds. Seeds from this mutant also showed distorted cells and smaller protein storage vacuoles than the Wt cells. The authors also found that AtVSR1 binds the C-terminal peptide of the 12S globulin storage protein, in a $Ca^{2+}$ dependent manner.

It is remarkable that putative VSRs for lytic and storage vacuoles apparently share similar recognition mechanisms specificities. Indeed, the pumpkin seed-specific receptor, PV72 was proposed to sort seed storage proteins to the PSV. PV72 bound *in vitro* the internal and C-terminal sequences from pumpkin 2S albumin. It is expressed at the seed maturation stage in association with the synthesis of storage proteins, but not expressed in vegetative tissues (Shimada et al., 1997; Shimada et al., 2002). Watanabe and coworkers (2004) found that PV72 bound to an NPIR-containing peptide (a typical vacuolar signal for LV) of the precursor of a cysteine proteinase (Watanabe et al., 2004). The authors hypothesized that an Arabidopsis homologue of PV72 functions as a sorting receptor for the NPIR-containing proteinase.

**Gene expression**

Expression analysis in various organs during plant development has been carried out using northern blot and RT-PCR approaches (Laval et al., 1999; Laval et al., 2003). AtVSR2, AtVSR2' and AtVSR6 were detected on roots, young and old leaves, floral stalk, flowers, immature or pre-mature siliques. AtVSR1 was transcribed in all the above tissues except in premature siliques. AtVSR3 was transcribed in roots, floral stalk, and young organs (leaves

and siliques), but not in old leaves, in old siliques or in flowers (Laval et al., 2003). AtVSR4 was only detected in flowers. AtVSR5 was expressed in every tissue except in pre-mature siliques. AtVSR6 was expressed in every tissue except dry seeds.

The expression of AtVSR1, AtVSR2 and AtVSR3 is well controlled during seed development since none of them is expressed continuously from embryogenesis to germination. For example, AtVSR2 and ATVSR3 are expressed during the two first days of embryogenesis while AtVSR1 is not (Laval et al., 1999).

Even if the *Arabidopsis* VSR proteins are well conserved at the amino acid level, the expression data so far obtained suggest that they may participate in specific development steps, and therefore, they could have different functions. For example, it has been proposed that the variant AtVSR1 could mediates the degradation of the seed storage content during germination by transporting lytic enzymes into the storage vacuole, while AtVSR2 and AtVSR3 could be important to prepare the vacuolar system for storage in the early stages of seed development (Paris and Neuhaus, 2002).

**Phylogenetic analysis**

A phylogenic study of the $VSR_{PS-1}$ homologues from pea, *Arabidopsis* and pumpkin revealed three main subfamilies (Paris and Neuhaus, 2002). Subfamily I contains AtVSR1 (AtELP), PV72 and AtVSR4. The second subfamily includes the original pea VSR protein ($VSR_{PS-1}$), and the two very close *Arabidopsis* homologues AtVSR2 and AtVSR2'. The third, more distinct subfamily is represented by the three remaining and less studied *Arabidopsis* homologues. It has been proposed that each of these subfamilies may be associated to a different vacuolar route. It is likely that the receptors have diverged in functionally distinct subfamilies, one involved in the well described lytic pathway, where aleurain is the model ligand, while another would be used for a less well characterized storage pathway.

Several features could be conserved between these sorting systems, such as the exit of the receptors from the TGN, the use of vesicles and the implication of inositol phospholipids. However, they could differ, e.g. in the use of different adaptins, such as AP-1, AP-2 or AP-3. As we described above, the direct transport of ALP from the Golgi to the yeast vacuole is dependent on the AP3 adaptin and apparently CCV-independent, while the CPY pathway uses AP1, AP2 and CCV to transport vacuolar proteases from Golgi to the PVC. The plant sorting systems could also use different isoforms of PI-3-kinases, differing in their sensibility to wortmannin (an inhibitor of phosphatidylinositol 3-and 4-kinase activities). Transport of proteins with C-terminal VSDs appeared to be more sensitive to this drug than the transport of proteins with ss-VSDs (Matsuoka et al., 1995b). However, further studies revealed that the sequence-specific vacuolar sorting pathway is rather stronger inhibited by wortmanin (Kim et al., 2001;

Pimpl et al., 2003). Very recently, da Silva and coworkers (2005) found that this drug inhibits the recycling of $VSR_{PS-1}$ from the PVC.

### The RMR family

A new family of putative receptors was identified by its homology to the first domain of the VSR proteins. These proteins are composed of an N-terminal domain related to VSRs, but lacking the EGF-repeats, a TMD, and a CT with a RING-H2 domain and a serine-rich region (Jiang et al., 2000). They were named ReMembR-H2 (Receptor Membrane Ring-H2) or RMR family. Antibodies to one RMR detected the same organelles as antibodies against the TIP isoform DIP, i.e. the crystalloid precursor compartment, in *Arabidopsis* and tomato (Jiang et al., 2000).

The luminal domain of the RMR proteins is homologous to a portion of the VSR proteins that is known to participate in ligand binding, the PA domain (Cao et al., 2000). This raises the possibility that RMR proteins could be vacuolar sorting receptors. However, whether these proteins could have a role in sorting soluble proteins carrying ct-VSD remains to be elucidated. The genome of Arabidopsis harbors 6 homologues (AtRMR1-6).

## Functionally distinct sorting pathways in plant cells

Related working models of the routes of soluble proteins to the different vacuoles have been proposed, based on the above discussed results (Neuhaus and Rogers, 1998; Vitale and Raikhel, 1999; Bassham and Raikhel, 2000a) (Figure 1.4). Each route is believed to end up in a different vacuolar compartment with either lytic or storage character. The route for the LV is associated with soluble cargo proteins carrying a ss-VSDs, which are sorted into CCVs by $VSR_s$ at the TGN and then released in a lytic PVC, which would be the last station before the LV. The route to the PSV apparently involves different systems depending on the cell type. In cotyledons of legumes, the storage proteins bud from the TGN in DV. The sorting of these proteins and their concentration in lateral buds already begins by an unknown condensation mechanism in the *cis*-Golgi. In pumpkin and castor bean, storage proteins bud from the ER in PAC vesicles, though a VSR-type of receptor is also involved. Condensation seems also to be part of the sorting mechanism in this transport type.

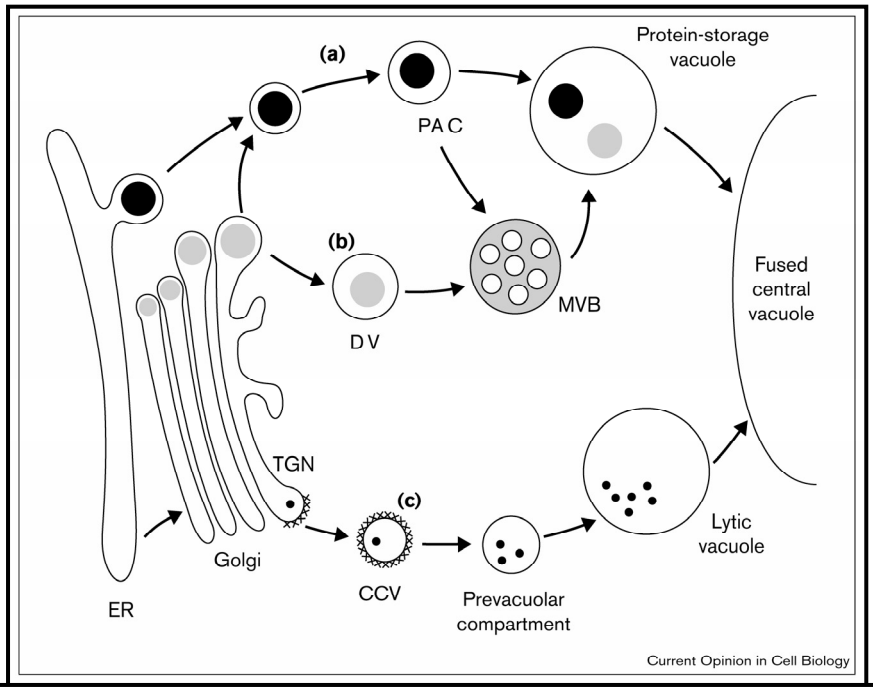



**Figure 1.4. A hypothetical model showing pathways of protein transport to the plant vacuole.** In some cell types, LVs and PSVs co-exist. After entry into the ER, proteins are transported to the vacuole by one of three major routes. **(a)** PACs and **(b)** DVs transport storage proteins to the storage vacuole, whereas CCV transport proteins to the LV. The relationship between the dense vesicle and precursor-accumulating vesicle pathways is unclear, as is the role of multivesicular bodies (MVB) (prevacuolar compartments) in these pathways. In mature cells, the lytic and the protein storage vacuoles fuse to produce a large central vacuole. This figure was taken from Bassham and Raikhel (2000a).

Some observations suggest that these major vacuolar sorting pathways are not as clearly separated as previously assumed. The first evidence for this comes from the frequent association of DV and CCV (Hohl et al., 1996; Robinson and Hinz, 1997; Robinson et al., 1998a). The second evidence comes from studies on the biogenesis of a complex storage vacuole in seed tissues of non-leguminous plants (Jiang et al., 2000; Jiang et al., 2001). This type of storage vacuole is surrounded by a membrane labeled with α-TIP (a marker for the storage vacuole) as in legume seeds (including pea) PSV but also δ-TIP. However, this vacuole contains three morphologically distinct regions: (1) a matrix that contains most of the soluble storage proteins (e.g. chitinase and ß-1,3 glucanase); (2) a crystalloid that contains integral membrane proteins and lipids (this compartment was also termed DIP organelle because it can be labeled by antibodies against this TIP isoform); (3) a globoid cavity that contains phytic acid, oxalate crystals, and is labeled by antibodies against vacuolar H+ -pyrophosphatase (V-PPase) and γ-TIP (Jiang et al., 2000; Jiang et al., 2001). The DIP

organelles are believed to be equivalent to the PAC vesicles. Like PACs, this complex prevacuolar organelle receives proteins both directly from the ER and via the Golgi complex (Jiang et al., 2000).

## Sorting of integral membrane proteins to the vacuoles

TIP and VSR proteins have been used as probes to understand integral membrane protein sorting in plant cells. TIP proteins are specifically localized to vacuolar membranes whereas VSR proteins could be implied in the different sorting pathways to the vacuoles. Several studies have used sequences from α-TIP, a marker for PSVs and the prototype VSR protein, $VSR_{PS-1}$.

### Transport of α-TIP to vacuoles

In 1992, the TMD and CT sequences of α-TIP were found to be sufficient to target a reporter protein to tonoplast in tobacco mesophyll cells (Höfte and Chrispeels, 1992). A truncated form of this chimeric protein that lacked the last 15 amino acids from the CT still trafficked to tonoplast, indicating that the α-TIP TMD and two amino acids of its CT were sufficient for vacuolar targeting. When the soluble vacuolar protein phytohemagglutinin was expressed in transgenic plants, it was prevented from reaching the vacuole by brefeldin A (BFA), while the transport of the intact α-TIP was not affected (Gomez and Chrispeels, 1993). This result indicated the presence of two different transport pathways for these two proteins. Because BFA blocks traffic to and through the Golgi, transport of α-TIP to tonoplasts in presence of BFA raised the possibility of a direct ER to vacuole pathway. It is not known, whether tonoplast proteins of the LV also follow this direct pathway. A modified N-glycosylated α-TIP was expressed in tobacco plants. It kept a high-mannose type of glycan. The cells were treated with BFA, the glycan was modified, showing that it was competent for modifications by Golgi enzymes. This result thus supports a Golgi-independent trafficking of α-TIP to its final destination (Park et al., 2004). On the other hand, α-TIP was also detected in the Golgi of legume cotyledons.

### Trafficking of $VSR_{PS-1}$

Trafficking of $VSR_{PS-1}$ has been studied in the plant secretory pathway. Paris et al. (1997) demonstrated that a truncated soluble form of $VSR_{PS-1}$ (lacking the TMD and the CT tail) was secreted by tobacco suspension cells. Jiang and Rogers (1998) reported that a chimeric protein containing a mutated form of the cysteine protease proaleurain that lacks its VSD, but is attached through its C-terminus to the $VSR_{PS-1}$ TMD and CT was able to reach the lytic PVC

where proaleurain was proteolytically processed into mature aleurain, indicating that the $VSR_{PS-1}$ TMD and CT were sufficient to direct the reporter to a proteolytic compartment. Additionally, the chimeric protein trafficked through the Golgi before reaching the prevacuole, it acquired complex modifications to Asn-linked glycans because BFA prevented its proteolytic processing. These results indicate that the $VSR_{PS-1}$ TMD and CT sequences could contain the necessary information for its proper trafficking within the secretory pathway.

**Roles of the $VSR_{PS-1}$ TMD and CT sequences**

To determine the role of the CT domain in prevacuolar targeting, a series of deletions were carried out in the same chimeric protein described above. Complete deletion of the CT still resulted in prevacuolar targeting and Golgi trafficking of the chimeric protein (Jiang and Rogers, 1998), indicating that the transmembrane domain of $VSR_{PS-1}$ is sufficient for targeting to the lytic PVC. To test whether the CT is important for the retrieval from the PVC to GA, the stability of the intact $VSR_{PS-1}$ and of the truncated $VSR_{PS-1}\Delta CT$ was compared (Jiang and Rogers, 1998). The half-life of the truncated protein was substantially reduced compared to that of the intact receptor. Both results are comparable to those obtained with the yeast Vps10p receptor, and indicated that the CT of $VSR_{PS-1}$ may be important for recycling the receptor back from the PVC to the TGN. In contrast, more recently, Brandizzi et al. (2002) showed that a chimeric protein made with the GFP fused to the TMD of $VSR_{PS-1}$ (19 amino acids) accumulated in Golgi-like structures rather than in the PVC. This indicated that the TMD $VSR_{PS-1}$ alone is not sufficient to accumulate GFP in the PVC. Additionally, by lengthening the chimera's TMD to 22 amino acids, the GFP accumulated in the PM and not in the PVC or in the tonoplast. Therefore, the authors concluded that the TGN and PVC informations do not reside within the TMD and suggested that the CT plays an important role in the localization of the protein.

The TMD appears to have some specificity in vacuolar targeting, since its replacing in a chimeric protein by the C-terminal TMD of α-TIP resulted in different targeting of the reporter: the protein was directed to the PSV, bypassing the Golgi complex, and therefore following the same route as the WT α-TIP (Jiang and Rogers, 1998). In contrast, the CT of γ-TIP did not prevent transport to the LV. Therefore, the CT of α-TIP seems to contain dominant information for sorting to the PSV that overrides the information contained in the TMD of $VSR_{PS-1}$ for sorting to the LV.

These results indicate the existence of three separate pathways to vacuoles for membrane proteins: a direct ER to PSV pathway, a pathway via the Golgi to the PSV and a pathway via the Golgi to the LV (Figure 1.5). Moreover, both TMDs and CTs could specify the destination of membrane proteins.

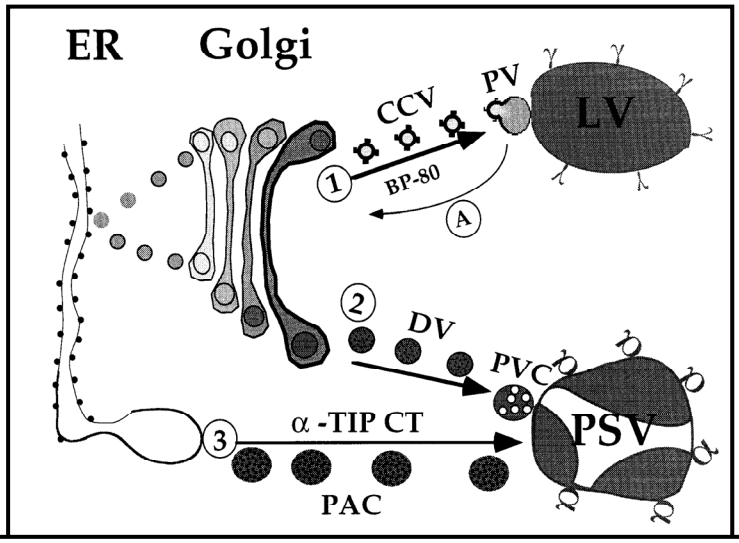



**Figure 1.5. Working model of membrane protein sorting to vacuoles in plant cells.** There are at least three distinct routes for membrane proteins to reach the vacuoles: the first (1) represents transport of BP-80 ($VSR_{PS-1}$) via Golgi-derived CCVs to the prevacuolar compartment (PV) of the LVs. The CT of BP-80 may be responsible for its recycling back from the PV to GA (A). The second route (2) is marked by Golgi-derived DVs that carry storage proteins such as provicilin and prolegumin to a prevacuolar compartment (PVC) before delivery to PSVs in pea cotyledons. The ER to PSV pathway (3) is responsible by the delivery of storage proteins including proglobulin and proalbumin via ER-derived PAC vesicles in pumpkin seeds; such an ER to PSV pathway is also marked by the α-TIP CT reporter protein that traffics directly from ER to a PSV-like compartment in tobacco cells. The tonoplasts of LV and PSV are marked by γ-TIP and α-TIP, respectively. This figure was taken from Jiang and Rogers (1998).

## Approaches for studying the ligand-receptor interactions

The identification of possible propeptides involved in vacuolar sorting and targeting is a key to study the vacuolar transport since these propeptides distinguish between secretion and the vacuolar transport.

A widely used approach is the transient expression in plant protoplasts of a reporter protein fused to the candidate peptide (Paris and Neuhaus, 2002). The vacuolar localization of the fusion protein is usually assessed by the intracellular presence of the reporter in a mature form. The big advantages of this method are that it is *in vivo*, sensitive, and allows measurements of the transport rate and the stability of the constructs by radioactive pulse-chase labeling. The main disadvantages are that it does not allow to study the receptor-ligand recognizition step alone and that it is not possible to find out which variant of VSR is involved.

In addition, protoplasts do not originate from seed tissue and therefore may lack some key factors in the transport machinery to separate the two vacuolar routes detected in seeds.

Affinity binding approaches are mainly used to studied the interaction between a vacuolar sorting peptide and a putative receptor (Paris and Neuhaus, 2002). In this case, the advantage is the possibility to test a single receptor molecule and a single signal and to address competition with other ligands. However, one disadvantage is that the two elements that are mixed together *in vitro* may never be in contact in a living cell. Also, the affinity column unnaturally exposes a given peptide that is no longer in its native context. Therefore, it is essential to control that the peptide is necessary and sufficient for vacuolar targeting in plants.

The second *in vitro* approach is classical binding assay, mixing a synthetic peptide with an extract containing the receptor which is then immunoprecipitated (Paris and Neuhaus, 2002). The complex is thus separated from the free peptide and quantified. The disadvantage of this procedure is that the availability of partially purified receptor could be a limiting factor; it is also hard to set up and requires radioactive labeling of the ligand.

The cross-linking approach also represents a tool to test ligand-receptor interactions, however it has a disadvantage. If cross-linking is needed to identify a complex, it is an indication that the interaction is weak and additional controls are required.

Other techniques such as the surface plasmon resonance and the yeast two-hydrid system could be also used to study ligand-receptor interactions (Paris and Neuhaus, 2002). The first is a very powerful and sensitive technique but requires expensive equipment. The idea is to bind one partner on a sensor surface, while the second is supplied in a flow-through buffer. Binding modifies the optical properties of the surface. Although this technique allows precise measurements, it is limited by the availability of highly purified partners and probably cannot address binding of the whole receptor. The second technique is used to identify binding partners of a chosen bait. It is necessary to remove the transmembrane domain of a receptor protein since the interaction must happen in the yeast nucleus. The disadvantage of this technique is that it gives little information in terms of affinity. In addition, posttranslational modifications of the secretory pathway will be lost. If disulfide bonds and/or glycans are needed for the function of one binding partner, the interaction will be lost.

## Comparison of the secretory pathway in yeast and plants

Plants and yeast are eukaryotes and therefore it can be expected that many biological processes are conserved or at least similar between them.

A comparison of the vacuolar sorting systems of yeast and plants could reveal many conserved or similar characteristics such as:

1) The existence of a similar sorting pathway for soluble vacuolar proteins to the LV (ss-VSD pathway, in plants; CPY pathway, in yeast), where many molecular components are common such as:

    - the requirement of peptidic informations for soluble proteins sorting to the vacuole (Horazdovsky et al., 1995; Bar-Peled et al., 1996; Neuhaus and Rogers, 1998).

    - the existence of sorting receptors (i.e. Vps10p, in yeast, VSRs, in plants) (Marcusson et al., 1994; Paris and Rogers, 1996; Paris et al., 1997).

    - the use of motifs contained in the CTs of the receptors for the assembly of the clathrin coat (Cereghino et al., 1995; Cooper and Stevens, 1996; Happel et al., 2004).

    - the existence of PVCs (Vida et al., 1993; Paris et al., 1997; Jiang and Rogers, 1998; Robinson et al., 1998b).

    - many homologous genes involved in vesicle trafficking have been found in both systems (Horazdovsky et al., 1995; Bar-Peled et al., 1996; Sanderfoot and Raikhel, 1999; Bassham and Raikhel, 2000a, b).

2) The existence of different pathways to target soluble and membrane proteins to the vacuole.

All these similarities could suggest that yeast and plants are very similar in the targeting and transport of proteins to the vacuole. However, some observations made over the past years have revealed features that seem to be unique to plants and differences between closely related proteins in yeast and plants (see below). Excellent reviews have already been published in this area (e.g. Bassham and Raikhel, 2000a, b). Plant cells contain distinct vacuolar types: LVs (equivalent to the yeast vacuole) and the PSVs. Therefore, plants must

generate biochemically and structurally different types of tonoplast membranes and luminal contents to maintain these organelles as separate entities. Additionally, plants must have distinct vesicles pathways leading to each vacuolar type. Finally, they must also have mechanisms that sort storage proteins to one pathway and hydrolytic enzymes to the other. The pathway to the storage vacuole appears to be unique to plants, and many components of its machinery are not yet known. The aggregation of storage proteins in early compartments of the secretory pathway are exclusive of this pathway, in particularly for the direct delivery from the ER to PSVs.

Whole genomic sequences revealed numerous plant proteins with similarity to proteins involved in vesicle trafficking in yeast. In some cases, they apparently have equivalent functions: e.g. the ER-to-Golgi trafficking proteins Sar1p and Sec12p (d´Enfert et al., 1992) and the prevacuolar t-SNARE AtSYP22 (Pep12p) (Bassham et al., 1995). However, several other examples indicate that the correlation between localization and /or function of the protein trafficking machineries of yeast and plant is not so clear.

One example is the *Arabidopsis* protein AtSYP22 (AtVAM3). The *AtSYP22* gene was identified as a cDNA that complements some phenotypes of a yeast *vam3* mutant (Sato et al., 1997). Vam3p is a t-SNARE on the yeast vacuole that functions in multiples transport pathways to this organelle (Wada et al., 1997). The AtSYP22 protein was originally reported to reside on the tonoplast in the shoot apical meristem. However, further examination of the localization of AtSYP22 demonstrated that in roots and leaves, AtSYP22 is found on the PVC, where it co-localizes with AtSYP21, and it is not detectable on the tonoplast (Sanderfoot et al., 1999). The tonoplast localization was then assumed to be restricted to specialized cell types in plants, and in most tissues AtSYP22 is probably prevacuolar. The authors suggested that the complementation of the yeast mutant reflected the mistargeting of AtSYP22 due to differences in the endomembrane system and in protein targeting between yeast and plants.

Two or more plant genes may appear to correspond to a single yeast vesicle transport gene (Sanderfoot and Raikhel, 1999). At least three genes in *Arabidopsis* are closely related to the yeast Pep12p gene (Becherer et al., 1996): AtSYP21 (Bassham et al., 1995), AtSYP22 (t-SNARE at the PVC, Sato et al., 1997), and AtSYP23 (AtPLP) (Zheng, 1999). AtSYP21 resides on the *Arabidopsis* PVC and forms a complex with characteristics of fusion complexes (Bassham and Raikhel, 1999). Despite the ability of AtSYP22 to functionally replace Vam3p in yeast, its sequence is more closely related to AtSYP21 and yeast Pep12p. Despite this sequence conservation, AtSYP22 is not able to complement a *pep12* mutant, indicating that AtSYP21 and AtSYP22 have distinct functions. Therefore, it is likely that the duplication in plants correspond with a functional specialization rather than being a simple redundancy.

*Arabidopsis* also has multiple homologues of the yeast v-SNARE VTI1. Yeast VTI1 is a multifunctional protein involved in vesicle fusion in several different transport pathways (Fischer von Mollard and Stevens, 1999). There are vti1 mutant alleles that are defective in only a subset of these pathways but are able to function normally in others. Two *Arabidopsis* isoforms related to VTI1 have been identified, AtVTI11 (AtVTI1a) and AtVTI12 (AtVTI1b). Interestingly, they function in distinct transport pathways when expressed in yeast mutants, as they complement the phenotypes of different mutant alleles. This suggests that each Arabidopsis protein may be specialized for a particular subset of functions of yeast Vti1p. This hypothesis remains to be tested in plants.

AtVPS45 also differs from its yeast homologue Vps45p (Bassham and Raikhel, 1998) which is required for transport to the vacuole via two distinct pathways: the CPY (carboxypeptidase Y) pathway (Cowles et al., 1994; Piper et al., 1994) and the Cvt (cytoplasm-to-vacuole transport) pathway (Abeliovich et al., 1999). Vps45p seems to be a multi-functional protein, interacting with the t-SNARE Pep12p at the PVC in the CPY pathway and with the t-SNARE Tlg2p at the late Golgi in the Cvt pathway. In contrast, AtVPS45 is localized exclusively to the TGN in Arabidopsis roots and interacts with two Tlg2p-like proteins: AtSYP41 (AtTLG2a) and AtSYP42 (AtTLG2b). AtVPS45 does not interact with the Arabidopsis Pep12p homologue AtSYP21, and AtVPS45 cannot be detected at the PVC (Bassham et al., 2000). The function of AtVPS45 thus appears to have diverged from that of its yeast equivalent.

The yeast TGN/endosomal t-SNARE Tlg2p also has multiples homologues in *Arabidopsis*. At least two of these, AtSYP41 and AtSYP42, are expressed. Although both proteins interact with AtVPS45, they are localized to distinct domains of the TGN, indicating that they may have different functions (Bassham et al., 2000).

Finally, the plant vacuolar receptor $VSR_{PS-1}$, is a member of the vacuolar sorting receptor family. Although some VSR homologues have a similar subcellular localization, they could have distinct roles in vacuolar targeting. VSR family members have no homologues in yeast. Despite the similar function of $VSR_{PS-1}$ and yeast Vps10p, they do not share sequence homology.

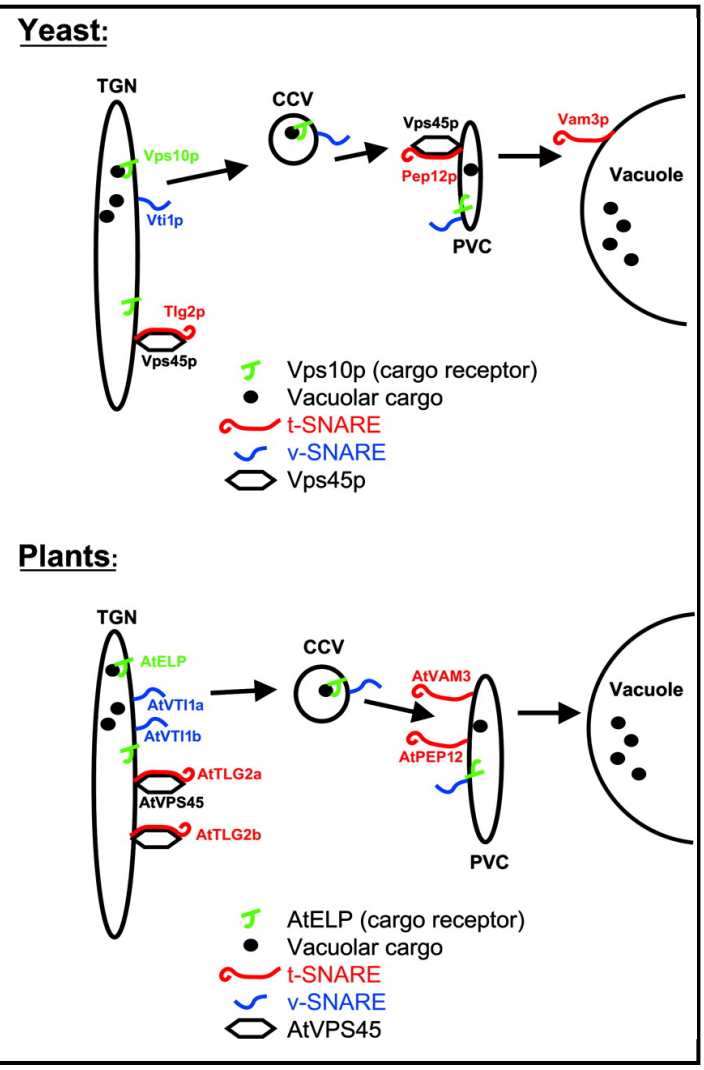
In summary, the plant secretory pathway resembles by many features the yeast secretory pathway. In particular, sorting of hydrolytic enzymes to the plant LV appears closely related to the CPY pathway of vacuolar sorting in yeast. However, the plant vacuolar system is more complex than the yeast system. In order to reproduce a vacuolar sorting mechanism for plant proteins in yeast, we must cautiously interpret the data since (a) the localization of a protein in yeast does not necessarily correspond to the localization of the same protein in its native plant (Figure 1.6), and (b) proteins can reside in different organelles in different cell types within the same species, due to differences in the endomembrane system (Sato et al., 1997; Sanderfoot

et al., 1999). Therefore, it is now clear that results obtained in yeast need to be confirmed in plants.

**Figure 1.6. Comparison of the localization of proteins thought to be involved in clathrin-coated vesicles-mediated transport to the vacuole in yeast and plants.** Two of the Arabidopsis proteins have different localizations compared with yeast. AtVAM3 is found on the PVC in Arabidopsis, whereas yeast Vam3p is found on the tonoplast. AtVPS45 is present only at the TGN, whereas yeast Vps45p is localized to both the TGN and the PVC. This figure was taken from Bassham and Raikhel (2000b).

**Nomenclature:**

| Previous name | New Name |
|---|---|
| AtPEP12: | AtSYP21 |
| AtVAM3: | AtSYP22 |
| AtTLG2a: | AtSYP41 |
| AtTLG2b: | AtSYP42 |
| AtVTI1a: | AtVTI11 |
| AtVTI1b: | AtVTI12 |




## Can yeast be used as mode for study plant vacuolar sorting and targeting?

Attracted by the practical advantages to work with yeast and the conservation of a part of the vacuolar sorting machinery between yeast and plants, several groups have used yeast to study plant proteins. Screening cDNA expression libraries for complementation of yeast mutant phenotypes is a valuable technique to identify plant gene products with a specific biochemical activity or involved in a particular pathway. In some cases, testing a plant gene with sequence similarity to a yeast gene by complementation of the corresponding yeast mutant has allowed to attribute a function to this gene product when functional analysis was extremely difficult in plants (Lee et al., 1993; Bassham et al., 1995).

Several studies showed that yeast can be used to identify ER-retention signals in plant secretory proteins and to elucidate the function of a receptor used for the ER-retention in plants. Kaletta et al. (1998) showed that the peptide HDEF at the C-terminus of the tomato RNAase LX is a new ER-retention signal. Lee and coworkers (1993) demonstrated that the Arabidopsis Erd2 receptor can complement the lethal phenotype of the *erd2* deletion mutant of *S. cerevisiae*, indicating that this protein may have a similar function in plants. The yeast Erd2 receptor has two main functions, to bind the ER-retained proteins and to retrieve these proteins back to the ER (Lee et al., 1993).

The fact that both yeast and plants use peptide sequences as targeting signals for vacuolar proteins initially led researchers to use yeast to identify VSDs within the plant proteins. Tague and Chrispeels (1987) showed that bean phytohemagglutinin (PHA) was targeted to the yeast vacuole. This targeting was mostly due the LQRD sequence located near the N-terminus of the protein (Tague et al., 1990). However, this sequence is not a vacuolar-targeting signal in plants (Chrispeels, 1991). Matsuoka and Nakamura (1992) showed that sporamin from sweet potato was targeted to the yeast vacuole, independently of the presence of its propeptide, which contains a sequence specific ss-VSD, suggesting that this plant VSD was not recognized by yeast. Gal and Raikhel (1994) showed that the ct-VSD from barley lectin was not recognized by yeast, because it was not able to target the secreted invertase to the vacuole. In contrast, the complete barley lectin (with or without the C-terminal propeptide) was able to target invertase to the vacuole. In all these cases, the localization in yeast was independent of the plant VSD but another cryptic signal was found. The C-terminal VSD from Brazil nut 2S albumin was also not recognized by yeast (Saalbach et al., 1996), while it was sufficient for efficient targeting of the yeast invertase to the vacuoles of transformed tobacco plants. The tobacco chitinase A was sent to the yeast vacuole independently of its VSD (Humair, 2000).

Yeast was also used to demonstrate the function of the plant vacuolar sorting receptor $VSR_{PS-1}$ (Humair et al., 2001). However, as shown in this thesis, this finding must be carefully reconsidered.

Despite the fact that many advances have been made using yeast, several other observations suggest that this simple eukaryotic organism should be only used as a starting point for studying plant processes. We now think that the targeting information for vacuolar localization and many components of the vacuolar sorting machinery are not conserved enough between these two systems to allow either the identification of plant VSDs or to demonstrate the function of VSR proteins.

## Some problems when expressing heterologous proteins in yeast

### Protein folding

In contrast to E.coli, where most foreign cytoplasmic proteins accumulate as insoluble aggregates, in yeast they can be correctly folded. However, normally secreted proteins encounter an abnormal environment in the cytoplasm and often become insoluble, though many are soluble and active (Romanos et al., 1992). Factors such as the lack of correct cofactors and/or chaperone functions could influence the folding of a heterologous protein. In the case of membrane proteins (MPs), overexpression often leads to saturation of the cell's protein folding capacity and to accumulation of inactive unfolded or misfolded protein in intracellular membranes (Abdulaev et al., 1997; Lenoir et al., 2002). These proteins can become substrates for proteolytic degradation ("Quality Control system").

### Post-translational processing

Another advantage of yeast over bacterial is that yeast can perform many of the post-translational modifications typically found in higher eukaryotes, such as processing of signal sequences, disulfide bridge formation, certain types of acylation and N-glycosylation of asparagine (Asn) and O-glycosylation at Serine or Threonine residues (Romanos et al., 1992). However, there are some differences between yeast and higher eukaryotes regarding these post-translational modifications. While mammalian and plant N-glycans can contain various monosaccharides such as: fucose, xylose, galactose and sialic acid, the N-glycans of yeast are only modified with additional Mannose (Man) residues. In plants, two types of N-glycans are known: high Man glycans (with 5 or more), and complex glycans that have fewer Man residues but additional residues such as xylose or fucose. The complex glycans can be small or large (carrying several fucoses). In yeast, two different types of N-glycans structures are found: (1) with a small number of Man residues and (2) with 50 to 200 Man residues (so called mannans).

Some foreign proteins secreted in *S. cerevisiae* appear to be hyperglycosylated. N-linked high-mannose glycans represent a significant problem in the production of foreign glycoproteins for the pharmaceutical industry. They are extremely antigenic in mammals. Additionally, these large glycans can interfere with folding or function of a foreign protein.

### Protein stability

The protein half-life is of major importance when expressing heretologous proteins in yeast. Proteins that fail to acquire the proper conformation can be substrates for the different degradation pathways in yeast. Many examples document the instability of heterologous

proteins in yeast. The major intracellular proteolytic pathways are: (1) the ER-associated hydrolytic degradation by proteasomes (ERAD) and, (2) the degradation in the vacuole via either the GA or the endocytic pathway. The degradation of the cystic fibrosis transmembrane conductance regulator (CFTR) protein was sensitive to the proteasome inhibitor lactacystin β-lactone, indicating degradation by the proteasome (Kiser et al., 2001). The instability of the human transferrin receptor (TfR) depends on vacuolar proteases, indicating that it is degraded in the vacuole (Prinz et al., 2003). Hong et al. (1996) also reported that hybrid proteins containing mutant forms of the $NH_2$-terminal domain of the λ repressor fused to the secreted form of invertase were found in the vacuole, where the repressor moiety was degraded by vacuolar proteases (Hong et al., 1996).

Ubiquitination often plays an important role in protein degradation. It has been shown that attachment of ubiquitin facilitates the binding of heterologous proteins to the proteasome (Ishida et al., 1993). It also serves as a signal for internalization of membrane proteins via the endocytic pathway for subsequent degradation in the vacuole (Hicke, 1997).

**Specific lipid requirement**

Specific lipids may also be necessary for correct folding and intracellular transport. Therefore, the phospholipid, glycolipid and sterol content of the yeast host cell could be important. For instance, a limiting factor in the expression of mammalian membrane proteins could be due the absent of cholesterol (and of plant-specific sterols in the case of plant proteins). The main yeast sterol, ergosterol, might replace plant or animals sterols, but often not resulting in fully functional heterologous proteins (Opekarová and Tanner, 2003).

**Toxicity**

A high level of a foreign gene places a metabolic burden on the host cell, reducing its growth rate and the efficiency of gene expression (Romanos et al., 1992). Expression of some genes causes a more acute effect, either through a severe defect in metabolism or by direct toxicity. This can result in the inadvertent selection of variants expressing lower levels of the heterologous or of a mutated form protein, particularly when constitutive expression systems are used. Even with regulated promoters, gradual accumulation of a toxic protein through leaky expression may have the same effect.

Toxicity is common with membrane proteins or complex secreted proteins: when they accumulate as malfolded aggregates they block the secretory pathway (Beilharz et al., 1998). The over-expression of transcriptional *trans*-activators may also result in toxicity, through their

ability to sequester transcriptional factors. Toxicity can be an intractable problem and it may then be more efficient to use a transient expression system.

## The use of reporter proteins

Studies of the tissue localization of gene expression as well as the intracellular protein localization *in vivo* are essential to understand the function of a protein in the organism. The first reports of localization of gene expression used immunocytochemistry with fluorescently labelled antibodies. Later, scientists used the fluorescence *in situ* hybridization (FISH) to determine precisely in which cells genes are transcribed. More recently, reporter gene systems have been well developed to detect *in vivo* and *in situ* the presence and the activity of a gene product. Reporter proteins are normally fused to the protein of interest and their expression is placed under control of its promoter.

Two types of reporter proteins are possible: endogenous and exogenous proteins. Endogenous reporters are for example the invertase encoded by the SUC2 gene in yeast and the β-galactosidase encoded by the *lacZ* gene, in E.coli. The invertase is normally secreted, but fusion with an intracellular protein can retarget it elsewhere within the cell. In the yeast secretory pathway, CPY is another good endogenous reporter protein since its localization is easily determined from its maturation state (Stevens et al., 1982).

Among exogenous reporters the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is a particularly popular example (Chalfie et al., 1994). This protein is a simple universal reporter in living tissues as it requires only blue light or UV light for its green fluorescence, without any exogenous substrate (Chalfie et al., 1994; Chalfie, 1995; Heim and Tsien, 1996). Indeed, the polypeptide itself is fluorescent, and thus all problems related to substrate penetration (as for luciferase) or reaction product diffusion (as for ß-glucoronidase) are avoided. Sufficient production, proper post-translational folding, oxidative formation of the fluorophore and correct targeting within the cell are important steps for the successful use of GFP as a compartment marker. Fusion proteins can easily be constructed without disturbing the native function and compartmentation (Grebenok et al., 1997). GFP tolerates both N-and C- terminal extensions (Di Sansebastiano et al., 1998, 2001).

Several modifications have been performed on GFP in order to increase its thermostability (Siemering et al., 1996), or to alter its fluorescent properties (emission shifted to other colors, enhancemented brightness) (Heim and Tsien, 1996). Mutants with shifted absorption and emission peaks allow the multiple labeling necessary for protein colocalization experiments (Rizzuto et al., 1996). GFP can be used to study the plant and the yeast secretory pathways (Kunze et al., 1999; Kiser et al., 2001; Li et al., 2002; Saint-Jore et al., 2002). The GFP is one of the most used reporters.

## Aim of the thesis

The yeast *Saccharomyces cerevisiae* has been extensively used to study intracellular transport of heterologous proteins. Previously, we demonstrated the *in vivo* function of the plant vacuolar sorting receptor $VSR_{PS-1}$ (from pea, *Pisum sativum*) in yeast (Humair et al., 2001). In a yeast strain disrupted for the Vps10p receptor (*Δvps10*), $VSR_{PS-1}$ redirected the reporter GFP to the vacuole but only when the petunia aleurain VSD was fused to GFP (aleu-GFP). A family of genes with high homology to $VSR_{PS-1}$ has been identified in *Arabidopsis thaliana* (AtVSR1, 2, 2', 3, 4, 5 and 6). The existence of several homologues in one plant species suggests that these VSRs could have different ligand specificities, and might therefore be involved in different vacuolar sorting pathways and/or function at different stages of plant development (Paris and Neuhaus, 2002).

The main objective of this thesis was to investigate the functional roles and specificities of the putative AtVSRs by the same yeast complementation assay mentioned above. For specificity tests, we fused several additional plant VSDs to GFP (VSD-GFP fusion proteins). We also cloned most AtVSRs in yeast expression vectors under the control of the GAL inducible promoter. To corroborate the fluorescence microscopy observation, we quantified by fluorometry the amount of VSD-GFP that was intracellularly retained. Unfortunately, we were unable to assess the capacity of the AtVSRs to interact with their putative ligands. More surprisingly, we were also unable to quantitatively demonstrate the function of $VSR_{PS-1}$ as a sorting receptor in yeast as previously quantitatively described. This discrepancy prompted us to re-examine the expression and localization of $VSR_{PS-1}$ in yeast. The main objective of this thesis thus became the analysis of the fate of a plant VSR in the yeast secretory pathway.

## Outline of the thesis

This thesis is organized in three main chapters. After this introductory chapter, chapter 2 describes the material and methods used for the experiments. Chapter 3 evaluates the merits of a yeast approach to study the function of the AtVSR family. This includes the demonstration that two plant additional ss-VSDs (the *Nicotiana alata* proteinase inhibitor (NAPI) and the *Bertholletia excelsis* 2S albumin (2Sa)) are functional *in planta*, a statistical analysis of the $VSR_{PS-1}$ effect on retention, the numerous controls done to rule out problems with either the protocol of induction or the yeast strain, the interaction of $VSR_{PS-1}$ and aleu-GFP. Moreover, we report the stability and localization of $VSR_{PS-1}$ and a comparison between the cytosolic tail domains of $VSR_{PS-1}$ and Vps10p. This chapter also compiles the stability and localization of the hybrid protein $VSR_{PS-1}$-Vps10p and its interaction with aleu-GFP. Chapter 4 presents a general discussion of the results of this thesis.

Finally, three appendixes are included, 1) The publication: “The Yeast *Saccharomyces cerevisiae* is Not an Efficient Tool for *in Vivo* Studies of Plant Vacuolar Sorting Receptors” (The Plant Cell. Vol. 7, 1-4, May 2005), 2) the cDNA sequences of the VSRs and 3) the sequence of the hybrid protein $VSR_{PS-1}$-Vps10p-HA.

# Chapter 2. Materials and Methods

Most of the molecular biology methods used in this work are described in Sambrook's laboratory manual (1989). Protocols for basic manipulations of yeast can be found in the books of Ausubel and coworkers (1987-1995) and Glover and Hawes (1995) and the web site of the Faculty of Medicine, University of Manitoba (see Gietz and Wood's protocols). Many other good protocols were given by the yeast specialist, Dr. O. Deloche at the Department of Microbiology and Molecular Medicine, Universitary Medical Center, University of Geneva.

## Water and sterilization

We used distilled de-ionized water (dd $H_2O$) in all recipes. dd $H_2O$, media and solutions were sterilized by autoclave at 15 psi (1.05 kg/cm$^2$) for 15 - 20 minutes (min) or filtration.

## Bacterial strains

*Escherichia coli* XL-1 Blue: *rec*A1, *end*A1, *gyr*A96, *thi, hsd*R17 ($r_k$-,$m_k$+), *sup*E44, *rel*A1, *lac*, [F', *proAB*+, *lacI*$^q$ZΔM15::Tn10(Tet$^r$)] (Bullock et al., 1987) was used to replicate the different recombinant plasmids. Wild type XL-1 Blue grows on LB medium containing 12.5 µg/ml tetracycline (Tet). When XL-1 Blue strain is transformed with a plasmid (Ampicillin resistance, Amp$^r$), it can grow on LB medium containing 100 µg/ml Amp. When pBluescript II SK(+/-) (Stratagene) and PGEM®-T Easy vectors (Promega) were used, the F′ episome, in presence of 40 µg/ml IPTG and 40 µg/ml X-Gal, allows the selection of recombinant colonies by a change of color (blue - white).

## Bacterial growth and maintenance

All bacterial cultures were grown in LB (Luria-Bertani) medium (per liter: 10 g Bacto tryptone, 5 g Bacto yeast extract, 10 g NaCl). For LB plates, the medium was supplemented with 15 g/L Bacto-agar. After autoclaving, it was cooled down to 60°C and antibiotics were added. The plates were then poured under sterile conditions and a flame was used to remove bubbles.

### Bacterial stocks maintenance

Bacterial strains, either wild type or transformed with a plasmid, were stored in 15% of glycerol at -70°C. To recover a strain, a small amount of cells was scraped from the frozen stock with a platine loop and streaked onto LB plates containing the appropriate antibiotics.

## Preparation of competent bacterial cells

Competent XL-1 Blue cells were prepared using either $CaCl_2$ or RbCl. In both cases, LB was inoculated with a single colony from a freshly grown LB plate containing 12.5 µg/ml Tet. $CaCl_2$ method: 150 ml of LB were inoculated with 1.5 ml of an O.N. culture. Cells were grown with agitation to 0.4 - 0.6 $OD_{600}$, harvested at 4500 x g for 10 min in sterile Sorval bottles (4°C) and resuspended slowly in 5 ml of RF-1 buffer (100 mM KCL, 50 mM $MnCl_2.4H_2O$, 30 mM KOAc pH 7.5, 10 mM $CaCl_2.6H_2O$, 15% glycerol, pH 5.8, filter sterilized). Cells were then incubated on ice for 15 min and pelleted at 4500 x g (4°C) for 10 min. The pellet was resuspended in 1.2 ml of RF-2 buffer (10 mM MOPS pH 6.8, 10 mM KCl, 50 mM $CaCl_2.6H_2O$, 15 % glycerol, pH 6.8, filter sterilized) and kept on ice for 15 min. Cells were aliquoted, 100 µl per Eppendorf tube and frozen in liquid nitrogen, and stored at -70°C.
RbCl method: 250 ml of LB were inoculated with 2.5 ml of an O.N. culture. Cells were grown with agitation to 0.4 - 0.6 $OD_{600}$ and harvested at 4500 x g for 10 min in sterile Sorval bottles (4°C). The pellet was slowly resuspended in 100 ml of TFB1 buffer (30 mM KOAc, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 100 mM RbCl, 15% glycerol, pH 5.8, filter sterilized) and incubated on ice for 5 min. Cells were then pelleted at 4500 x g (4°C) for 10 min, resuspended in 10 ml of TFB2 buffer (10 mM MOPS pH 6.5, 75 mM $CaCl_2$, 10 mM RbCl, 15% glycerol, pH 6.5, filter sterilized) and kept on ice for 15 - 60 min. Cells were aliquoted, 100 µl per Eppendorf tube, and frozen in liquid nitrogen, and stored at -70°C.

## Bacterial transformation by heat shock

The DNA (less than 1 µg DNA in less than 10 µl) and bacteria were gently mixed (but not by pipetting up and down), incubated on ice for 30 min and subjected to a heat shock for 30 sec to 2 min (at most) at 42°C in a water bath. The tubes were immediately transferred to ice and incubated for 10 min. Finally, the suspension was spreaded on LB agar plates containing antibiotics at the appropriate concentration. Plates had been incubated for 5 - 10 min at RT° before the spreading. Plates were then incubated O.N. at 37°C.

## Nucleic acid isolation and manipulations

### Small scale isolation of DNA from bacteria

This protocol is a modified version of the alkaline protocol according to Sambrook et al. (1989). With this protocol one can obtain a good quantity of pure plasmid DNA for sequencing, cloning, yeast transformation and other usual experiments. First, 3 ml of LB containing 100 µg/ml Amp was inoculated with a single colony and incubated with agitation O.N. at 37°C.

Cells were pelleted, resuspended in 100 µl of ice-cold Solution I (25.0 mM Tris-HCl pH 8, 50.0 mM Glucose, 10.0 mM EDTA) and then incubated at RT° for 3 min. 200 µl of freshly prepared Solution II (0.2 N NaOH, 1% SDS) were added and mixed rapidly by inversion to obtain a homogenous transparent suspension. The tube was incubated for 10 min on ice. 150 µl of ice-cold Solution III (4 M KOAc, 2M glacial acetic acid) were added, mixed rapidly by moderate vortexing and incubated on ice for 10 min. Cell debris and chromosomal DNA were pelleted (5 min at 15,000 rpm). The supernatant (450 µl) was then transferred to a fresh tube, the same volume of chloroform was added and mixed vigorously by vortexing. The aqueous phase was recovered and was subjected to a second chloroform treatment. 900 µl of ice-cold 95% EtOH was added to precipitate the DNA. The tube was incubated for 15 min at RT°. The pellet was washed twice with 70% EtOH (RT°), dried at RT° for 15 min and resuspended in 50 µl TE pH 8 (10 mM Tris-HCl pH 8, 1 mM EDTA) containing RNAase A (20 µg/ml). Finally, the DNA yield was checked by loading 1 µl on an agarose gel.

**Large scale isolation of plasmid DNA from bacteria by a CsCl density gradient**

To isolate larger quantities of plasmid DNA, I followed the above alkaline lysis protocol but using 400 ml of starter culture. I purified DNA on a CsCl density gradient for protoplast transformation. First, 4.3 g CsCl and the DNA pellet were each dissolved in 3 ml and 1 ml TE, respectively. Both solutions were mixed, 500 µl of EtBr (10 mg/ml) were added and the mixture was incubated at RT° for 1hr in the dark. After centrifugation at 3700 rpm for 10 min, the supernatant was transferred to an ultracentrifuge tube (NVT 865 or NVTI90) and subjected to an ultracentrifugation at 65000g for 15hrs. The EtBr was extracted with three times with isoamylalcohol. Then, 2 volumes of TE and 6 volumes of absolute EtOH were added, mixed well and incubated an O.N. at 20°C. After centrifugation, the pellet was resuspended in 450 µl TE, 50 µl of 3M Na-acetate. 1 ml of absolute EtOH was then added to precipitate the DNA, the tubes were centrifuged again and the pellet was washed with 1.5 ml of 70% EtOH. The pellet was resuspended in 20 to 30 µl TE.

**DNA digestion, ligation, PCR, electrophoresis and sequencing**

Restriction enzymes and buffers were purchased from New England Biolabs, Promega and Boehringer Mannheim, following their instructions regarding the reaction temperature, DNA amount and reaction time. For DNA ligation, we used the T4 DNA ligase from Promega. The ratio vector: insert was about 1:3. For PCR experiments, we used a PTC-100 apparatus from MJ Research with standard programs.

PCR reactions were performed in 50 µl using 10 - 50 ng of template DNA, 0.5 - 1 µM each primer, 200 µM dNTPs, 1 unit of *Pfu* DNA Polymerase or Taq Polymerase from Promega, and 1X Taq polymerase buffer containing 2.5 mM $MgCl_2$.

When cDNAs were used as template, the DNA was first denaturated (2 - 5 min at 94°C). Then, 35 cycles of denaturation (30 sec at 94 °C), annealing (30 sec at 52 - 55°C) and extension (4 min at 72°C) were carried out followed by a final extension at 72°C for 10 min. When a plasmid was used a template, the DNA was first denaturated (3 min at 94°C), followed by 30 cycles of denaturation (30 sec at 94°C), annealing at the corresponding specific temperature (specific T°C) and extension (45 sec at 72 °C) and a final extension at 72°C for 10 min.

If the primers were not totally homologous to the template, for example, in case of addition of a restriction site to the PCR fragment, we first run 10 cycles at the annealing T calculated only for the homologous part of the primer. We then continued with 20 cycles at the annealing T calculated for the full-length primer.

DNA fragments were separated and visualized on standard agarose gel (0.6 - 3%), depending on the length of the fragments. Expected fragments were extracted by pumping the DNA-fragment out of the gel with a pipette (“Sophie” method). PCR fragments were cloned in the EcoRV-digested, T-tailed pBluescript II SK(+/-) (Stratagene) or in the PGEM®-T Easy (Promega) vectors. DNA constructs were sequenced using the Labstation thermo sequenase labeled primer cycle sequencing kit 7-deaza-dGTP (Molecular Dynamics-Amersham Life Technologies alliance) with IRD800-labelled primers (MWG-biotech) and the Licor 4000L apparatus (MWG-biotech).

## Yeast strains

Table 2.1 Yeast strains used in this study

| Strain | Genotype | Source |
|---|---|---|
| SEY6210 | *MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9* | (Robinson et al., 1988) |
| EMY3 | *MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 vps10Δ1::HIS3* | (Marcusson et al., 1994) |
| RSY372 | *MATα leu2-3, 112 ura3-52 sec18-1 (ts)* | R.S collection |
| CCY120 | *MATα leu2-3, 112 ura3-52 his3-Δ200 trp1- Δ901 lys2-801 suc2-Δ9 vps45Δ2::HIS3* | (Burd et al., 1997) |
| EHY191 | *MAT**a** ade2-1 his3-11,15 leu2-3,112 trp1-Δ ura3-1 pep4Δ::TRP1 TPI::SUC2::HIS3* | (Harsay and Schekman, 2002) |

The *sec18-1* mutations is temperature-sensitive mutant allele.

I thank Prof. Scott D. Emr (University of California, San Diego) for providing me with the SEY6210 and EMY3 yeast strains, Prof. R. Schekman (University of California, Berkeley) for providing me with the RSY372 yeast strain, Dr. O. Deloche (University of Geneva) for

providing me with the CCY120 yeast strain, and Dr. E. Harsay (University of California, Berkeley) for providing me with the EHY191 yeast strain.

## Yeast media

The YPD medium (per liter: 10 g yeast extract (US Biological, Y2010), 20 g peptone (US Biological, P3300)) was supplemented with 15 g/L Bacto-agar for solid medium (US Biological, A0930). After autoclaving (15 min), 2% dextrose was added.

Three different selective or minimal media (SC) were used: **(I)** From DIFCO. Per liter: 6.7 g yeast nitrogen base without amino acids (DIFCO) was supplemented with 20 g agar (DIFCO) for solid medium. After autoclaving (15 min), the medium was supplemented with 2% dextrose (DIFCO, 215530) for normal growth conditions while it was supplemented with 2% galactose and 1% - 2% raffinose (DIFCO, 217410) for the induction under the yeast GAL promoter. Additionally, 1X drop-out solution from the appropriate sterile 20X stock solution was added. For example, to prepare SD-His-Trp, we used a 20X drop-out solution lacking His and Trp. For this selection, we used the following 20X drop-out: 400 mg/l L-Adenine hemisulfate salt, 3000 mg/l L-Valine, 400 mg/l L-Arginine HCl, 600 mg/l L-Isoleucine, 600 mg/l L-Lysine HCl, 600 mg/l L-Tyrosine, 400 mg/l L-Methionine, 1000 mg/l L-Phenylalanine, 4000 mg/l L-Threonine, 2000 mg/l L-Leucine and 400 mg/l L-Uracil. When the selection required L-Tryptophan and L-Histidine HCL monohydrate amino acids, they were added to the medium from 20X stocks (400 mg/l). **(II)** The premix amino acid powder was prepared in our laboratory. Per liter: 1.7 g of yeast nitrogen base minus amino acids and minus ammonium sulphate (DIFCO, 0335-15-9), 1.115 g from the premade package of amino acid premix –HUTL, 5 g of $(NH4)_2SO_4$ (Fluka, 09978), pH = 5.5 (NaOH). The SC liquid medium was supplemented with 20 g of Bacto-agar (DIFCO, 0140074) for solid medium. After autoclaving (20 min), the medium was supplemented with 2% dextrose (DIFCO, 215530) for normal growth conditions while it was supplemented with 2% galactose (Glucose free, Sigma, G0750) and 1% - 2% raffinose (DIFCO, 217410) for the induction under the yeast GAL promoter. Additionally, 1X of each amino acid stock solution from 100X stock solutions was added depending on the desired selection (40 mg/l L-Histidine (monohydrochloride monohydrate, Sigma H9511), 40 mg/l L-tryptophane (Sigma, T0254), 60 mg/l L-Leucine (Sigma, L8912) and 20 mg/l Uracil (Sigma, U1128)). The premix amino acid powder was prepared by weighting the following quantities: 40 mg/l Adenine hemisulfate salt (Sigma, A-9126), 20 mg/l L-Arginine HCl (Sigma, A3909), 100 mg/l L-aspartic acid (Sigma, A4534), 100 mg/l L-glutamic acid monosodium salt (Sigma, G5889), 30 mg/l L-Lysine mono-HCl (Sigma, L1262), 20 mg/l L-methionine (Sigma, M2893), 50 mg/l L-phenylalanine (Sigma, P5030), 375 mg/l L-serine (Sigma, T-8625), 200 mg/l L-Threonine (Fluka, 89180), 30 mg/l L-Tyrosine (Sigma, T3754) and 150 mg/l L-Valine (Sigma,

V0500). The total amount of powder (55.75 g) was homogenized using a pillar and mortar and then filtered using a metallic filter (100 µm stainless steel sieve), before mixing into a glass beaker. The homogenated powder was then aliquoted in sealed bags of 2.23 g each (stock for 2 L of medium of –HUTL selection). The bags were kept at a dry place in the dark at RT° **(III)** From US Biological. Per liter: 1.7 g yeast nitrogen base minus amino acids and minus ammonium sulphate (US Biological, Y2030), 2 g Drop-out Mix Synthetic minus His, Leu, Trp, Ura, Adenine Rich w/o Yeast Nitrogen Base (US Biological, D9541), and 5 g ammonium sulphate (Fluka, 09978)) was supplemented with 15 g of Bacto-agar (US Biological, A0930) for solid medium. After autoclaving (15 min), the medium was supplemented with 2% dextrose (DIFCO, 215530) for normal growth conditions while it was supplemented with 2% galactose (Glucose free, Sigma, G0750) and 1% - 2% raffinose (DIFCO, 217410) for the induction under the GAL promoter. Additionally, 1X of each amino acid stock solution from 100X stock solutions was added depending on the desired selection. The 100X stocks were: 10 mg/ml L-Histidine monohydrochloride monohydrate (Sigma, H9511), 5 mg/ml L-tryptophane (Sigma, T0254), 10 mg/ ml L-Leucine (Sigma, L8912), and 2.5 mg/ml Uracil (Sigma, U1128).
The carbon sources and amino acids drop-out stock solutions were filter sterilized and stored at RT°. Erlenmeyer flasks with 3 to 4 times the capacity of the volume of the culture were used since yeast cells needed more “aeration” than E. coli.

### Yeast stocks maintenance

Yeast strains were stored in 15% - 20% glycerol at -70°C. To recover a strain, a small amount of cells were scraped from the frozen stock with a platine loop and streaked onto YPD medium or SC plates containing the desired amino acid selection. The plates were incubated at 30°C for 1 to 3 days until colonies appeared. If you cannot recover the strain by scraping the frozen stock, the cells may have settled to the bottom of the tube before the stock was frozen. In this case, thaw the frozen culture on ice and vortex it before restreaking. Make a new frozen stock.

### Yeast transformation

Three protocols for yeast transformation were used: **(I)** This protocol yields between $10^5$ to $10^6$ transformants per µg of plasmid DNA (Ausubel et al., 1987-1995). A single colony was inoculated into 5 ml of either YPD or SC mediums with agitation for O.N. at 30°C. The starter culture was then diluted to a concentration of 5 x $10^6$ cells/ml ($OD_{600}$ of 0.3 - 0.5). The culture should have reached at least an $OD_{600}$ of 1.0 to be diluted. You need 10 ml of culture per transformation and 10 ml for a control in order to calculate the volume of the starting culture.

Cells were grown with agitation for 4 - 5 hrs at 30°C, pelleted in a table top centrifuge at 3000 rpm for 5 min and resuspended in 1 ml of sterile dd $H_2O$. Cells were then transferred to an Eppendorf tube and resuspended in Lithium acetate-TE (0.1 M Lithium acetate, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) (50 µl per starting culture). Cells were dispatched in aliquots of 50 µl. Then, 20 - 50 µg of single-stranded (ss) salmon sperm carrier DNA and 5 µl of plasmid DNA (1 - 2 µg of DNA to be on the safe side) were added. The plasmid DNA and the ss salmon sperm carrier DNA were mixed before they were added to the cells. The tubes were flipped several times and 350 µl of PEG-Li-TE solution (40% PEG 4000, Fluka), 0.1 M Lithium acetate, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) were added. The tubes were again flipped several times and incubated for 30 min at 30°C. Cells were then subjected to a heat shock at 42°C for 20 min and subsequently pelleted at RT° at 4000 rpm for 3 min. After washing with 500 µl of sterile dd $H_2O$, cells were resuspended in 1 ml of TE or sterile dd $H_2O$. 100 µl of cells were plated on SC plates supplemented with the adequate amino acids. One can expect $10^4$ - $10^5$ transformants per mg of plasmid DNA. **(II)** This procedure was taken from Quick and Easy TRAFO Protocols (After Gietz and Woods, 1994). It is very flexible, however, for better results, one should use cells from a freshly grown plate. 20 - 50 µl of yeast cells were scraped from the plate with a sterile toothpick and resuspended in 1ml of sterile dd $H_2O$. Each transformation reaction required about 25 µl of yeast cells. Therefore, if you scrape about 100 µl of yeast cells into 1ml, this represents 4 transformation reactions. Cells were pelleted, resuspended in 1 ml of 100 mM Lithium acetate and incubated for 5 min at 30°C. The volume representing a single transformation reaction was aliquoted into a microcentrifuge tube and spinned down at top speed for 5 sec. The supernatant was removed and 240 µl of PEG (50% w/v), 36 µl of 1.0 M Lithium acetate, 25 µl of ss salmon sperm carrier DNA (2.0 mg/ml), 5.0 µl of plasmid DNA (100 ng to 5 µg) and 45 µl of sterile dd $H_2O$ were added on the top of the cell pellet (in this order) and mixed by vortexing for at least 1 min. After incubation at 42°C for 20 min, the cells were pelleted at top speed in a microcentrifuge for 10 sec and washed with 500 µl of sterile dd $H_2O$. A brief centrifugation was performed and 300 µl were removed. The cell suspension was plated onto 1 or 2 SC plates. Colonies were visible in 2 to 4 days after incubation at 30°C. The yield ranged from a few hundred to a few thousand transformants **(III)** This protocol requires frozen competent cells. The protocol for the preparation of the competent cells was the following. A single colony from a freshly grown plate was grown in liquid culture for 12 hrs at 30°C with agitation. The starter culture was then diluted with the same medium to an $OD_{600}$ of 0.3 - 0.5 (400 ml). Once the culture density reached 0.6 - 0.8 $OD_{600}$, the cells were pelleted in sterile tubes and washed with 80 ml of sterile dd $H_2O$ and subsequently with 80 ml of Lithium acetate-TE. The washed pellet was resuspended in 20 ml of Li-TE and incubated for 1 hr at 30 °C with gently agitation. The cells were harvested and resuspended in 4 ml of G-Li-TE solution

(12% Glycerol, 0.1 M Lithium acetate, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) at a density of 0.6 $OD_{600}$ (the volume may have to be adjusted). This led to approximately 3 $x10^8$ cells per 100 µl fraction. The competent cells were then distributed into sterile tubes in 100 µl aliquots and frozen slowly to -70°C.

For transformation, the competent cells were thawed and 20 µl of 0.5% ss salmon sperm carrier DNA, 0.1 to 10 ng of plasmid DNA in a volume smaller than 10 µl (~ 1 µg) and 10 µl of 96% EtOH were added (the order was respected). Cells were incubated at RT° for 5 min and 700 µl of PEG-Li-TE were added and mixed with a cut blue tip in order to minimize the loss of solution on the wall of the pipette (mix well until obtaining a homogenous solution, do not vortex). The cells were then incubated for 1 hr at 30°C and subjected to a heat shock for 20 min at 42°C. After a brief centrifugation, 650 µl of supernatant were discarded and the pellet was resuspended with pipette. Finally, the cells were plated and grown for 3 to 5 days at 30°C.

**Notes:** **-** The plates were warmed at 30°C before starting any transformation procedure.
**-** The protocol (I) gave the best results.

***Yeast transformation solutions***

The SS salmon sperm carrier DNA was prepared at 2 mg/ml (TRAFO's protocol). 200 mg of high molecular weight DNA Sodium Salt Type III from Salmon Testes (Sigma, D-1626) were weighed out and dissolved with 100 ml of TE buffer (10 mM Tris-HCl pH 8, 1mM EDTA). The DNA was mixed by pipetting up and down repeatedly using a 10 ml pipette (do not sonicate: the bigger the DNA, the more efficient the transformation). The solution was then vigorously mixed on a magnetic stirrer for 2 - 3 hrs until fully dissolved. The DNA was aliquoted and stored at -20°C. Prior to use, an aliquot was placed in a boiling water bath for 5 min and quickly cooled in ice water slurry. We made sure that the ss salmon sperm carrier DNA was cold and homogenous before use. It can be stored at 4°C and reused 3 or 4 times, however, if the transformation efficiencies decrease it should be boiled again or a new aliquot used.

Polyethylene glycol (PEG 50% w/v)

The polyethylene glycol (PEG), MW 4000 (Fluka, 81240) was dissolved at 50% (w/v) in dd $H_2O$ and filter sterilized. For optimal transformation efficiencies make sure that the PEG solution is at the proper concentration. It is important to store the PEG in a tightly capped container to prevent evaporation of water and subsequent increase in PEG concentration. Small variations above or below the optimum PEG concentration during transformation reactions, which is 33% (w/v), can reduce the number of transformants. 50 g of polyethylene glycol (MW 4000) were added to a 150 ml glass beaker and mixed with 35 ml of dd $H_2O$. The

solution was stirred with a magnetic bar until it was dissolved. The beaker was rinsed with a small amount of dd $H_2O$, after the solution had been transferred to a 100 ml graduated cylinder, and the rising solution was added to it. The volume was brought to exactly 100 ml and mixed well by inversion. The solution was sterilized by filtration using a 0.45 µm filter unit (Steritop, Millipore, SCGPT01RE) and stored in a secure capped bottle.

## VSR constructions

The pGAL-BgIII-VSR-PS1 plasmid has been previously described (Humair et al., 2001). The following constructs were prepared with the technical help of S. Marc-Martin, and the cDNAs libraries (Ecotype Columbia) were kindly provided by Dr. M. Kessler (University of Zurich). Six of the seven *A. thaliana* VSRs (AtVSR1, 2, 3, 5 and 6) were cloned under the control of the GAL inducible promoter. The following primers were used (restriction sites in bold):

| | | |
|---|---|---|
| T7 short | (5' side of AtVSR2): | 5' taatacgactcactataggg 3' |
| N4A | (3' side of AtVSR2, adding **SacI** site): | 5' tc**gagctc**ttaggcacg 3' |
| AT3-sense | (5' side of AtVSR3): | 5' ttggtggattcttcagagatggg 3' |
| AT3-anti | (3' side of AtVSR3): | 5' ttaattgatctcaaacatttctttgtg 3' |
| AT5 sense | (5' side of AtVSR5, adding **NotI** site): | 5' ga**gcggccgc**gaaaagaatgtctccgagc 3' |
| AT5-anti | (3' side of AtVSR5): | 5' caagagttcctatatatttactta 3' |
| AT6 sense | (5' side of AtVSR6, adding **NotI** site): | 5' ga**gcggccgc**gatcagaaacaatgtctttgattc 3' |
| AT6-anti | (3' side of AtVSR6): | 5' caagatttataaaattaggctgcag 3' |

The construct containing AtVSR1 (pGAL-BgIII-AtVSR1) was obtained by introducing the KpnI-SacI fragment from pBluescript II SK(+) NP432 (Z38123) (Paris et al., 1997) plasmid corresponding to the full coding sequence with 242 base pairs (bp) upstream and 172 bp downstream into the same sites of the previously cut pGAL-BgIII.

To prepare the pGAL-BgIII-AtVSR2 plasmid, we first amplified by PCR the open reading frame (ORF) of AtVSR2 with 132 pb downstream using the pSPORT1 MJ447 plasmid (Paris et al., 1997) as a template and the T7 short and N4A primers. The PCR fragment was cloned into the EcoRV-digested, T-tailed pBluescript II SK(+). The KpnI-SacI fragment was then removed and introduced into the equivalent sites of the previously digested pGAL-BgIII-VSR-PS1.

To construct the pGAL-BgIII-AtVSR3 plasmid, we amplified by PCR from a cDNA library of roots the ORF of AtVSR3 with 60 bp upstream and 18 bp downstream using the AT3-sense and AT3-anti primers. The PCR fragment was cloned into the EcoRV-digested, T-tailed pBluescript II SK(+). The KpnI-EcoRI fragment was then removed and introduced into the same sites of the previously digested pGAL-BgIII-AtVSR2.

To construct the pGAL-BgIII-AtVSR5 plasmid, we amplified by PCR from a cDNA library of roots the ORF of AtVSR5 with 17 bp upstream and 7 bp downstream using the AT5-sense and AT5-anti primers. The PCR fragment was cloned into the pGEM-T Easy vector. The pGEM-T Easy-AtVSR5 was linearized with SphI and blunt ended with T4 DNA Polymerase. After a SacI digestion, the SphI/Blunt-SacI fragment was introduced into the SmaI-SacI previously digested pGAL-BgIII-AtVSR2 plasmid.

To prepare pGAL-BgIII-AtVSR6 plasmid, we amplified by PCR from a cDNA library of roots the ORF of AtVSR6 with 14 bp upstream and 11 bp downstream using the AT6-sense and AT6-anti primers. The PCR fragment was cloned into the pGEM-T Easy vector. The pGEM-T Easy-AtVSR6 was linearized by SphI and blunt ended with T4 DNA Polymerase. After a SacI digestion, the SphI/Blunt-SacI fragment was cloned into the SmaI-SacI previously digested pGAL-BgIII-AtVSR2 plasmid.

All constructs were confirmed by sequencing. The cDNA sequences of the VSRs are presented in Appendix 2.

## Truncated and hydrid constructions for $VSR_{PS-1}$

A truncated form and a hybrid of $VSR_{PS-1}$ were also cloned under the control of the GAL inducible promoter. The following primers were used (restriction sites in bold and stop codons underlined):

GFP-S65T (5' side of GFP):

5' ccatggccaacacttgtcactactctcactta 3'

Sac-anti2 (3' side of $VSR_{PS-1}$ CT, adding **SacI** site and a stop codon):

5' **gagct**<u>ca</u>cctaattctatatttataca 3'

SphIHA (5' side of $VSR_{PS-1}$ NT, start at the codon 421(NG)):

5' caacggtggttgttggcaggat 3'

SacINotHA (3' side of $VSR_{PS-1}$ CT, adding **SacI**, **NotI** sites and a codon stop):

5'agc**gagctc**<u>cta</u>**gcggccgc**cacctctttgatgattgacgtgatt 3'

SalIVps10 (5' side of Vps10p (start at the codon 1389 (VSAGP), adding **SalI site**):

5'aa**gtcgac**agtaagtgccggtccctttgca3'

SacIVps10 (3' side of Vps10p CT, adding **SacI** site and a stop codon):

5'aa**gagct**<u>c</u>**t**actggttttcgttagatggcgc3'

To construct the pGAL-BgIII-VSR-PS1ΔCT plasmid, we amplified by PCR the sequence encoding the $VSR_{PS-1}$ TMD and 4 carboxyl-terminal residues using the NAD24 plasmid (Lab collection) as template and the GFP-S65T and Sac-anti2 primers. The PCR fragment was then

cloned into the pGEM-T Easy vector. The SalI-SacI fragment was removed and introduced into the same sites of the previously digested pGAL-BglII-PS1-AtVSR1 plasmid (the VSR-PS1 luminal domain (NT) fused to the AtVSR-AT1 TMD and CT, Lab collection). This truncated protein is devoided of 32 carboxy-terminal residues and its C-terminus codifies for YKYRIR and a stop codon.

To prepare the pGAL-BglII-VSR-PS1-HA plasmid, we amplified by PCR the sequence encoding a part of the $VSR_{PS-1}$ NT domain, the TMD and CT domain using the pGAL-BglII-VSR-PS1 as template and the SphIHA and SacINotIHA primers. The PCR fragment was cloned into the pGEM-T Easy vector followed by the addition of a NotI fragment containing three copies of the HA epitope (YPYDVPDYA) digested from pRS306VPS10HA plasmid (Deloche et al., 2001) resulting in plasmid pGEM-T Easy PS1 (NT(431))-TMD-CT-HA. The full sequence of VRS-PS1 tagged with the HA tag was obtained by a triple ligation, downstream the XmaI-SphI fragment from the pGAL-BglII-VSR-PS1 plasmid and upstream the SphI-SacI fragment from the pGEM-T Easy PS1 (NT(431))-TMD-CT-HA in the SmaI-SacI digested pBluescriptII SK(-) vector. The XmaI-SacI fragment was removed and introduced into the corresponding sites of the previously digested pGAL-BglII-PS1.

To construct the pGAL-BglII-PS1-Vps10pHA plasmid, we first amplified by PCR the last 189 carboxyl-terminal residues of Vps10p using the pKE50vps10p (Deloche's collection) as template and the SalIVps10 and SacIVps10 primers. The PCR fragment was cloned into the pGEM-T Easy vector. The SalI-SacI fragment was introduced into the same sites of the previously digested pGAL-BglII-PS1-AtVSR1 plasmid to obtain pGAL-BglII-PS1-Vps10p ($VSR_{PS-1}$ NT domain fused to the Vps10p TMD and CT). The junction is KST/VSAG. To add the HA tag, the BclI-SacI fragment was replaced by BclI-SacI fragment from the pRS306VPS10HA plasmid (Deloche et al., 2001) which contains three copies of the HA epitope (see Appendix 3). All constructs were confirmed by sequencing.

## GFP constructs with new VSDs

Three plant VSDs independently fused to GFP were cloned under the yeast GAL promoter. These VSDs were the *Nicotiana alata* proteinase inhibitor (NAPI) ct-VSD, the *Bertholletia excelsis* 2S albumin (2Sa) ct-VSD, and the *Phaseolus vulgaris* phaseolin (Ph) ct-VSD. The following primers were used (restriction sites in bold, overlap region in italics and stop codons underlined):

NAPI sense (5' side of the NAPI VSD (EYASS.), adding **BglII** site):

5' **agatct**tgaatatgccagcaaagtggatgaatatgtt*ggtgaagtggag* 3'

NAPI reverse (3' side of NAPI VSD (SVAV…), adding **PstI** site and a stop codon):

5’ **ctgcag**tta*ggaaacagcaaccttagacttctggagatcattct**ccacttcacc** 3’

GFP-NcoI (5’ side of GFP5 (start at the codon 74 (LPVP.)):

5’ ctacctgttccatggccaac 3’

2Sa-NcoI (3’ sides of 2Sa-VSD and GFP; adding **PstI** site and a stop codon, the internal NcoI site is destroyed (changed base in italics and bold, t instead of g):

5’gggg**ctgcag**tcagaacccggcaatggagccacccat***t***ggtttgtatagttcatccatg3’

2Sa VSD GFP

To obtain the pYWG1-GFP5-NAPI plasmid, a second-strand DNA based on the *N. alata* NAPI ct-VSD was synthesized with DNA polymerase I, Large (Klenow) fragment using the NAPI sense and NAPI reverse primers. The resulting DNA fragment (NAPI-VSD) is the following:

```
 BglII
AGATCTTGAATATGCCAGCAAAGTGGATGAATATGTTGGTGAAGTGGAGAATGATCTCCAG
TCTAGAACTTATACGGTCGTTTCACCTACTTATACAACCACTTCACCTCTTACTAGAGGTC
  D   L   E   Y   A   S   K   V   D   E   Y   V   G   E   V   E   N   D   L   Q

                                PstI
AAGTCTAAGGTTGCTGTTTCCTAACTGCAG
TTCAGATTCCAACGACAAAGGATTGACGTC
 K   S   K   V   A   V   S   *
```

After phosphorylation with T4 Polynucleotide Kinase, the synthesized fragment was cloned into the pGEM-T Easy vector. The resulting BglII-PstI fragment was introduced into the corresponding sites of the previously digested PGY1-GFP5-Chi (Di Sansebastiano et al., 1998) to obtain the PGY1-GFP5-NAPI plasmid. We introduced the BamHI-SacI fragment into the equivalent sites of the previously digested pYWG1 (Lab collection).

To prepare the pYWG1-GFP5-2Sa plasmid, we first amplified by PCR the sequence encoding a part of the GFP5 and the *B. excelsis* 2Sa ct-VSD using PGY1-GFP5-Chi (Di Sansebastiano et al., 1998) as template and the GFP-NcoI and 2Sa-NcoI primers. The PCR fragment was cloned into the pBluescript II SK(+) vector. The NcoI-PstI fragment from pBluescript II-GFP5 (74)-2Sa was introduced in the equivalent sites of the previously digested PGY1-GFP5-Chi to obtain PGY1-GFP5-2Sa. The BamHI-SacI fragment was cloned into the same sites of previously digested pYWG1 (Lab collection).

The pYWG1-GFP-Ph plasmid was obtained by introducing the BamHI-PstI fragment from pDHA-GFP-AFVY (Frigerio et al., 2001) which encodes the *P. vulgaris* Ph ct-VSD fused in frame with the GFP5 sequence, into the corresponding sites of the previously cut pYWG1-GFP5-2Sa. All constructs were confirmed by sequencing. The construction of pYWG1-sec-

GFP, pYWG1-aleu-GFP, pYWG1-GFP-Chi plasmids has been previously described (Humair et al., 2001).

For protoplast transient expression, the *N. alata* NAPI ct-VSD and the *B. excelsis* 2Sa ct-VSD independently fused to GFP were cloned in the same expression vector used for the controls. The construct containing the GFP6 fused to the ct-VSD of NAPI (PGY1-GFP6-NAPI) was perfomed by cloning the BglII-PstI fragment from PGY1-GFP5-NAPI into the equivalent sites of previously digested PGY1-GFP6-Chi (Di Sansebastiano et al., 1998). The construct containing the GFP6 fused to the ct-VSD of 2Sa (PGY1-GFP6-2sa) was obtained following the same procedure as for PGY1-GFP5-2Sa but using PGY1-GFP6-Chi (Lab collection) as template. Both constructs were confirmed by sequencing. The construction of pGY1-GFP5-Chi and pGY1-aleu-GFP6 plasmids has been previously described (Di Sansebastiano et al., 1998; Humair et al., 2001).

## Induction under the GAL promoter

Transformed yeast cells were grown at 30°C using different induction procedures: **(I)** Cells were grown in SC medium containing 2% galactose for 24 hrs or 48 hrs (used for fluorescence microscopy and GFP measurements) **(II)** Cells were grown in SC medium containing 2% dextrose ($OD_{600}$ of 2.5 - 3) and washed twice with SC medium containing 2% raffinose. Subsequently, the cells were diluted in this medium to an $OD_{600}$ of 0.4. After 3 hrs, 2% galactose was added and cells were observed after 24 hrs (used for fluorescence microscopy) **(III)** Cells were grown to exponential phase ($OD_{600}$ of 0.7) in SC medium containing 2% dextrose and washed with SC medium supplemented with 2% raffinose and 2% galactose. Cells were then diluted in the same induction medium to an $OD_{600}$ of 0.35 and incubated until they reached the exponential growth phase ($OD_{600}$ of 0.8-1.5) (used for fluorescence microscopy and all the experiments related to the degradation and localization of $VSR_{PS-1}$-HA and $VSR_{PS-1}$-Vps10p-HA) **(IV)** Cells were grown to exponential phase ($OD_{600}$ of 0.7) at 30°C in SC medium containing 2% dextrose. Cells equivalent to 1 $OD_{600}$ unit were collected, washed twice with SC medium containing 2% galactose and subsequently spotted (4µl) on plates containing the same medium of induction (used for colony blot assay).

**Note: -** Protocol (III) gave the more homogeneous vacuolar signal in wild type cells.

## GFP measurement

After induction (18 to 24 hrs), cells were subjected to a glass beads protein extraction. Cells equivalent to 10 $OD_{600}$ units were transferred into an Eppendorf tube. Samples were centrifuged and the pellet was resuspended in 100 µl of extraction buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA). Two-thirds of volume of acid washed glass beads (0.45 – 0.5 mm, Sigma, G 8772) was then added and vortexed four times at full speed for 2 min, between each cycle, cells were frozen in liquid nitrogen and thawed at 40ºC. Samples were kept on ice between each cycle. 50 µl of extraction buffer were used to wash the beads. After a quick centrifugation, the lysate was recovered using an ultra-thin Pasteur pipette and the beads were washed again with 50 µl of extraction buffer. The final volume (200 µl) was centrifuged at 14:000 rpm for 5 min and the supernatant was transferred into a fresh tube. Samples were kept on ice.

For the Bradford protein assay, a standard curve of BSA was produce. From a 10% BSA stock solution (100 µg/µl, stored at -20 ºC), approximately 1 ml set of dilutions of BSA was performed to have either 2 – 5 – 10 – 15 - 20 µg in 100 µl of extraction buffer. A negative control tube with 100 µl of extraction buffer was prepared. 20 µl from the protein extract were transferred into a new plastic tube and diluted with 80 µl of extraction buffer. In each tube (negative, standard and sample), 1 ml of diluted Bradford reagent was added and mixed by vortexing. The tubes were first stored in the dark for 5 min at RTº and the $OD_{595}$ was measured. Finally, the protein concentration was calculated. According to the standard curve reached approximately $OD_{595}$ = 1 for 20 µg BSA. The amount of proteins recovered per 20 µl of yeast extract averaged 10 to 20 µg (700 µg to 1.5 mg for 1 ml of a 10 $OD_{595}$ culture of yeast).

To prepare the Bradford stock, 300 mg Coomasie Brilliant Blue G250 (Serva blue) were dissolved in 150 ml 95% Ethanol (142.5 ml of absolute EtOH and 7.5 ml H20) and 300 ml of 85% phosphoric acid. The solution was filtered through 2 paper filters and stored at RTº in a brown bottle. In order to freshly dilute the Bradford reagent, 1.5 ml from the stock solution and 8.5 ml 1X PBS were mixed.

Using a spectrofluorimeter (Luminescence Spectrometer, LS50B, Perkin Elmer), relative fluorescence unit (RFU) values from each sample were obtained. 150 - 200 µl were directly loaded in a cuvette (73.3-F Q 3 mm, Starna GmbH) for fluorescence measurements. The excitation and emission wavelengths were set to 485 nm and 508 nm, respectively. The amount of recovered GFP averaged from 60 to 200 ng of GFP based on a Clontech reference.

Quantification of the intracellularly retained GFP in yeast cells

In order to quantify the GFP intracellularly retained by the VSRs, the positive control is given by the wild type strain (SEY6210) expressing aleu-GFP (in the plasmid pYWG1). In this case, the yeast vacuolar sorting receptor Vps10p efficiently sends the reporter to the vacuole. The negative control is the mutant strain (EMY3), lacking Vps10p and expressing aleu-GFP, by which the reporter is secreted. To determine the targeting efficiency by the VSRs, we coexpressed aleu-GFP with each VSR (in the plasmid pGAL-BglII).

We determined the background fluorescence (B) for each of the above strains. The background for the positive and negative controls is given by the wild type and mutant strains containing the empty plasmid pYWG1 which was used to express aleu-GFP. The background for the VSR expressing strain was determined for the mutant strain containing both empty plasmids pYWG1 and pGAL-BglII.

After induction, 10 $OD_{600}$ units of cells of each transformant were harvested and subjected to a glass beads protein extraction. For each sample, we measured the RFU and the protein concentration by spectrofluorimetry and Bradford assay, respectively. As shown in table 2.2 (column 3, 4), the RFU values were first normalized by the protein concentration (F and B values) and then the background was substrate (F-B). The indexes indicates the positive control (WT), the negative control (M) and VSR expressing strain (VSR).

The receptor-mediated retention due to the plant VSR was expressed as percentage (%) of the retention mediated by Vps10p in the wild type strain (Table 2.2, column 5). The receptor-mediated retention is the difference between the values for the strain with and without receptor ($\Delta F_{WT} - \Delta F_M$, for Vps10p, $\Delta F_{VSR} - \Delta F_M$, for the VSR). In addition to $VSR_{PS-1}$, AtVSRs (1, 2 and 3) were also tested.

**Table 2.2 Calculation of the intracellular retention of GFP**

| Transformants | Pattern | RFU/[protein] | RFU/B | Receptor-mediated retention |
|---|---|---|---|---|
| WT + aleu-GFP (positive control) | Vacuolar | $F_{WT}$ | $F_{WT} - B_{WT} = \Delta F_{WT}$ | $\Delta F_{WT} - \Delta F_M = 100\%$ |
| WT + pYWG1 | - | $B_{WT}$ | | |
| M + aleu-GFP (negative control) | Secreted | $F_M$ | $F_M - B_M = \Delta F_M$ | 0% |
| M + pYWG1 | - | $B_M$ | | |
| M + aleu-GFP + VSR | - | $F_{VSR}$ | $F_{VSR} - B_{VSR} = \Delta F_{VSR}$ | $\frac{\Delta F_{VSR} - \Delta F_M}{\Delta F_{WT} - \Delta F_M} = X\%$ |
| M + pYWG1 + pGAL-BglII | - | $B_{VSR}$ | | |

WT: SEY6210 strain

M: EMY3 (*Δvps10*) strain

RFU: Relative Fluorescence Unit
Excitation = 475nm (Ext)
Emission = 508nm (Em)

[protein]: mg protein/ml

$B_{WT}$: Background fluorescence for WT strain transformed with empty plasmid pYWG1 (no aleu-GFP)

$B_M$: Background fluorescence for M strain transformed with empty plasmid pYWG1 (no aleu-GFP)

$B_{VSR}$: Background fluorescence for VSR expressing strains: M strain transformed with empty plasmid pYWG1 (no aleu-GFP) and empty plasmid pGAL-BglII (no VSR)

## GFP Colony Blot Assay

Transformed cells were grown to exponential phase ($OD_{600}$ 0.7) at 30°C in SC medium containing 2% dextrose. Cells equivalent to 1 $OD_{600}$ unit were collected, washed twice with the SC medium containing 2% galactose and 4µl were spotted on plates containing the same medium. The plates were overlaid with nitrocellulose filters (Schleicher and Schuell, Dassel Germany, Protan BA 85, 0.45µM Ø 82mm) and incubated at 30°C for 18 - 24 hrs. The nitrocellulose membranes were then removed and subjected to immunostaining with anti-GFP antibodies as described in Roberts et al. (1991). Briefly, the membranes were washed several times with sterile dd $H_2O$ and then twice with standard phosphate-buffered saline. The immunostaining assay was performed using the anti-GFP antibody (Molecular Probes) at 1:5,000 dilution. Anti rabbit IgG-horseradish peroxidase (HRP) (Santa Cruz Biotechnology, CA) at 1:10,000 dilution was used as the secondary antibody, followed by visualization with the

ECL chemiluminescent protein detection system (Amersham Biosciences, England, RPN2108).

### Cycloheximide treatment, whole cell extracts and Western blot analysis

Transformed cells were grown to exponential phase in SC medium containing 2% raffinose and 2% galactose at 30°C. Cycloheximide (CHX, 1 µg/ml, Sigma-Aldrich Co, St. Louis, MO) was added to cultures at $OD_{600}$ 1. At the indicated time points (see results), 3 $OD_{600}$ units of cells were harvested and subjected to post-alkaline protein extraction (Kushnirov, 2000). Briefly, the pellet was resuspended in 100 µl of dd $H_2O$ and 100 µl 0.2 M NaOH was added. After incubation for 5 to 10 min at RT°, cells were pelleted and resuspended in 60 µl SDS sample buffer (60 mM Tris-HCl pH 6.8, 5% glycerol, 2% SDS, 4% ß-mercaptoethanol, 0.0025% [w/v] bromophenol blue). The protein extracts were boiled for 3 min in a water bath and centrifuged again before loading. For the *sec18-1$^{ts}$* mutant, cells were grown at 22°C and shifted to 37°C for 30 min before the addition of CHX. For these blots, the cells were lysed by a glass bead procedure (modified from Romanos et al., 1992), transferred to Eppendorf tubes and resuspended in 60 µl extraction Buffer (50 mM Tris-HCl pH 7.5, 10mM EDTA and 1X protease inhibitor cocktail from 25X stock solution (Roche, 1 697 498)). Two-third volume of acid washed glass beads was added and cells were lysed by vortexing four times for 90 seconds at full speed. Between each cycle, cells were frozen in liquid nitrogen and thawed at 40°C. Lysates were subsequently centrifuged for 1 min at 14.000 rpm in an Eppendorf microfuge and the supernatant was mixed with 1 volume of Laemmli 2X buffer without ß-mercaptoethanol (Laemmli, 1970). Samples were loaded on a 7.5% SDS-PAGE gel and immunoblots to detect all epitopes were prepared as described by Towbin and coworkers (1979).

Mouse 12CA5 anti- hemagglutinin (HA) antibodies were purchased from Berkeley Antibodies (Richmond, CA) and were used at a dilution of 1:10,000. Anti-VSR monoclonal (used at 1:5,000) antibodies [17F9; (Cao et al., 2000)] only recognize the native $VSR_{PS1}$ protein and were kindly provided by John C. Rogers (Pullman, WA). Mouse anti-Pep12p (used at 1:5,000), mouse anti-Vph1p (used at 1:5,000) and rabbit anti-GFP (used at 1:10,000) antibodies were purchased from Molecular Probes Inc. (Eugene, OR). Secondary goat anti-mouse (used at 1:10,000) antibodies coupled to horseradish peroxidase (HRP) were purchased from BIO-RAD (CA). Secondary goat anti-rabbit (used at 1:10,000) antibodies coupled to horseradish peroxidase (HRP) were purchased from BIO-RAD (CA) and Santa Cruz Biotechnology (CA). The bands were visualized using the ECL chemiluminescent protein detection system (Amersham Biosciences, England, RPN2108).

## Labeling of yeast cells

The yeast vacuole membrane was labeled with FM4-64 (*N*-[3-triethylammoniumpropyl]-4-[*p*-diethylaminophenylhexatrienyl] pyridinium dibromide; Molecular Probes, T-3166) (Vida and Emr, 1995). Cells were grown in SC medium supplemented with 2% galactose to early mid-log phase. Cells equivalent to 0.2 - 0.8 $OD_{600}$ units were collected, resuspended in 1 ml of SC medium without galactose containing 8 µM FM4-64 (diluted from a 16 mM solution in DMSO (Dimethyl sulfoxide, Fluka, 41650) and incubated for 15 min at 30°C with agitation. Cells were pelleted, resuspended in 1 ml of SC medium with 2% raffinose and 2% galactose and FM4-64 but without the amino acids for the selection. After incubation for 90 min at 30°C with agitation and washing twice with 1 ml 1X PBS, cells were resuspended in 0.1 ml SC medium with the corresponding amino acids but without a carbon source. 5 µl of cells were immobilized on a glass slide (0.4% low melting agarose was usually used) and observed with appropriate optics.

## Cell fractionation by differential centrifugation

EMY3 cells expressing $VSR_{PS-1}$-Vps10HAp were fractionated by differential centrifugation after osmotic lysis (Horazdovsky and Emr, 1993). Cells were grown to exponential phase ($OD_{600}$ of 0.7) at 30°C in SC medium containing 2% dextrose without uracil and induced as described above. Cells equivalent to 10 $OD_{600}$ units were harvested and converted to spheroplasts by treatment with zymolyase 20T (20 µg/OD, Seikagaku Corporation) in 1 ml of 0.8 M sorbitol, 10 mM Tris pH 7.5, 1 mM DTT and 20 mM $NaN_3$ at 30°C for 45 minutes. To check the spheroplasting efficiency, 20 µl were taken before and after the addition of zymolyase. They were diluted with 800 µl of dd $H_2O$ and the $OD_{600}$.was measured. For correct spheroplasting, the $OD_{600}$ value should go down at least 50% after treatment with zymolyase.

Spheroplasts were washed once in 0.5 ml of ice cold MES/0.8 M sorbitol buffer (0.1 M MES, pH 6.5, 0.8 M sorbitol, 0.5 mM $MgCl_2$, 1 mM $NaN_3$, 1 mM EGTA, 0.2 mM DTT, 1 mM PMSF) and lysed in 0.5 ml of MES/0.25 M sorbitol Buffer (100 mM MES pH 6.5, 0.25 M Sorbitol, 0.5 mM $MgCl_2$, 1 mM $NaN_3$, 1 mM EGTA, 0.2 mM DTT, 1 mM PMSF + 1X protease inhibitor cocktail (1 µg/ml Pepstatin A, 1 µg/ml Leupeptine, 0.15 µg/ml Benzamidine, Roche)). The stock solutions were prepared earlier and stored at 4°C. However, the DTT, PMSF and the protease inhibitors were added at the end.

Whole cell extracts were centrifuged at 13:000 g for 10 min and the pellet was resuspended in 30 µl MES 0.2 M Sorbitol solution and 30 µl loading buffer to yield P13 (pellet). The supernatant fraction was centrifuged at 100:000 g for 60 min and the pellet was resuspended in 30 µl MES 0.2M Sorbitol solution + 30 µl Loading buffer 2X to yield P100 (pellet). Proteins from the supernatant were precipitated with 10% trichloroacetic acid (TCA) and then washed

with acetone. The pellet was resuspended in 30 µl 2X Loading Buffer, 6 µl 1M NaOH, 24 µl 0.2M MES Sorbitol solution to yield S100 (supernatant). 10 µl of each fraction were subjected to immunoblot analysis after SDS-PAGE on a 7.5% acrylamide gel (Laemmli, 1970). All centrifugations were performed at 4°C.

## Plant Material and protocols

*A. thaliana* (ecotype Columbia) cell suspension cultures were a gift from Dr. L. Sticher (University of Fribourg, Switzerland). Cell suspension cultures were grown under sterile conditions on MSMO (Sigma M 6899) medium supplemented (per liter) with: 30% sucrose, 0.05 µg/ml NAA (2-naphtalene acetic acid), 0.5 µg/ml kinetin, pH 5.7, at 26°C with 16 hrs of daily light. The flasks were gently agitated on a rotary shaker at 110-130 oscillations $min^{-1}$ to facilitate the dissociation of the calli. Every 10 days, about 2 g of mother cell suspension were transferred into 50 ml of fresh culture medium.

### Protoplast isolation from cell suspension cultures

50 ml volume of a 10 days-old cell suspension was filtered through a 1-1.5 mm stainless steel sieve and the clumps remaining on the filter were discarded. An aliquot of the filtrate containing 4 g of fresh cells was filtered through a 100 µm stainless steel sieve. The collected cells were washed on the filter with K3 solution (per liter: 100 ml K3 Macro, 5 ml from $Na_2EDTA$ (200x): 40 mM, 5 ml 20 mM $FeCl_3*6H_2O$ (200x), 1 ml B5-micro, 10 ml K3-Vit, 102.7 g Sucrose, 0.25 g Xylose, 0.5 ml 2,4-D; 5 ml NAA and 1 ml BAP pH 5.8) containing 0.3 M sucrose. The cells were transferred into a 150 ml Erlenmeyer flask containing 15 ml of enzyme solution (per 100 ml: 0.3 g Onozuka R10 Cellulose, 0.1 g Macerozyme R10 and 3.43 g sucrose in K3 solution, filter sterilized) and incubated for 2 or 3 hrs with gentle agitation. The protoplast (pps) suspension was collected with a sterile pipette with cut-off tip and filtered through a 100 µm stainless steel sieve and divided into 6 ml aliquots. K3 solution was used to adjust the volume. The suspension was carefully overlaid with 1ml of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM Glucose). After centrifugation at 80 g (570 rpm) for 10 min, good pps floated at the interphase. The pps were collected with a sterile pipette with cut-off tip taking as little as possible of the lower phase aliquoted in 4ml portions and washed twice with 10 ml W5 solution (mix gently). After centrifugation at 80 g for 5 min, the pps were resuspended in a total volume of 11 ml of W5 buffer and stored at 4°C in the dark for 2 hrs. 1 ml of pps was diluted in 9 ml of W5 buffer to count in a 0.2 µl hematocytometer (Obs. pps*500000=tot. pps). The diluted pps were recovered by centrifugation and added to the rest of pps fraction. The pps were resuspended in MMM (0.5 mM Mannitol, 15 mM $MgCl_2$, 0.1%

MES) at a concentration of $2.5*10^6$ pps/ml. After isolation, the pps were healthy and ready for transformation.

## Protoplast transformation for transient expression

### *Solutions for protoplasts preparation and transformation*

For 1 liter K3 Macro: 25.3 g $KNO_3$, 2.5 g $NH_4NO_3$, 1.5 g $NaH_2PO_4*H_2O$, 9 g $CaCl_2*2H_2O$, 2.5 g $MgSO_4*7H_2O$, 1.34 g $(NH_4)_2SO_4$.

For 100 ml liter B5 Micro: 0.3 g $H_3BO_3$, 0.075 g KI, 1 g $MnSO_4*H_2O$, 0.2 g $ZnSO_4*7H_2O$, 2.5 g $CuSO_4*5H_2O$, 25 g $Na_2MoO_4*2H_2O$, 2.5 g $CoCl_2*6H_2O$.

For 100 ml K3 100x-Vit: 1 g Inositol, 10 mg Pyridoxine HCl, 100 mg Thiamine HCl, 10 mg Nicotinic acid.

For 100 ml of either 2,4-D or NAA, 20 mg were dissolved in 1 ml absolute EtOH and completed with dd $H_2O$ to 100 ml. For 100 ml BAP: 20 mg were dissolved in 1 ml KOH 1M and completed to 100 ml with dd $H_2O$. All solutions were filter sterilized.

The DNA (5 µl plasmid DNA, ~10 µg) was dispatched in sterile tubes and mixed with 300 µl of the pps suspension. 300 µl PEG solution (40% PEG4000, 0.4 M Mannitol, 0.1 Ca $(NO_3)_2$, pH 8.0, filter sterilized) was added and pipetted slowly (high viscosity). The solution was gently shaked and incubated for 5 min. 2 ml K3 solution were then added and after mixing the pps were incubated for 2 hrs at 26°C in the dark. To remove the PEG, 5 ml of W5 solution were added. The pps were pelleted at 70 g for 5 min and resuspended in 2 ml of K3. pps were incubated O.N. at 26°C in the dark. To check the state of the pps, 5 µl were placed on a glass slide and observed with appropriate optics.

## Confocal Microscopy

Images were collected with a Leica (Wetzlar, Germany) confocal laser scanning microscope using the Leica TCS 4D operating system. FITC or TRITC settings were used to detect GFP and FM4-64, respectively. Digital images were pseudocolored using Adobe Photoshop version 7.0, and green was attributed to GFP and red to FM-4-64. Images with transmitted light were also collected using the confocal microscope.

# Chapter 3. Evaluation of a yeast system for studying the function of plant Vacuolar Sorting Receptors (VSRs)

A specific interaction between the vacuolar sorting receptor $VSR_{PS-1}$ (previously named BP-80) and the petunia aleurain VSD fused to GFP (aleu-GFP) was demonstrated in the yeast *S.cerevisiae* (Humair et al., 2001). $VSR_{PS-1}$ expressed in the yeast mutant strain lacking the *VPS10* gene was able to redirect the aleu-GFP fusion protein to the yeast vacuole. These results showed that $VSR_{PS-1}$ could functionally replace its yeast counterpart Vps10p to mediate the transport of aleu-GFP to the vacuole. This sorting was specifically dependent on the interaction between $VSR_{PS-1}$ and the aleu-VSD since $VSR_{PS-1}$ was unable to transport to the yeast vacuole GFP alone (Sec-GFP) or GFP fused either to the yeast targeting signal of the carboxypeptidase Y (CPY-GFP) or to the VSD of another type of plant vacuole (GFP-Chi).

***GFP fusion proteins with different VSDs tested in plants***

By the same yeast complementation approach, we wanted to test whether the family of vacuolar sorting receptors identified in *Arabidopsis* (AtVSR1, 2, 2', 3, 4, 5 and 6) and showing a high homology to $VSR_{PS-1}$, was able to redirect to the yeast vacuole the GFP reporter fused to different VSDs. For this purpose, we cloned various plant VSDs independently fused to GFP, in the same yeast expression vector used to express the previously used GFP fusion proteins. These VSDs were the *Nicotiana alata* proteinase inhibitor (NAPI) ct-VSD, the *Bertholletia excelsis* (Brazil nut) 2S albumin (2Sa) ct-VSD, and the *Phaseolus vulgaris* (Common bean) phaseolin (Ph) ct-VSD. Table 3.1 shows the GFP fusion proteins used in this study. First, we checked whether these VSDs were sufficient for vacuolar targeting in plants. Therefore, we cloned each of these VSD-GFP fusions, into the same plant expression vector used for the positive controls (aleu-GFP6, Humair et al., 2001 and GFP5-Chi, Di Sansebastiano et al., 1998). *A. thaliana* protoplasts from cell suspension cultures were transformed with these constructions and after 24 hours of transient expression, we observed the fluorescence by confocal microscopy (The GFP-Ph construct was no tested because it was designed later and we had problems with the yeast assay).

**Table 3.1 GFP constructions with different VSDs tested in plants and/or yeast**

| Construct | | Source |
|---|---|---|
| Sec-GFP | s.p-**GFP** | Humair et al, 2001 |
| aleu-GFP | $s.p_{CPY}$-RTANFADENPIRQVVSDSFHELES-**GFP** | Humair et al, 2001 |
| GFP-Chi | s.p-**GFP**-DLLVDTM | Humair et al, 2001 |
| GFP-NAPI | s.p-**GFP**-EYASKVDEYVGEVENDLQKAKVAVS | This study |
| GFP-2Sa | s.p-**GFP**-PMGGSIAGF | This study |
| GFP-Ph | s.p-**GFP**-AFVY | This study |

| | |
|---|---|
| s.p: *A. thaliana* chitinase B signal peptide | NAPI: *N. alata* proteinase inhibitor ct-VSD |
| 2Sa: *B. excelsis* 2S albumin ct-VSD | Ph: *P. vulgaris* phaseolin ct-VSD |
| s.pCPY: CPY signal peptide | GFP: the green fluorescent protein |
| aleu: Petunia aleurain ss-VSD | Chi: Tobacco chitinase A ct-VSD |

In many cells, we found GFP6-NAPI and GFP6-2Sa fluorescence (Figure 3.1, green) accumulated in the large central vacuole that occupies almost of the cell volume as in the positive controls. These results showed that the ct-VSDs of *N. alata* NAPI and the *B. excelsis 2Sa* are sufficient for vacuolar sorting in plants. Thus, we concluded that they could be used as ligands to study plant VSD-receptor interactions in yeast as well as the previously characterized aleurain and chitinase VSDs.

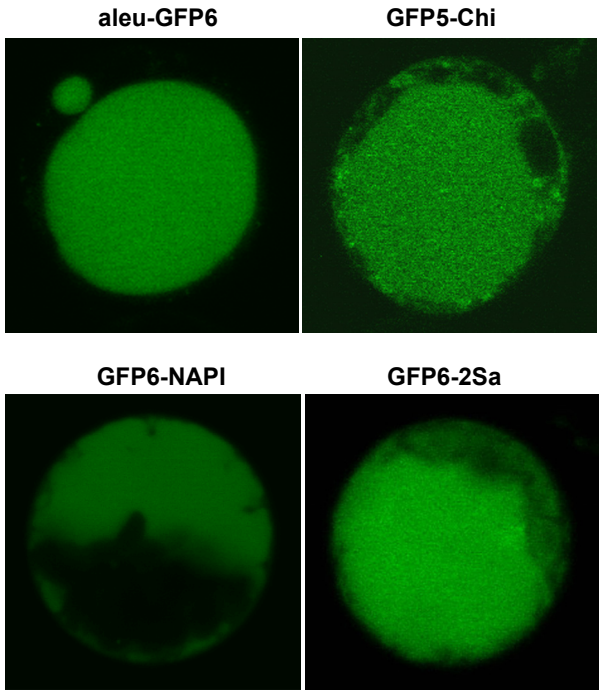



**Figure 3.1. Demonstration of the vacuolar sorting properties of the *N. alata* proteinase inhibitor and *B. excelsis* 2S albumin propeptides in *A. thaliana* cell suspension cultures protoplasts.**

Live protoplasts transiently expressing aleu-GFP6, GFP5-Chi, GFP6-NAPI and GFP6-2Sa were visualized using a confocal microscope 24 hr after transformation. One cell from each transformation is shown with the fluorescein isothiocyanate fluorescence (FITC) filter. These images use the pseudocolor green for GFP.

### *Fluorimetric assay*

For the comparison of binding affinity and sorting efficiency study in yeast, we cloned most AtVSRs (1 to 3, 5 and 6) in the same yeast expression vector used for $VSR_{PS-1}$. The AtVSR2 and 2' are so highly homologous that we did not clone the later receptor. We first tested whether the AtVSRs were able to redirect to the yeast vacuole the aleu-VSD or Chi-VSD. Each GFP fusion protein was expressed alone in the wild type and *⊿vps10* mutant strain or together with each putative *Arabidopsis* VSR in the *Δvps10* mutant strain. The GFP retained in the yeast vacuole by AtVSRs was then detected by fluorescence microscopy. Using the same growth conditions previously described (Humair et al., 2001), we found that none of the five tested AtVSRs were able to significantly retain either GFP construct intracellularly (data no shown). More surprisingly, we were unable to demonstrate quantitatively the function of $VSR_{PS-}$

$_1$ as a sorting receptor in yeast as described before (Humair et al., 2001). As previously shown, the aleu-GFP accumulated in the vacuole in a Vps10p-dependent manner (Humair et al., 2001). Vps10p has been shown to interact with and transport to the vacuole misfolded proteins and many foreign proteins (Hong et al., 1996). Therefore, Vps10p probably mediates the transport of aleu-GFP to the vacuole by interacting with a yet unfolded structures of GFP, which was shown to fold slowly in the secretory pathway (Wooding and Pelham, 1998). In contrast, no significant accumulation of aleu-GFP in the vacuole was observed in *Δvps10* cells expressing $VSR_{PS-1}$ (data no shown). Considering that I did not have enough prior experience in handling with yeast together with the high variability of the vacuolar fluorescence intensity observed from these experiments led me to make unclear conclusions about the efficiency of the assay.

Because confocal observations were too unreliable to draw objective conclusions in this type of assay, we set up a fluorometric method to quantify the intracellularly retained GFP in crude yeast extracts. We determined the percentage of aleu-GFP retained by $VSR_{PS-1}$ and three other AtVSRs (1, 2 and 3) in yeast cells. aleu-GFP was expressed alone in the wild type and *Δvps10* mutant strains or together with each of the three AtVSRs in the *Δvps10* mutant strain. Cells were grown in selective medium containing 2% galactose for 24 hrs. Results from one single experiment are shown in table 3.2.

**Table 3.2 Intracellular retention of aleu-GFP (single experiment)**

| Transformants | RFU | [protein] (mg/ml) | RFU/[protein] | Intracellular fluorescence | | Receptor-mediated retention (%) | |
|---|---|---|---|---|---|---|---|
| | | | | RFU/B | % | | |
| WT + aleu-GFP (positive control) | 57.74 | 3.06 | 18.87 | 9.78 | 100% | 7.17 | 100% |
| M + aleu-GFP (negative control) | 31.84 | 2.85 | 11.17 | 2.61 | 27% | 0 | 0% |
| M + aleu-GFP + $VSR_{PS-1}$ | 34.11 | 2.82 | 12.09 | 3.46 | 35.38% | 0.85 | 11.85% |
| M + aleu-GFP + AtVSR1 | 36.59 | 2.89 | 12.66 | 4.03 | 41.21% | 1.42 | 19.80% |
| M + aleu-GFP + AtVSR2 | 37.36 | 3.28 | 11.39 | 2.76 | 28.22% | 0.15 | 2.1% |
| M + aleu-GFP + AtVSR3 | 33.53 | 2.85 | 11.76 | 3.13 | 32.0% | 0.52 | 7.25% |
| $B_{WT}$ | 28.56 | 3.14 | 9.09 | - | - | - | - |
| $B_M$ | 24.92 | 2.91 | 8.56 | - | - | - | - |
| $B_{VSR}$ | 24.78 | 2.87 | 8.63 | - | - | - | - |

WT: SEY6210 strain

M: EMY3 (*Δvps10*) strain

RFU: Relative Fluorescence Unit

[protein]: mg protein/ml

$B_{WT}$: Background fluorescence for WT strain transformed with empty plasmid pYWG1 (no aleu-GFP)

$B_M$: Background fluorescence for M strain transformed with empty plasmid pYWG1 (no aleu-GFP)

$B_{VSR}$: Background fluorescence for VSR expressing strains: M strain transformed with empty plasmid pYWG1 (no aleu-GFP) and empty plasmid pGAL-BgIII (no VSR)

Compared to the positive control, the percentage of retention of aleu-GFP by $VSR_{PS-1}$ was low and only one of the three tested AtVSRs gave a higher value. These results clearly indicated that none of the VSRs are able to retain the aleu-GFP intracellularly as efficiently the yeast receptor. In order to exclude unidentified genetic differences in the wild type and mutant strains that could affect the sorting efficiency of the receptors, we tested the intracellular retention mediated by Vps10p in the same mutant strain, expressing Vps10p from a plasmid. We found that Vps10p was able to retain the aleu-GFP with the same high efficiency (95%) as in the wild type strain confirming a clear difference in the sorting efficiency between the plant VSRs and the yeast receptor Vps10p.

In repeats of these experiments, a high variability of the percentage of retention of aleu-GFP by the VSRs was observed. The fluorimetric technique presents evident limitations. As shown in table 3.2, we are at the limit of detection, since the positive control has only a 2X signal compared to the negative control (57, 74 versus 31, 84). Moreover, we could not detect

with any precision the GFP secreted into the medium, which may vary depending on the induction efficiency.

Despite the low percentages of intracellular retention of the aleu-GFP by the plant VSRs, they were regularly higher than the negative controls, but we needed to determine whether this retention was significant. For this purpose, we subjected data from six independent experiments of $VSR_{PS-1}$-aleu-GFP sorting efficiency to an Oneway Anova analysis. Although the effect of $VSR_{PS-1}$ on the aleu-GFP accumulation was significant ($P = 0.035 < 0.05$), the results of $VSR_{PS1}$ (PS1) was not only very different from that of the wild type strain (Wt), but it also overlapped with the results of the mutant strain (M) (Figure 3.2). This indicates that the activity of $VSR_{PS-1}$ is low compared to the yeast receptor Vps10p and cannot be given a reliable quantitative value.

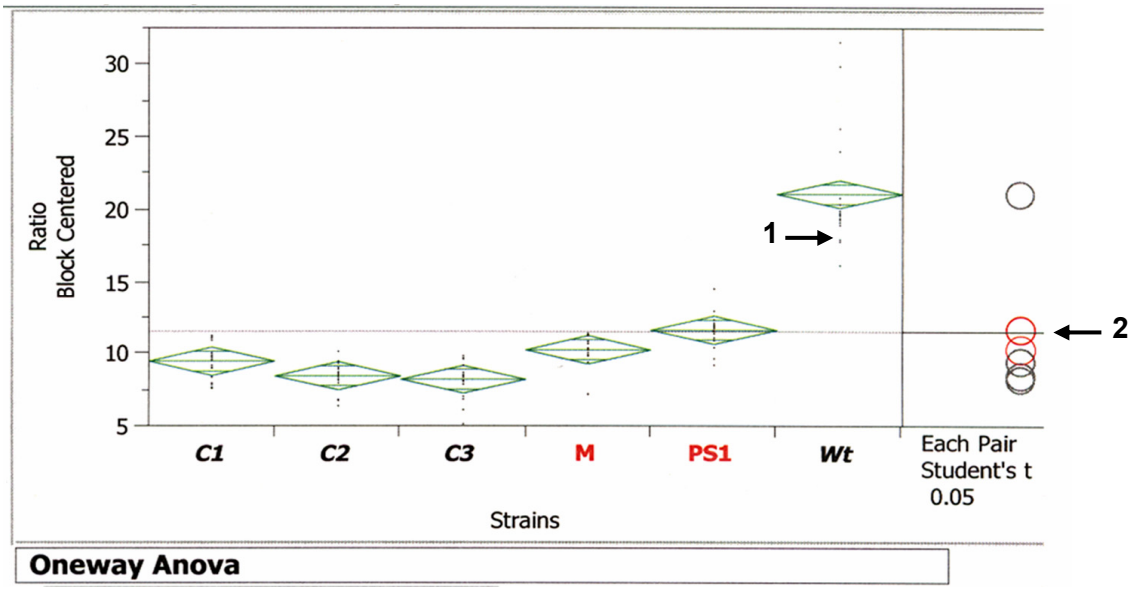



**Figure 3.2. $VSR_{PS-1}$ does not retain significant aleu-GFP in yeast cells.**

The $VSR_{PS-1}$ data from six different experiments were subjected to a Oneway- Anova analysis. Wt and M correspond to the positive (WT strain + aleu-GFP) and negative (M strain + aleu-GFP) controls, respectively (see page 82). PS1 corresponds to the $VSR_{PS-1}$ expressing strain (M strain + aleu-GFP + $VSR_{PS-1}$). C1, C2 and C3 correspond to the background fluorescence for the wild type ($C_{WT}$), mutant ($C_M$) and for $VSR_{PS-1}$ expressing strain ($C_{VSR}$) respectively. (1)Outliers (2) Grand median.

***Optimization of growth and induction conditions***

These above results were consistent with our previous observations by fluorescence microscopy. We therefore decided to optimize the growth and induction conditions, to obtain more significant fluorescence vacuolar retention. We tested many parameters such as the use of freshly transformed yeast, the time between transformation and inoculation for induction, the age of the competent cells used for the transformations (protocol (III), page 72), the temperature range, various providers of growth medium (see page 70) and the type of induction procedure (see protocols, page 78) (Table 3.3).

| **Table 3.3 Parameters that did not influence the induction efficiency** |
|---|
| Freshly transformed yeast versus strain maintained on plates |
| Time between transformation and inoculation for induction (between 3 and 5 days) |
| Age of the competent cells (fresh versus frozen stocks (up to 4 months at -70 °C)) |
| Temperature range (28-32°C) |
| Various providers of growth medium |

None of the parameters shown in table 3.3 influenced the vacuolar fluorescence. The only parameter that influenced the induction efficiency was the induction procedure. The optimal efficiency of induction was obtained from cells in exponential phase of cell growth rather than late exponential or stationary phase as previously used. We found the highest percentage of fluorescence vacuolar signal in wild type cells, where almost 100% of the cells consistently display a vacuolar GFP signal and the lowest fluorescence background in the *Δvps10* mutant strain (see Figure 3.3) Furthermore, less variability on the intensity of the fluorescence signal and less dead cells were observed. Under these new growth conditions, no significant accumulation of aleu-GFP in the vacuole was observed in the *Δvps10* mutant cells expressing $VSR_{PS-1}$ compared to Vps10p (Figure 3.3) and in addition, none of the five tested AtVSRs (AtVSRs1, 2, 3, 5 and 6) significantly retained intracellularly either aleu-GFP and Chi-GFP (data no shown).

These results suggest that these new conditions were more physiologically relevant for studying protein trafficking compared to those previous used (Humair et al., 2001), where the fluorescence detection was performed on cells in the late stationary phase ($OD_{600}$ between 5 to 10). However, the retention efficiency of the VSRs remained non significant.

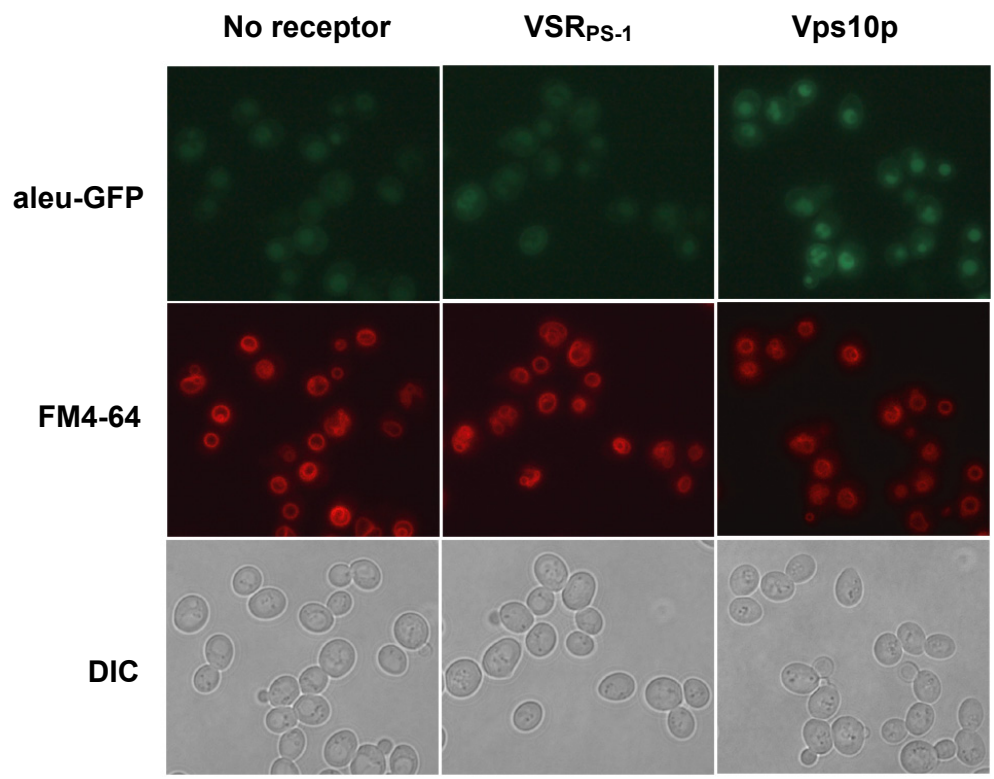



**Figure 3.3. $VSR_{PS-1}$ does not cause significant accumulation of aleu-GFP in the vacuole.** The Δ*vps10* mutant containing the aleu-GFP construct was transformed with *VPS10* or $VSR_{PS-1}$ under the control of a GAL promoter. Cells were visualized by fluorescence microscopy during the exponential growth phase, after 24 hours of induction in galactose medium at 30°C. Pictures of GFP fluorescence, FM4-64 staining of vacuolar membranes and intact cells by differential interference contrast (DIC) are shown.

In parallel, we also checked the *Δvps10* mutant strain. By PCR, we confirmed that the *VPS10* gene was disrupted by the insertion of the *HIS3* gene (Marcusson et al., 1994). We found that this strain has reduced growth phenotype and displays the typical vacuolar sorting defects. Additionally, a large amount of secreted aleu-GFP was detected in the *Δvps10* mutant strain using the GFP blot assay (Figure 3.4) (see the principle of the assay below), confirming the absence of the Vps10p sorting activity. We showed that re-introducing a functional Vps10p restores a normal growth phenotype and the transport of aleu-GFP to the vacuole (Figure 3.3). This was convincing evidence that the trafficking factors associated to the Vps10p machinery

are present and therefore confirmed that the strain was suitable for testing $VSR_{PS-1}$ sorting activity. It is important to mention that after having some problems repeating the positive test as previously described using the batch we had in the laboratory, we obtained two more independent *Δvps10* strain batches. All the batches come from Prof. Scott D. Emr.

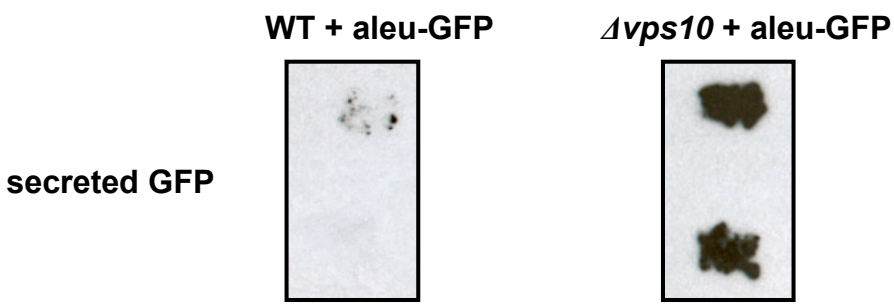



**Figure 3.4. The mutant strain efficiently secretes aleu-GFP.**
The aleu-GFP fusion protein was expressed in the wild type and *Δvps10* mutant strains. Colonies from the transformed plates were directly streaked on galactose induction medium plates and coverered with a nitrocellulose filter. After 24 hours at 30°C, the filters were removed and the secreted GFP was detected by immunostaining.

***Plate assay for GFP secretion***

Because it was difficult to detect the sorting activity of $VSR_{PS-1}$ by the amount of intracellularly retained GFP, we decided to test a different approach to investigate the merits of this *in vivo* yeast approach for studying plant vacuolar sorting interactions. We continued our investigation with the optimized growth conditions since they are more physiologically relevant for studying protein trafficking and the above tested parameters did not gave satisfying results. For this purpose, we set up an alternative approach to demonstrate a functional interaction between the plant VSRs and our different VSD-GFP constructs. In this approach, we took advantage of the fact that most soluble yeast vacuolar proteins are secreted by default in the absence of functional Vps10p. Thus, instead of detecting the GFP that is retained intracellularly by plant VSRs, we estimated the amount of secreted GFP, detecting its binding to a nitrocellulose filter in a colony blot assay (Figure 3.5). As expected, a large amount of secreted Sec-GFP and aleu-GFP was detected in the absence of Vps10p. The expression of $VSR_{PS-1}$ in the *Δvps10* mutant did not lead to a detectable reduction of secreted aleu-GFP compared to cells expressing Sec-GFP or to the control expressing Vps10p. As previously reported, $VSR_{PS-1}$ did not prevent the secretion of GFP-Chi either. We also showed that expression of neither of

the five tested *A. thaliana* VSRs (AtVSR 1, 2, 3, 5 and 6) significantly prevented the secretion of either aleu-GFP or GFP-Chi (Figure 3.5).

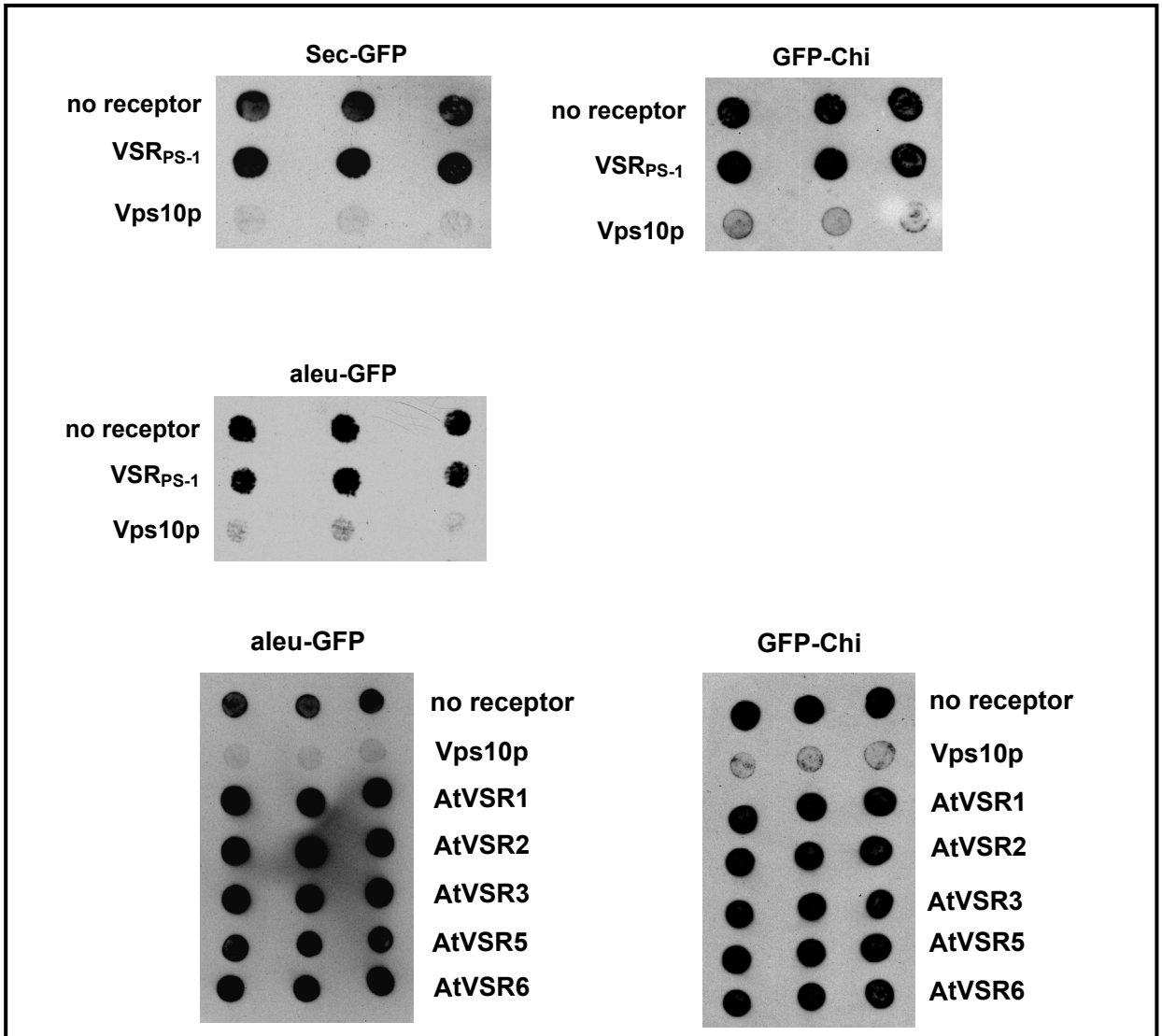



**Figure 3.5. $VSR_{PS-1}$ expression does not lead to a detectable reduction of aleu-GFP secretion.**

Sec-GFP, aleu-GFP or GFP-Chi fusion proteins were co-expressed with a plant VSR or Vps10p in the *Δvps10* mutant using a GAL promoter, as indicated. Equal amounts of each transformant were spotted in triplicates on inducing galactose medium plates and covered with a nitrocellulose filter. After 24 hours at 30°C, the filters were lifted from the plates and the secreted GFP hybrids were detected by immunostaining.

Unfortunately, we cannot deduce from these pictures the ratio of secreted/retained aleu-GFP when $VSR_{PS-1}$ is expressed in yeast. It was previously estimated that $VSR_{PS-1}$ had the potential to retain approximately 30% of aleu-GFP when compared to the wild type strain (Figure 5C of Humair et al., 2001). Therefore, the proportion of secreted aleu-GFP would be expected to vary between 70% and 100%. If we consider that the colony blot assay cannot detect a 10 to 30% diminution of aleu-GFP secretion, these new results are not contradictory with the previous quantitative data (Humair et al., 2001). However, they are consistent with our fluorescence data and confirm that yeast is not a good model system for *in vivo* studies of plant VSRs.

***Comparison of the cytosolic tail domains of Vps10p and $VSR_{PS-1}$***

The failure to observe massive retention of GFP coupled to any plant VSD raises the possibility that the expressed VSRs were not properly sorted in the yeast cells, thus preventing an appropriate interaction with ligands. Vsp10p mediates the transport of soluble vacuolar proteins by cycling between the *trans*-Golgi network (TGN) and the prevacuolar compartment (PVC) (Marcusson et al., 1994; Cereghino et al., 1995; Cooper and Stevens, 1996). The cytosolic tail domain (CT) of Vps10p plays a critical role in this transport cycle since it harbors multiple signals that are necessary for the sorting and the stability of the receptor (Cooper and Stevens, 1996) (Figure 3.6). These signals are recognized by cytosolic components of the vesicular machinery ensuring that the receptor protein follows its proper intracellular itinerary.

Vps10p was found to be associated with clathrin proteins by native coimmunoprecipitation (Deloche et al., 2001), which correlates with previous results showing a rapid inactivation of temperature-sensitive clathrin to result in secretion of a significant amount of CPY (Seeger and Payne, 1992b). In addition, the Golgi form of CPY (p2CPY) was detected in purified clathrin coated vesicles (CCVs), confirming the role of clathrin in the anterograde transport of vacuolar protein from the TGN to the PVC (Pishvaee et al., 2000). Clathrin interacts with adaptor proteins (APs), which are essential for the recruitment of transmembrane proteins into CCVs. The yeast AP-1 appears to function in clathrin-dependent trafficking of Vps10p, as it is the only yeast AP physically associated with clathrin and copurified with CCVs containing Vps10p (Yeung et al., 1999; Deloche et al., 2001). Furthermore, TGN protein sorting defects in temperature-sensitive clathrin mutants were enhanced by genetic disruption of any of the four subunits of AP1, but not of any subunit of the other APs.

The Vps10p CT can also bind the VHS domain of the mammalian GGAs (Golgi-localizing γ-adaptin ear homology domain ADP-ribosylation factor binding protein), other (monomeric) adaptors implicated in TGN-to-PVC transport (Dennes et al., 2002). This interaction appears to be mediated by the acid cluster-dileucine signal, AC-LL which is also found in the Vps10p CT

(Figure 3.6). However, a direct recognition by the yeast GGAs proteins has not yet been reported.

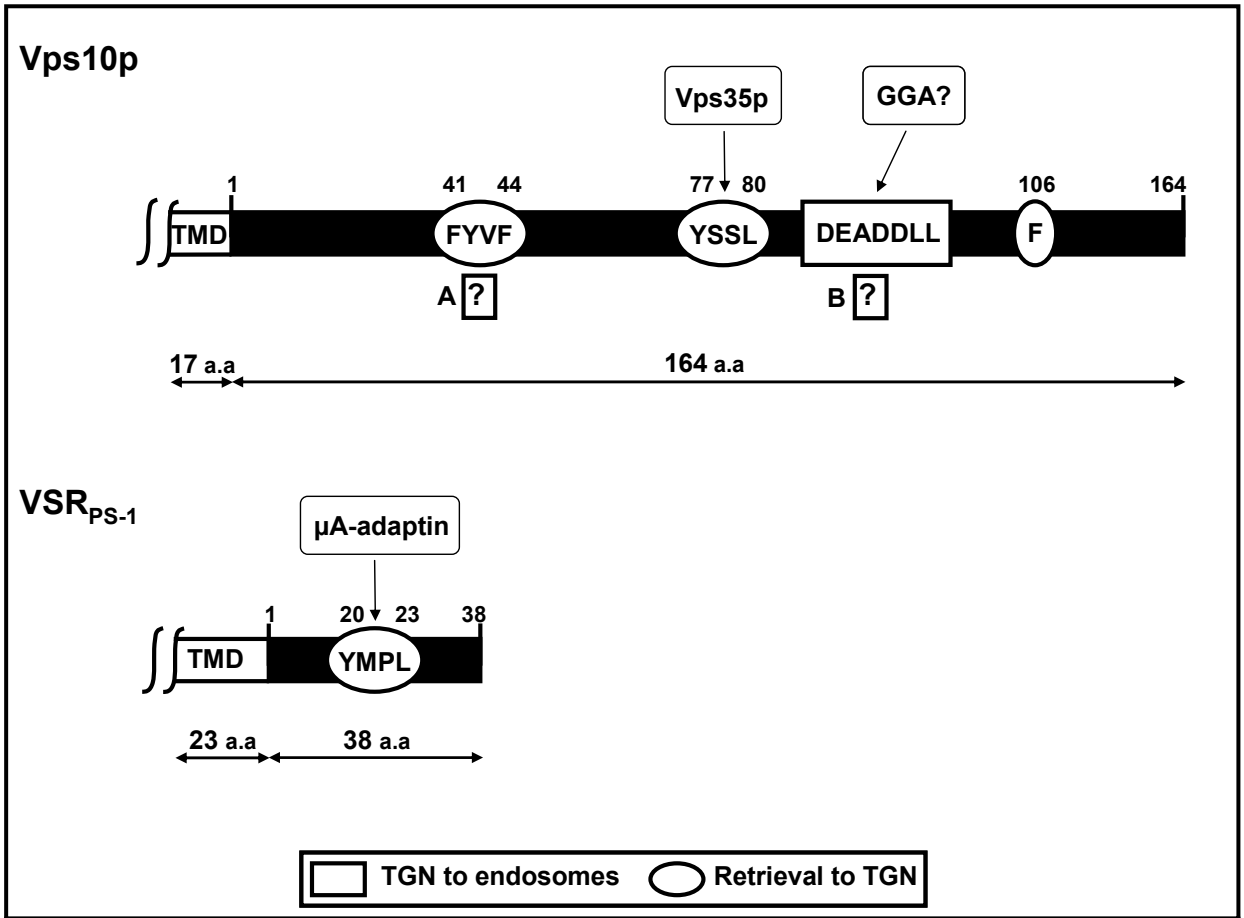



**Figure 3.6. Transmembrane and cytosolic tail domains from the yeast (Vps10p) and the plant (VSR$_{PS-1}$) vacuolar sorting receptors.**

The two receptors are type I integral membrane proteins with a large luminal domain (not shown), a single transmembrane domain, and a cytosolic tail domain (CT). Motifs identified as critical for specific sorting events are indicated. Arrows indicate the binding sites for the tail-binding proteins discussed in the text. Question mark (A) means that the role of the FYVF is not yet clear. Question mark (B) means that even though the CT of Vps10p can bind the VSH domain of mammalian GGAs, which are important participants in TGN-to-PVC transport, a direct recognition by the yeast GGAs has not yet been reported. The schematic representation of Vps10p is a modified version from Dell'Angelica and Payne (2001).

The interaction of the Vps10p CT with the trafficking machinery is mainly required for retrograde transport. Using pulse-chase experiment with radioactively labeled amino acids, it was demonstrated that Vps10p mutants lacking the CT (Vps10p$\Delta C_t$p) or with mutated key

residues travel to the PVC before being rapidly degraded in the vacuole (Cooper and Stevens, 1996).

The tyrosine motif (YSSL) is one of the required signals for Vps10p recycling and therefore for its efficient function. Previous reports showed that a mutant form of Vps10p in which the tyrosine residue is substituted by an alanine leads to a rapid degradation of the receptor in the vacuole (Cereghino et al., 1995; Cooper and Stevens, 1996). Vps35p is a subunit of the retromer complex, which is required for the retrieval of Golgi proteins from the PVC to the TGN, and it directly binds to Vps10p (Nothwehr et al., 2000). A single phenylalanine (F) residue located in the last region of the CT of Vps10p is another signal required for the recycling of the receptor. Its substitution by an alanine (A) results in secretion of 20% of CPY (Cooper and Stevens, 1996). Finally a cluster of aromatic amino acids (FYVF) has been implicated in targeting (Cereghino et al., 1995) although this motif is believed to play only a structural role (Cooper and Stevens, 1996).

The CT of $VSR_{PS-1}$ also appears important in recycling to the TGN and for the stability of the receptor since a truncated form lacking the CT ($VSR_{PS-1}\Delta CT$) was substantially less stable than the intact receptor in tobacco protoplasts (Jiang and Rogers, 1998). The TMD of $VSR_{PS-1}$ alone was reported to be sufficient for targeting to the lytic PVC in tobacco cells (Jiang and Rogers, 1998). In another study, though, it lead to the accumulation of a membrane-bound GFP in the GA (Brandizzi et al., 2002). No evidence for $VSR_{PS-1}$ recycling has been provided in plants so far.

The CTs of Vps10p and $VSR_{PS-1}$ display no sequence homology, except for a shared tyrosine motif, and they markedly differ in length (164 residues versus 38) (Figure 3.6). As already mentioned, the tyrosine motif (YMLP) of $VSR_{PS-1}$ may facilitate the recruitment of the receptor in CCVs since it was shown to interact *in vitro* with mammalian adaptins (Kirsch et al., 1994; Sanderfoot et al., 1998; Ahmed et al., 2000), and was crucial for the *in vitro* binding of $VSR_{PS-1}$ to the medium chain (μA-adaptin) of the *A. thaliana* AP-1 complex, which is associated with clathrin proteins (Happel et al., 2004). Although these data implicate the tyrosine motif, the direction of transport in which it participates was not elucidated by these studies. In contrast to yeast and mammals, no equivalent for the GGA family has been found in plants to date.

Altogether, these data indicate that the CT of $VSR_{PS-1}$ lacks most sorting signals to selectively interact with the yeast trafficking machinery and that $VSR_{PS-1}$ is unable to cycle efficiently between the TGN and the PVC. This shows that there is clear divergence on the vacuolar trafficking machinery among plants and yeast.

### *Localization and stability of $VSR_{PS-1}$ in yeast*

The available monoclonal antibody against $VSR_{PS-1}$ is not convenient for Western blot analysis, as it only recognizes non-reduced $VSR_{PS-1}$. We expressed a hemagglutinin (HA) epitope tagged-$VSR_{PS-1}$ ($VSR_{PS-1}$-HA) under the control of the GAL promoter in the *Δvps10* strain. Cells were grown to early exponential phase at 30°C and after 24 hours induction in the presence of galactose, cells were treated with cycloheximide (CHX) to block protein synthesis. An equivalent amount of cells was harvested after 0, 15, 30 and 60 minutes of CHX treatment and subjected to Western blot analysis. In contrast to Vps10p-HA, which was not degraded after several hours of CHX treatment, overexpressed $VSR_{PS-1}$-HA was rapidly degraded (half-life <15min) (Figure 3.7A). A similar rate of degradation was also observed for an untagged version of $VSR_{PS-1}$ using the specific monoclonal antibodies against VSRs (Figure 3.7B), thus ruling out a negative effect of the HA tag on $VSR_{PS-1}$ stability.

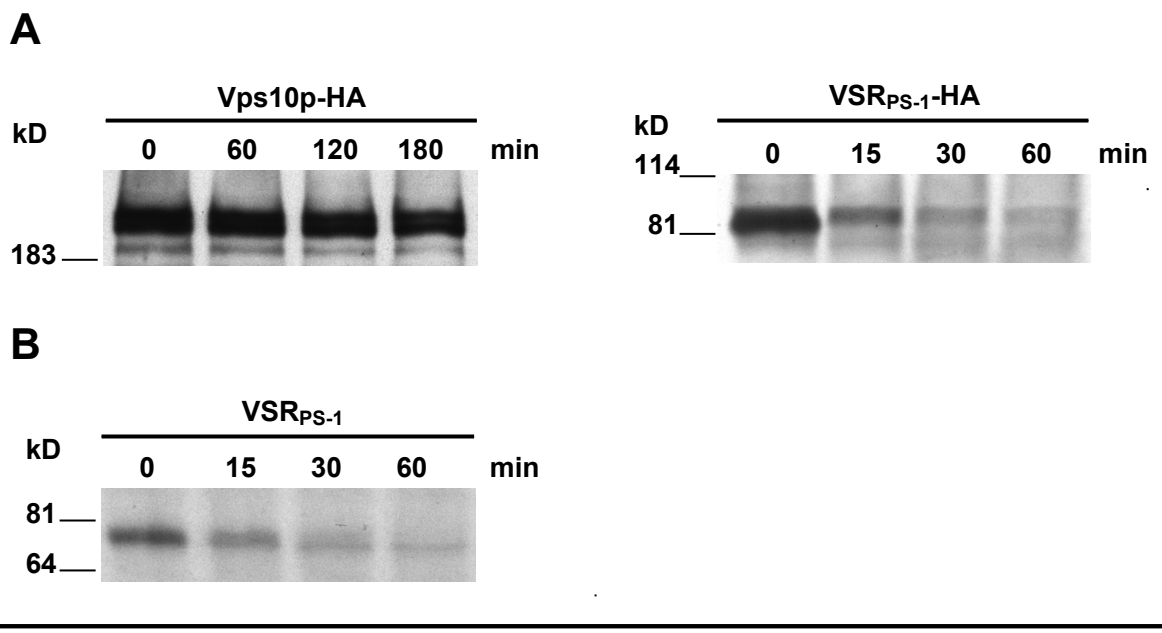



**Figure 3.7. $VSR_{PS-1}$ is rapidly degraded in yeast cells.**

**(A)** The HA-tagged $VSR_{PS-1}$ and HA-tagged Vps10p were expressed in the *Δvps10* mutant. After 24 hours of induction, protein synthesis was blocked by the addition of CHX. At the indicated times, cells were harvested, whole cell extracts were prepared and subjected to immunoblot analysis. Proteins were detected by monoclonal HA antibodies.

**B)** $VSR_{PS-1}$ was expressed in the *Δvps10* mutant. Proteins were detected by anti-VSR monoclonal antibodies (17F9; \Cao et al., 2000) that recognize the native form of the luminal domain of $VSR_{PS-1}$.

It is noteworthy that although we do no have information about the possible secondary effects (if there are any) of the CHX on yeast cells, a similar degradation pattern of $VSR_{PS-1}$-HA in Δ*vps10* cells was observed when glucose was added as repressor of the GAL promoter. We thus assumed that the degradation behavior of $VSR_{PS-1}$ could be monitored using both conditions and we used for convenience the CHX.

Misfolded proteins that leave the ER or mutated proteins that are not recycled from the PVC to the TGN are usually delivered to the vacuole where they are rapidly degraded. This type of degradation is prevented either in vacuolar protease-deficient cells or in mutants blocking protein transport to the vacuole. Figure 3.8 shows a schematic representation of all the mutants used in this work.

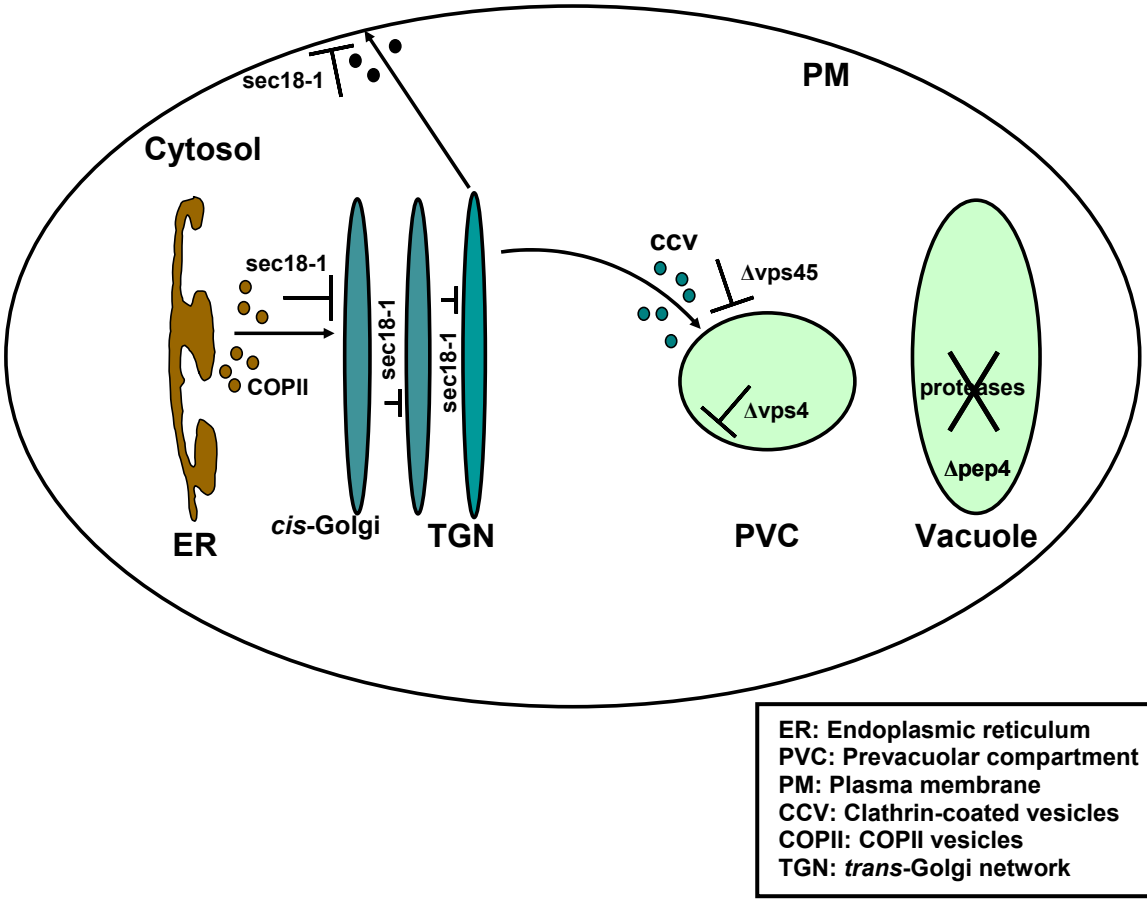



**Figure 3.8. Schematic representation of the different yeast mutants blocking the biosynthetic transport to the vacuole, and in vacuolar protease deficient cells.**
The *sec18-1*$^{ts}$ (yeast NSF) mutant prevents the fusion of ER-derived vesicles with the *cis*-Golgi, the vesicular fusion throughout the GA compartments, and the fusion of Golgi-derived vesicles with the PM; the *Δvps45* mutant blocks the transport from the GA to the PVC, and the *Δvps4* mutant blocks the transport out of the PVC (e.g. from the PVC to the TGN and from the PVC to the vacuole) and in the *Δpep4* mutant the activities of major vacuolar proteases are reduced.

We found that the degradation of $VSR_{PS-1}$-HA was completely blocked in the *sec18-1* (yeast NSF) mutant (Figure 3.9 A), which prevents the fusion of ER-derived vesicles with the *cis*-Golgi, indicating that $VSR_{PS-1}$ enters the secretory pathway and is able to reach the *cis*-Golgi. By contrast, degradation of $VSR_{PS-1}$-HA still occurred in the *Δvps45* and *Δvps4* mutants, which block transport from the Golgi to the PVC and the transport out from the PVC, respectively (Figure 3.9 A) meaning that the receptor was unable to reach the PVC. Consistently, the degradation of $VSR_{PS-1}$-HA was not delay in *Δpep4* cells, a mutant that reduces the activities of the major vacuolar proteases (Jones et al., 1982). As a control, we showed that the unstable Vps10p form lacking the CT ($Vps10pC_tp$-HA) is not degraded in the same *Δpep4* strain (Figure 3.9 B). Although, *pep4* gene (proteinase A) is required for the processing of five vacuolar hydrolases (the known until now) (Jones et al., 1982), we believed that at least we should have observed an inhibition of the degradation of $VSR_{PS-1}$-HA in the *Δpep4* mutant. All these findings mean that the receptor's degradation and its inability to reach the PVC are linked.

Because we assumed that the degradation of $VSR_{PS-1}$-HA is not *Δvps10* strain dependent since it was also obtained in other strains with different genetic backgrounds (wild type for Vps10p), we did not compare the stability of $VSR_{PS-1}$-HA in the SEY6210 strain.

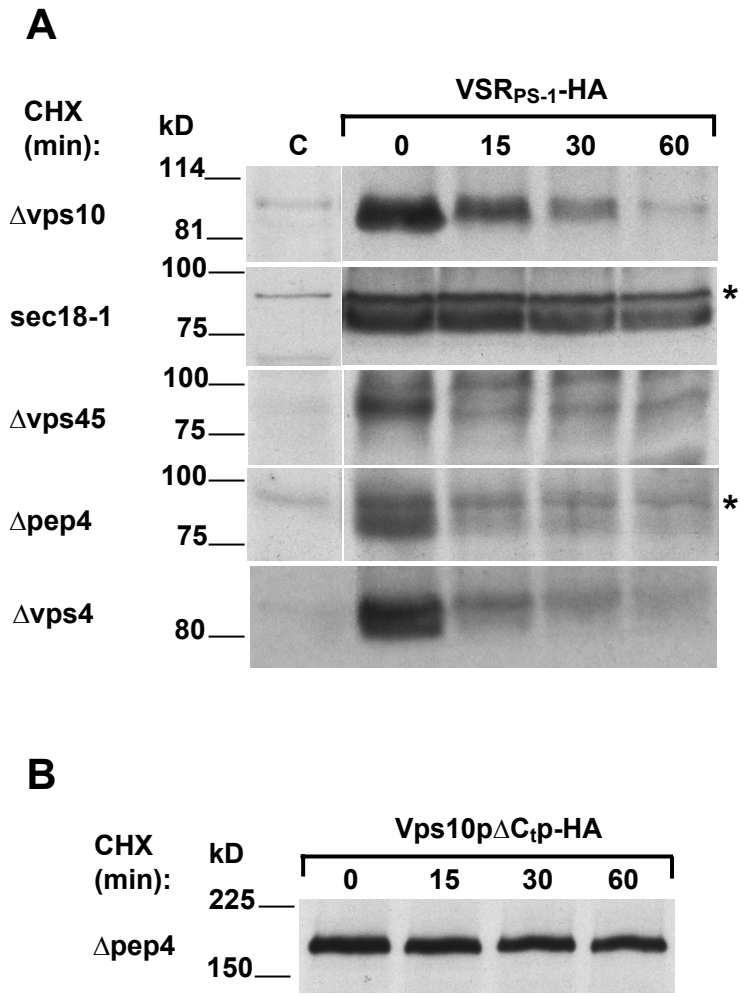



**Figure 3.9. VSR$_{PS-1}$ does not travel through the PVC.**

**(A)** The degradation of VSR$_{PS-1}$-HA is not prevented in mutants blocking the biosynthetic transport to the vacuole, the transport out of the PVC, and in vacuolar protease deficient cells. VSR$_{PS-1}$-HA was expressed in the *Δvps10*, *sec18-1*$^{ts}$, *Δvsp45*, *Δvps4* and *Δpep4* strains. Protein expression was induced at 30°C (or at 37°C for the *sec18-1* mutant); cells were then treated with CHX and analyzed as described in the experimental procedures. C is the control of mutant cells transformed with the vector alone; the asterisks indicates the presence of nonspecific bands.

**(B)** Vps10pΔCtp-HA was expressed in the *Δpep4* strain.

We then carried out a subcellular fractionation to further investigate the localization of VSR$_{PS-1}$-HA in yeast. Osmotically lysed yeast cells were subjected to sequential fractionation by centrifugation to yield a 13,000 X g pellet (P13), a 100,000 X g pellet (P100), and the supernatant (S100). Figure 3.10 A shows a schematic representation of the procedure used.

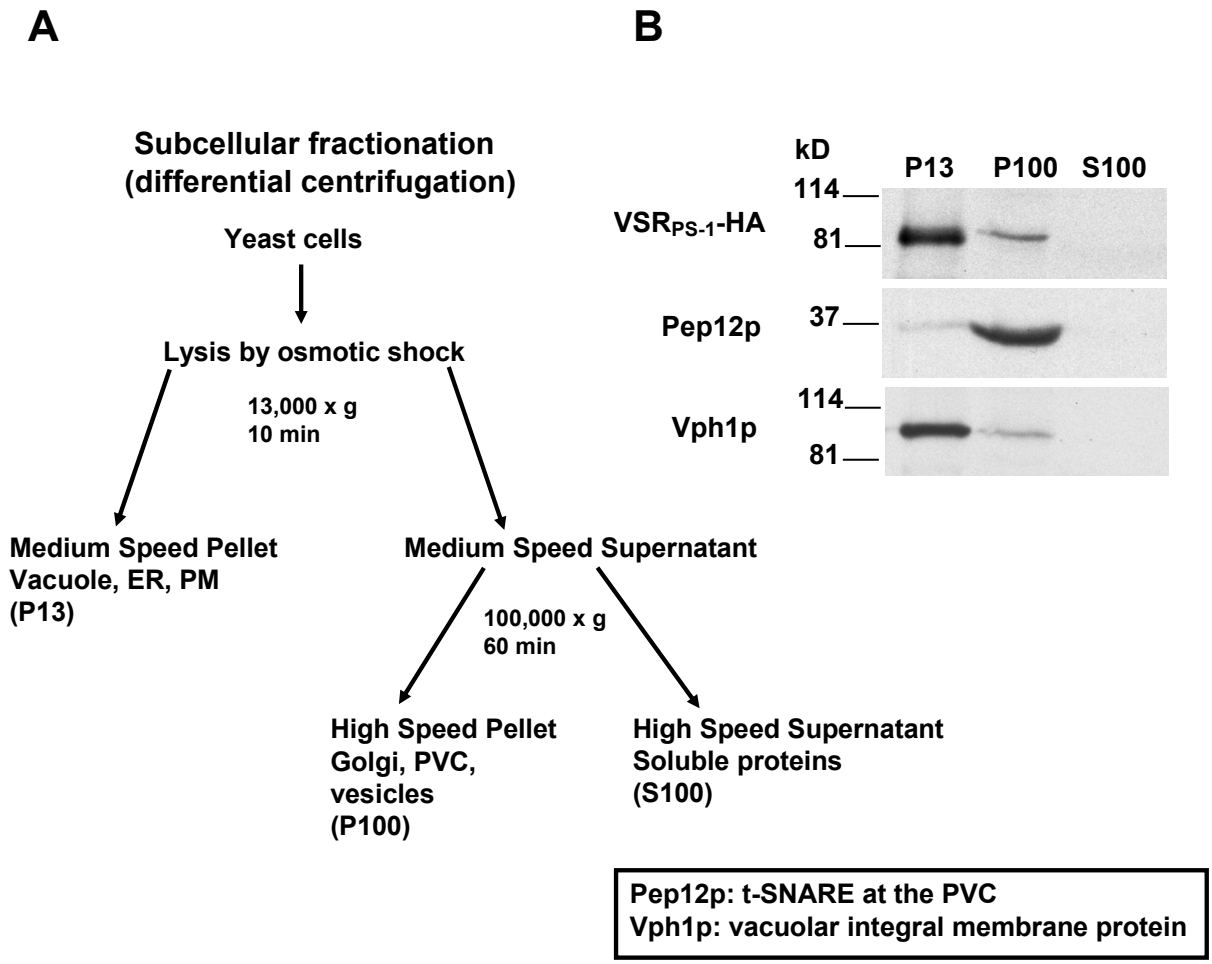



**Figure 3.10. $VSR_{PS-1}$ is not associated with Golgi and prevacuolar compartments.**

**(A)** Schematic representation of the sequential centrifugations perfomed to localize $VSR_{PS-1}$-HA. Osmotically lysed cells were subjected to sequential fractionation by centrifugation to yield a low speed pellet (P13), a high speed pellet (P100), and the supernatant (S100).

**(B)** *⊿vps10* spheroplasts expressing $VSR_{PS-1}$-HA were gently lysed by osmotic shock. Subcellular fractionation was then perfomed by differential centrifugation as described above. The amount of $VSR_{PS-1}$-HA, Pep12p (a PVC marker) and Vph1p (a vacuolar marker) in each of the fractions was determined by immunobloting.

Previous studies showed that Golgi and endosomal proteins such as Vps10p predominantly sediment in the high speed pellet (P100) (Marcusson et al., 1994; Becherer et al., 1996). As shown in figure 3.10 B, we found that most $VSR_{PS-1}$-HA did not sediment with the PVC Pep12p marker (P100) but instead accumulated in larger compartments (P13) including ER, vacuole, and the PM. Interestingly, we also detected some signal for $VSR_{PS-1}$-HA in the P100 fraction containing the Golgi and PVC in spite of the very short half-life of the protein. Unfortunately, we cannot provide a more precise localization of Vps10p. These findings demonstrated that $VSR_{PS-}$

$_1$ mainly does not accumulate in Golgi/PVC compartments suggesting that it is not properly sorted in yeast. It is possible that the small amount of $VSR_{PS-1}$ detected in the P100 fraction properly folded and reached the PVC. This pool of $VSR_{PS-1}$ may have been detected in the previous immunolocalization experiments (Humair et al., 2001). There, wild type cells showed a partial colocalization of $VSR_{PS-1}$ with Vps10p (Figure 6, Humair et al, 2001).
It is noteworthy that while figure 3.10 B suggests that $VSR_{PS-1}$-HA might go to the vacuole, the figure 3.9 A indicates that $VSR_{PS-1}$ does not reach the vacuole to be degraded.

Because the localization of certain proteins does not only depend on their CTs but also on the length and the amino acid composition of their transmembrane domains (TMDs) (Sato et al., 1996; Rayner and Pelham, 1997), the mislocalization of $VSR_{PS-1}$ could also be due either to the longer TMD (23 versus 17 residues for Vps10p) (see Figure 3.6, this chapter) or to its sequence. Thus, to improve the stability and sorting of $VSR_{PS-1,}$ we constructed a hybrid protein in which the luminal domain of $VSR_{PS-1}$ was fused to the Vps10p-HA TMD and CT (Figure 3.11 A). In addition, there was a possibility that the hybrid protein would traffic like Vps10p, having all its trafficking signals, but with the sequence specificity of $VSR_{PS-1}$.

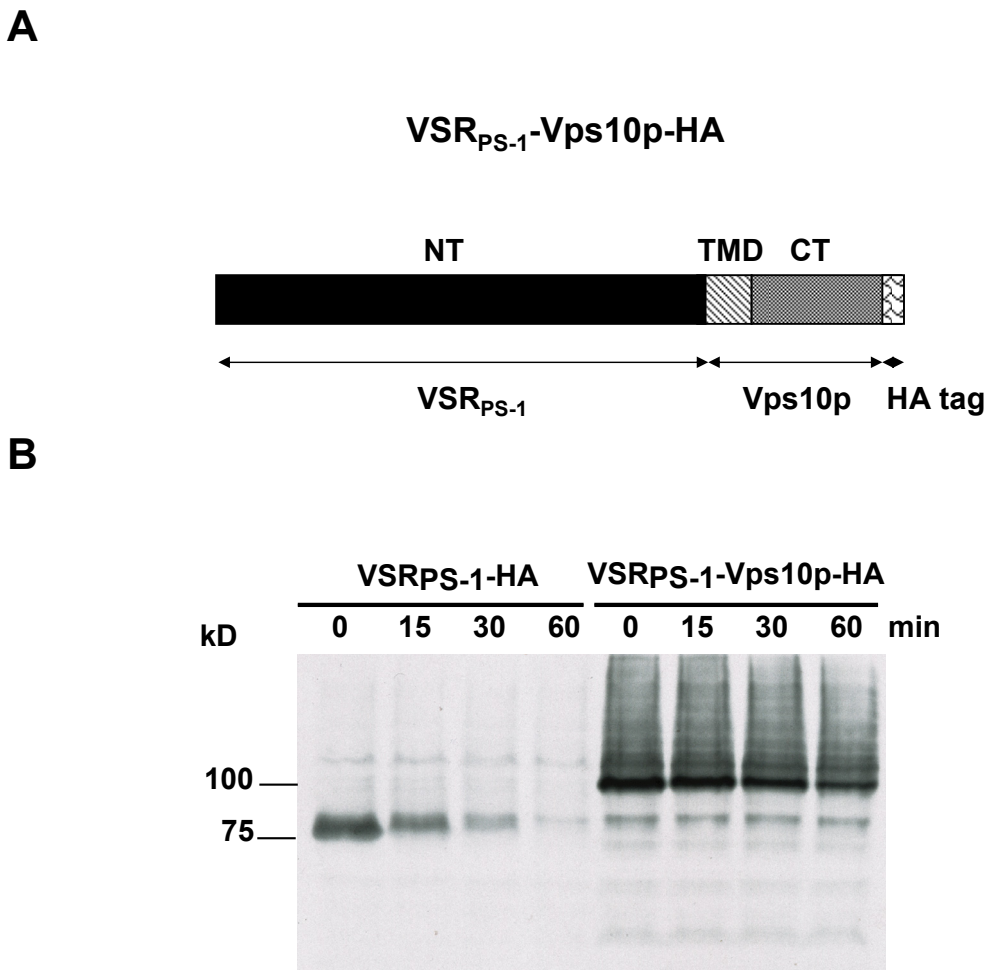



**Figure 3.11. VSR$_{PS-1}$-Vps10p-HA is also degraded in yeast cells.**

**(A)** Schematic representation of the VSR$_{PS-1}$-Vps10p-HA protein. The luminal domain of VSR$_{PS-1}$ was fused to the Vps10p-HA TMD and CT.

**(B)** VSR$_{PS-1}$-Vps10p-HA was expressed in the *Δvps10* mutant. After 24 hours of induction, protein synthesis was blocked by the addition of CHX. At the indicated times, cells were harvested and whole cells extracts prepared and subjected to immunoblot analysis. Proteins were detected by monoclonal HA antibodies.

Although this hybrid protein was less rapidly degraded than VSR$_{PS-1}$ (Figure 3.11 B), it still did not redirect the aleu-GFP to the vacuole as judged by fluorescence (data no shown). Consistent with this, we found that the expression VSR$_{PS-1}$-Vps10p in the *Δvps10* mutant did not lead to a detectable reduction of secreted aleu-GFP compared to cells expressing aleu-GFP alone or to the control expressing Vps10p by colony blot assay (Figure 3.12).

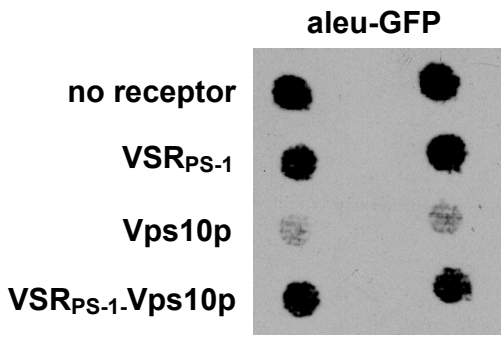



**Figure 3.12. $VSR_{PS-1}$-Vps10p does not lead to a detectable reduction of the secreted aleu-GFP.**

aleu-GFP fusion protein was coexpressed with $VSR_{PS-1}$ or Vps10p or $VSR_{PS-1}$-Vps10p in the *Δvps10* mutant using a GAL promoter. Equal amounts of each transformant were spotted on inducing galactose medium plates and covered with a nitrocellulose filter. After 24 hour at 30°C, the filters were removed and the secreted aleu-GFP was detected by immunobloting.

We also found that the degradation of the hybrid protein was completely blocked in the *sec18-1* mutant but it still occurred in the *Δpep4* mutant (Figure 3.13 A) indicating that $VSR_{PS-1}$-Vps10p also enters the secretory pathway and is able to reach the *cis*-Golgi but it does not reach the vacuole to be degraded. Additionally, $VSR_{PS-1}$-Vps10p-HA mainly did not sediment in the P100 fraction in a differential centrifugation experiment (Figure 3.13 B). Considering the above results, we assumed that the luminal domain of $VSR_{PS-1}$ is responsible for the $VSR_{PS-1}$ instability. This would be in agreement with previous observations showing that luminal domains of membrane proteins can affect their stability and localization. For instance, various deletions in the luminal domain of Vps10p result in either instability or mislocalization (Jorgensen et al., 1999). In addition, increasing evidence indicates that proteins with minor conformational defects that transit through the Golgi are retrieved to the ER to be either properly refolded or degraded by proteasomes (Caldwell et al., 2001; Vashist et al., 2001). Therefore, it is possible that the majority of $VSR_{PS-1}$ does not pass the quality control in the *cis*-Golgi compartment and is transported back to the ER to be retro-translocated to the cytosol and then degraded by the proteasomes.

In summary, we showed that the majority of $VSR_{PS-1}$-HA expressed in the yeast *Δvps10* mutant strain is rapidly degraded, most likely because of inefficient retention and/or transport to the PVC. This demonstrates that yeast is not well suited for study of the *in vivo* function of plant VSRs.

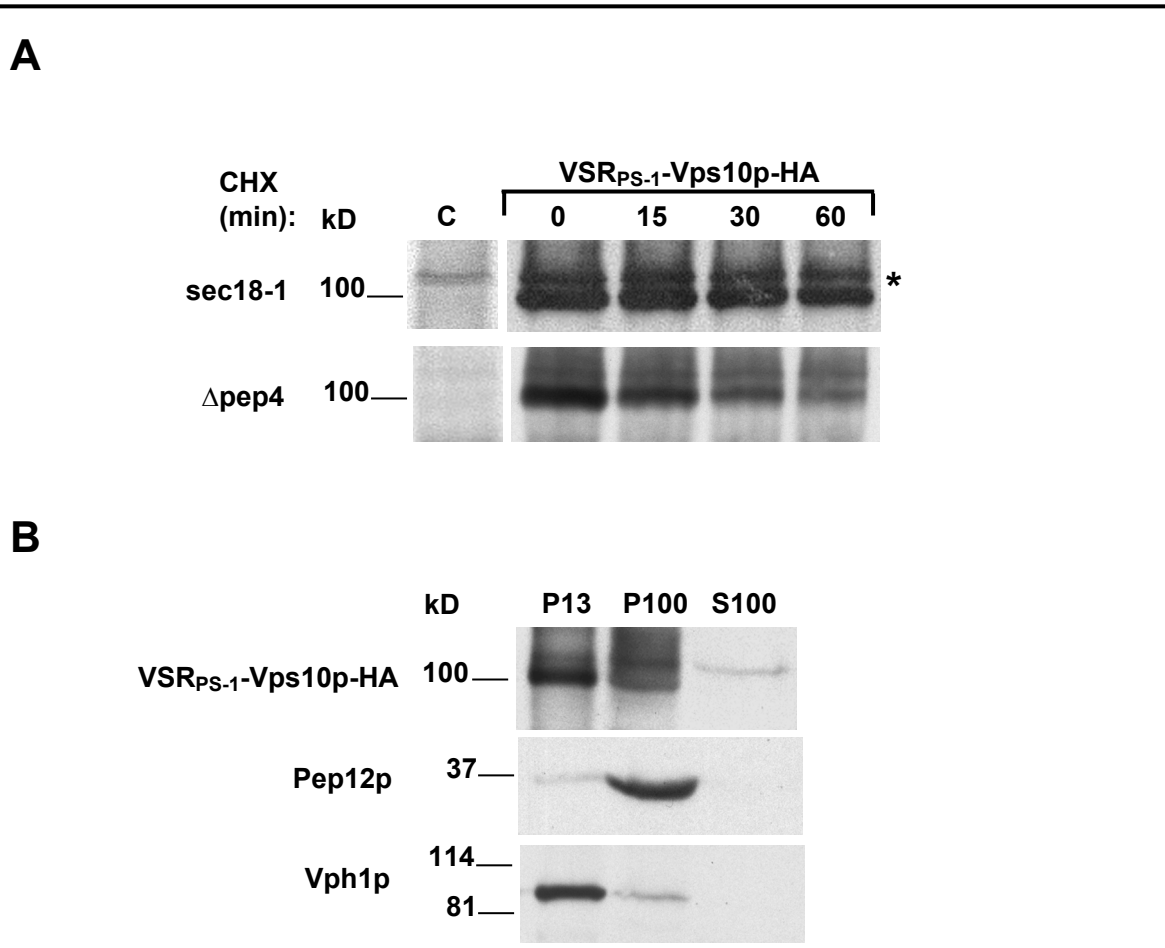



**Figure 3. 13. VSR$_{PS-1}$-Vps10p-HA is not associated with Golgi and PVCs.**

**(A)** The degradation of VSR$_{PS-1}$-Vps10p-HA is not prevented in mutants blocking the biosynthetic transport to the vacuole and in vacuolar protease-deficient cells. VSR$_{PS-1}$-Vps10p-HA was expressed in the *sec18-1$^{ts}$* and *Δpep4* strains. Protein expression was induced at 30°C or at 37°C for the *sec18-1* mutant, and cells were then treated with CHX and analyzed as described in the experimental procedure. C is the control of mutant cells transformed with the vector alone; the asterisk indicates the presence of nonspecific bands.

**(B)** *Δvps10* spheroplasts expressing VSR$_{PS-1}$-Vps10p-HA were gently lysed by osmotic shock. Subcellular fractionation was then perfomed by differential centrifugation as described in the experimental procedure. The amount of VSR$_{PS-1}$-Vps10p-HA, Pep12p and Vph1p in each of the fractions was assessed by immunobloting. The asterisk indicates the presence of nonspecific bands.

# Chapter 4. General Discussion

When I started this work, in November 1999, we considered that at least part of the plant vacuolar machinery was closely related to the yeast vacuolar machinery. Plant and yeast vacuoles are considered homologous organelles involved in hydrolytic functions. They are also essential for metabolite storage and for maintaining cytosolic ion and pH homeostasis (Klionsky et al., 1990; Wink, 1993). As in yeast, plant vacuolar targeting signals are part of the peptidic sequence of the soluble protein, rather than in a glycan side chain for lysosomal targeting in mammalian cells (Kornfeld, 1992). It has meanwhile become clear that, in contrast to yeast, the plant vacuolar sorting machinery is extremely complex, since some plant cells may have up to three functionally different vacuoles: the lytic vacuole (LV), the protein storage vacuole (PSV) (Hoh et al., 1995; Paris et al., 1996) and the neutral vacuole (Di Sansebastiano et al., 1998). In plants, transport analysis of soluble vacuolar proteins revealed three classes of vacuolar sorting determinants (VSDs), which likely interact with specific vacuolar sorting receptors (VSRs). This interaction allows the VSRs to direct their specific ligands to a specific vacuole. Indeed, protein transport to the LV depends on the sequence-specific-VSD (ss-VSD) and it is mediated by clathrin-coated vesicles (CCVs) (Kirsch et al., 1994; Hohl et al., 1996; Paris et al., 1997; Hinz et al., 1999). Transport to the neutral and (seed) storage vacuoles requires the C-terminal-VSD (ct-VSD) (Neuhaus et al., 1991) and the physical structure type VSD (ps-VSD) (Tague et al., 1990). The characterization of these sorting systems was reviewed by Neuhaus and Rogers (1998).

$VSR_{PS-1}$ (previously named BP-80) was the first VSR identified in plants. $VSR_{PS-1}$ was originally isolated from pea CCVs by its ability to bind the ss-VSD from barley proaleurain in a pH-sensitive manner in an *in vitro* assay (Kirsch et al., 1994; Paris et al., 1997). Since then, several VSRs have been cloned from different plant species (Ahmed et al., 1997; Paris et al., 1997; Shimada et al., 1997), and seven homologues (AtVSR1, 2, 2', 3, 4, 5 and 6) were identified in *Arabidopsis thaliana* (Laval et al., 1999; reviewed in Hadlington and Denecke, 2000). The existence of several homologues in one plant species suggests that these VSRs could have different ligand specificities, and might therefore be involved in the different plant vacuolar pathways and/or function at different stages of the plant development (Paris and Neuhaus, 2002). Fractionation of membranes from *Arabidopsis* cell suspension followed by a proteomics identification revealed that AtVSR1 and AtVSR2 sedimented together with the Golgi apparatus (GA) and the prevacuolar compartment (PVC), respectively (Paul Dupree, personal communication), suggesting that these two receptors could have different functions. Immunogold EM showed that both pea and *Arabidopsis* VSRs are predominantly localized in the PVCs, but also in the TGN (Paris et al., 1997; Sanderfoot et al., 1998; Hinz et al., 1999; Li et al., 2002). VSRs partially colocalized with AtSYP21, an homologue of the yeast t-SNARE

Pep12 that resides on PVCs, in Arabidopsis roots (Bassham et al., 1995; da Silva Conceicao et al., 1997; Sanderfoot et al., 1998) as well as in tomato and tobacco cells (Li et al., 2002). In addition, several proteins, such as t-SNARE AtSYP22 (Sato et al., 1997) and AtVPS45 (Bassham and Raikhel, 1998) that are also involved in vesicle transport to the LV have been identified by yeast complementation assays. Altogether the above findings suggested that VSRs traveling through the PVC could function like their yeast counterpart Vps10p, which mediates the transport from the *trans*-Golgi network (TGN) to the PVC of several vacuolar hydrolases such as carboxypeptidase Y (CPY, Johnson et al., 1987), proteinase A (Klionsky and Emr, 1989) and several misfolded or hybrid proteins (Hong et al., 1996).

Because genetic redundancy is frequent in plants and since there are remarkable conservation between the plant and yeast secretory pathways (see page 56), several plant cell biologists have attempted to use yeast either to identify a VSD of a plant soluble protein or to demonstrate the function of a plant receptors, with mixed results. Our group previously demonstrated the *in vivo* function of the plant vacuolar sorting receptor $VSR_{PS-1}$ in yeast (Humair et al., 2001). The $VSR_{PS-1}$ redirected the reporter GFP to the yeast vacuole only if fused to the petunia aleurain VSD (aleu-GFP) in a yeast strain disrupted for the Vps10p yeast receptor (*Δvps10*). This specific sorting was strictly dependent on the interaction between $VSR_{PS-1}$ and aleu-GFP since $VSR_{PS-1}$ was unable to transport GFP alone to the vacuole (sec-GFP) or GFP fused either to the yeast vacuolar targeting signal of the CPY (CPY-GFP) or to the ct-VSD of the chitinase A (GFP-Chi). This result demonstrated that the plant $VSR_{PS-1}$ is functional in yeast cells and capable of interacting, like Vps10p, with the yeast trafficking machinery. Considering the above results, we planned to further characterize plant vacuolar targeting and sorting using yeast.

Since very little was known about the functions of the seven *Arabidopsis* homologues VSRs (Laval et al., 1999; Hadlington and Denecke, 2000) and since the *in vivo* function of $VSR_{PS-1}$ had been successful demonstrated in yeast, we formulated as main goal, to determine the ligand specificity of the AtVSR family. This could reveal the functional role of each receptor in the different pathways to the plant vacuoles. Subsequently, we wanted to determine which yeast proteins were required for the vacuolar targeting of the reporter proteins and to identify their plant homologues.

To test the specificity of the receptors, we already had the reporters aleu-GFP and GFP-Chi. We cloned several additional plant VSDs. To check whether these new plant VSDs were functional *in planta*, the C-terminal propeptides of *Nicotiana alata* proteinase inhibitor (GFP-NAPI), *Bertholletia excelsis* 2S albumin (GFP-2Sa) and *Phaseolus vulgaris* phaseolin (GFP-Ph) were each fused to GFP and cloned in a plant expression vector. The first two of these propeptides were sufficient to target GFP to the vacuole (Ph-GFP was not tested) indicating

that they could be used as ligands to study protein VSR interactions in yeast. Additionally, we cloned most AtVSRs in a yeast expression vector under the control of the inducible GAL promoter.

To investigate the function of the AtVSRs, we coexpressed each VSD-GFP fusion protein together with each AtVSR in the *vps10* mutant strain. Our first experiments were based on qualitative observations by fluorescence microscopy, using $VSR_{PS-1}$ as positive control. We first wanted to determine which AtVSR (AtVSR1, 2, 3, 5 and 6) was able to efficiently redirect to the yeast vacuole either aleu-GFP or GFP-Chi. We found that none of these five AtVSRs were able to significantly retain intracellularly either GFP construct. More surprisingly, we encountered a problem, because we failed to detect a significant amount of aleu-GFP in the yeast vacuole in presence of $VSR_{PS-1}$, which was in discrepancy with our previous work (Humair et al., 2001). Consistently, we also obtained low percentages of retention of aleu-GFP by $VSR_{PS-1}$ and three of the AtVSRs (1, 2, and 3) by a quantitative fluorescence method. Although the effect of $VSR_{PS-1}$ on the aleu-GFP accumulation was significant by a statistical analysis, the activity of $VSR_{PS-1}$ was low compared to the yeast receptor Vps10p. We decided we had to optimize the growth and induction conditions and to verify the specificity of the *⊿vps10* mutant strain. To obtain a significant fluorescence vacuolar retention, we tested parameters like temperature, medium source, the use of freshly transformed yeast, induction procedure, time between transformation and inoculation for induction, and the age of the competent cells. We found that none of these parameters influenced the vacuolar retention of fluorescence except the induction procedure. However, under best growth conditions, no significant accumulation of the aleu-GFP in the vacuole was observed in the *⊿vps10* mutant strain expressing $VSR_{PS-1}$. Moreover, none of the above five AtVSRs was able to significantly retain intracellularly either aleu-GFP or GFP-Chi.

We verify that the *⊿vps10* mutant strain (EMY3, obtained from the laboratory of Dr. Scott Emr, University of California at San Diego), which has been used and cited in many publications has a reduced growth phenotype and displays the typical vacuolar sorting defects. We showed that re-introducing a functional Vps10p restores a normal growth phenotype and transport of the aleu-GFP to the vacuole. This indicated that the yeast strain used in this study was suitable for testing the VSRs activities. Therefore, assuming that VSRs requires part (if not all) of the Vps10p-associated trafficking machinery to be efficiently functional in yeast, we could expect this machinery to be available.

The discrepancy between our previous published results and the new results prompted us to re-investigate the activity of $VSR_{PS-1}$ in yeast and therefore, the merits of this *in vivo* yeast approach for studying plant vacuolar protein-protein interactions. For this purpose, we set up an alternative approach to estimate the amount of VSD-GFP secreted by the yeast cells when

a plant VSR is present. Although we were aware that this approach was not a quantitative technique, we wanted to use it as a complement to the fluorimetric measurements of retained VSD-GFP. In absence of receptors, we could show that all ligands (VSD-GFP fusion proteins) are secreted by default. We found that the expression of $VSR_{PS-1}$ did not lead to a detectable reduction of secreted aleu-GFP compared to the controls. The expression of each of five AtVSRs did not significantly prevent the secretion of either aleu-GFP or GFP-Chi. Unfortunately, we cannot assess the amount of retained aleu-GFP, and can therefore not calculate the ratio of secreted/retained aleu-GFP when $VSR_{PS-1}$ was expressed in yeast. It was previously estimated that $VSR_{PS-1}$ has the potential to retain approximately 30% of aleu-GFP when compared to wild type cells (Figure 5C of Humair et al., 2001). Therefore, the variation for secreted aleu-GFP should be expected to range between 70% to 100%. If we consider that the colony blot assay cannot detect 10 to 30% diminution of aleu-GFP secretion, these new results are not contradictory with the previous estimations (Humair et al., 2001) but indicate that the efficiency described before is likely to represent the maximum for this assay. On the other hand, the results obtained by the colony blot assay were consistent with the new fluorescence data and supported that yeast is not efficient for *in vivo* studies of plant VSRs.

We also considered to use Western blots to quantify the amounts of aleu-GFP retained within the cells or secreted into the medium. However, we realized that this approach is rendered difficult by the presence of several aleu-GFP forms (precursor, mature, partial degradation). Moreover, protein maturation could not be used to assess the vacuolar localization of the aleu-GFP because it has been found in yeast that some missorted soluble vacuolar proteases can also be processed in other compartments apart from the vacuole (Deloche and Schekman, 2002). Consistently, the previous study showed that CPY-GFP was recovered from the medium in a processed form in the absence of Vps10p (see figure 2 of Humair et al., 2001).

So, how do we explain that, in the previous study, $VSR_{PS-1}$ seemed to transport aleu-GFP to the vacuole? The previous conclusion of a positive assay was made by comparison with the mutant strain expressing the GFP alone and was based on both (1) on the intensity of the vacuolar signal obtained upon expression of the plant VSR and (2) on the number of cells showing fluorescent labeling (up to 60%). Despite the fact that the vacuolar signal was often variable in the negative controls and was on average much weaker in *Δvps10* cells expressing $VSR_{PS-1}$ than in wild-type strain, the retention of aleu-GFP by $VSR_{PS-1}$ was confirmed by immunoblot analysis (Figure 5C of Humair et al, 2001). In this present study, we used different growth conditions and observed GFP fluorescence during the late-exponential phase of cell growth, which showed a higher percentage of fluorescence vacuolar staining in wild-type cells (positive control) and a lower fluorescence signal background in the *Δvps10* mutant strain

(negative control). These growth conditions are more physiologically relevant for studying protein trafficking as they are routinely used for the yeast trafficking studies. It is possible that the growth conditions described in Humair et al. (2001), where the fluorescence detection was performed on cells in the late-stationary phase ($OD_{600}$ between 5 to 10), are more stressful, favoring an accumulation of aleu-GFP in the vacuole in the presence of $VSR_{PS-1}$, or by an alternative $VSR_{PS-1}$ independent transport mechanism. It is possible that the high variability of vacuolar fluorescence retention detected by this assay, could be due to the presence of this alternative pathway. Cells in the stationary phase trigger autophagy (Werner-Washburne et al., 1993; Werner-Washburne et al., 1996). Some early Sec proteins are involved in the autophagosome formation (Ishihara et al., 2001; Hamasaki et al., 2003; Reggiori et al., 2004) and some of the ER itself can be engulfed and delivered to the yeast vacuole by autophagy (Hamasaki et al., 2005). Not only ER membrane proteins are transported to the vacuole by this process but also soluble proteins. We can speculate therefore that aleu-GFP could have reached the vacuole by autophagy in a Golgi-independent pathway. Moreover, the regulation of gene expression is also tightly linked to the adaptation of cells at the stationary phase growth (Werner-Washburne et al., 1993). We also cannot rule out that the expression of the *VPS10*-like genes could have been affected. VTH1 and VTH2 encode Vps10p homologues able to act as sorting receptors (Cooper and Stevens, 1996), therefore, we can speculate that the VTH genes could also have been activated and would have been able to mediate the aleu-GFP transport to the vacuole. However, how do we explain the higher vacuolar retention observed in cells expressing aleu-GFP in presence of $VSR_{PS-1}$ compared to cells expressing aleu-GFP alone? It is possible that in cells expressing both proteins (i.e. aleu-GFP and $VSR_{PS-1}$) the autophagy process occurred faster than in cells expressing only one of the two proteins (i.e. aleu-GFP) since they had to produce a higher total level of foreign proteins. Nevertheless, we doubt that growth conditions alone can explain the observed differences because in this study we also again tested cells in the late-stationary phase (our first fluorescence data observations), and in the previous study, cells in late-exponential phase had also been tested. In both cases, no major influence of growth conditions on the fluorescence patterns had been observed.

The lack of retention of the GFP fusion proteins could be due to improper sorting of the VSRs themselves. Vps10p mediates the transport of vacuolar soluble proteins by cycling between the TGN and the PVC (Marcusson et al., 1994; Cooper and Stevens, 1996). The cytosolic tail domain (CT) of Vps10p plays a critical role in this cycle transport since it harbors multiple signals that are necessary for the sorting and stability of the receptor. Despite the conserved presence of a Tyr motif in Vps10p and $VSR_{PS-1}$, they differ in tail length (164 versus 38 residues) and have no other sequence homology. This suggests that the CT of $VSR_{PS-1}$

does lacks most signals necessary to selectively interact with the yeast machinery and that $VSR_{PS-1}$ is unable to efficiently cycle between the TGN and the PVC. We therefore investigated the stability and the localization of $VSR_{PS-1}$ in yeast cells. In contrast to Vps10p, $VSR_{PS-1}$ was rapidly degraded in Δ*vps10* cells. A similar rate of degradation was also observed for an untagged version of the $VSR_{PS-1,}$ indicating that the tag has no influence on $VSR_{PS-1}$ stability.

To elucidate whether $VSR_{PS-1}$ follows a typical degradation pathway like many misfolded proteins that can leave the ER or mutated proteins unable to be recycled from the PVC to TGN, we expressed $VSR_{PS-1}$ either in a set of vesicular mutants blocking the transport to the vacuole and in vacuolar protease-deficient cells. For instance, a mutant form of Vps10p lacking its CT is transported to the PVC and degraded in the vacuole because it is unable to recycle back to the Golgi (Cereghino et al., 1995; Cooper and Stevens, 1996).

We found that degradation of $VSR_{PS-1}$-HA was only prevented by the *sec18-1* mutant, indicating that the protein enters the secretory pathway, is rather stable in the ER and is normally able to reach the *cis*-Golgi. By contrast, the degradation of $VSR_{PS-1}$-HA still occurred in the *Δvps45* and *Δpep4* mutants meaning that the receptor's degradation and its inability to reach the PVC are linked. In agreement with this idea, we found that $VSR_{PS-1}$-HA did not sediment in a differential centrifugation experiment with the PVC Pep12p marker (P100) but instead accumulated in larger compartments (P13) including ER, vacuole and PM. Interestingly, we detected some signal for $VSR_{PS-1}$-HA in the high-speed pellet (P100) containing the Golgi apparatus (GA) and PVC despite the very short half-life of the protein. How do we interpret the cellular localization of $VSR_{PS-1}$? The previous immunolocalization of $VSR_{PS-1}$ in punctuate structures that also contained Vps10p in wild-type cells (Figure 6, Humair et al., 2001) is consistent with our new data. We showed that $VSR_{PS-1}$ exits the ER to reach the early Golgi. However, it is extremely difficult to distinguish between the early Golgi and the late-PVC by immunofluorescence in yeast cells. Double labeling of these structures is not easy to perform and must be analyzed on a large number of cells to be quantitatively significant. It is possible that a small fraction of $VSR_{PS-1}$ reaches the PVC. This pool of $VSR_{PS-1}$ may have been detected in the previous immunofluorescence experiment.

The localization of certain membrane proteins also depends on the length and the amino acid composition of their transmembrane domain (TMD) (Sato et al., 1996; Rayner and Pelham, 1997) and this suggested that the mislocalization of $VSR_{PS-1}$ could be due either to its longer TMD or to its sequence. In an attempt to make this yeast approach functional, we decided to prepare a hybrid protein construct in which the luminal domain of $VSR_{PS-1}$ was fused to the Vps10p-HA TMD and CT ($VSR_{PS-1}$-Vps10p-HA). Although this hybrid protein was less rapidly degraded than $VSR_{PS-1}$, it did not redirect the aleu-GFP to the vacuole as judge by fluorescence (data no shown). In agreement with this, we found that the expression $VSR_{PS-1}$-Vps10p in the

*⊿vps10* mutant did not lead to a detectable reduction of secreted aleu-GFP by colony blot assay.

The degradation of $VSR_{PS-1}$-Vps10p-HA was blocked in the *sec18-1* mutant but it still occurred in the Δ*pep4* mutant indicating that the protein also enters the secretory pathway and is also able to reach the PVC but it does not need to reach the vacuole to be degraded. Unfortunately, we have no clear information about the stability of this hybrid protein in mutants blocking the transport from the GA to the PVC. We also found that only little $VSR_{PS-1}$-Vps10p-HA was recovered in the high-speed pellet (P100) containing the GA and the PVC. Therefore, it is possible that the luminal domain of $VSR_{PS-1}$ is responsible for its instability, which would be in agreement with previous observations showing that luminal domains of membrane proteins can affect their stability and localization. For instance, various deletions in the luminal domain of Vps10p result in either instability or mislocalization (Jorgensen et al., 1999). Increasing evidence indicates that proteins with minor conformational defects that transit through the GA are retrieved to the ER to be either properly refolded or degraded by proteasomes (Caldwell et al., 2001; Vashist et al., 2001). Therefore, it is possible that the majority of $VSR_{PS-1}$ does not pass the quality control in the *cis*-GA compartment and is transported back to the ER to be translocated to the cytosol and then degraded by the proteasomes.

We believe that this yeast system is highly variable and tricky. However, we cannot exclude that (i) this system has previously worked under certain “non-physiological” conditions that we were later unable to reproduce and (ii) that $VSR_{PS-1}$ has a much lower activity than previously described and this activity is difficult to detect *in vivo* experiments.

Unfortunately, we did not identify which are the key factors inducing the high variability of this yeast assay. We can speculate that in absence of Vps10p, the associated trafficking factors would be downregulated, and as a result, $VSR_{PS-1}$ would be unable to traffic and be rapidly degraded in yeast, leading to a decrease in the efficiency of the assay. However, we do not favor this theory because we showed that $VSR_{PS-1}$ is not only highly unstable in *⊿vps10* mutant strain but also in several other strains (i.e. *⊿vps45*, *⊿pep4* and *⊿vps4,* wild-type strains for Vps10p) with different genetic backgrounds. Thus, there is evidence that the degradation is not strain-dependent. It is also noteworthy that we also checked the sequence of the $VSR_{PS-1}$ and aleu-GFP fusion protein to rule out any unidentified mutation that could also have affected the assay.

In summary, the majority of $VSR_{PS-1}$-HA expressed in the yeast *⊿vps10* mutant is rapidly degraded, most likely because of retention and/or inefficient transport to the PVC indicating that it is unlikely that this yeast assay would be very efficient. Therefore, we conclude that yeast is not an appropriate system to study the *in vivo* function of plant VSRs.

This work is a small contribution to a complex investigation. The functional role of the VSR proteins is an important issue for plant cell biologists. Therefore, several approaches have been used to study their possible functions and their ligand specificity. So far, *in planta* only two results have recently been reported. The first supports the role of AtVSR1 as a receptor for the storage vacuole pathway (Shimada et al., 2003). Using the genetic "knock-out" approach, the authors observed that *atvsr1* mutant plants missorted a proportion of the seed storage proteins, but without detectable effects on proteins in other tissues. The second result indicates that the presumed pumpkin PV72, seed-specific receptor, acts as a receptor for the sequence-specific type of VSD (Watanabe et al., 2004). By a competition experiment, the authors demonstrated that a PV72-HDEL fusion protein specifically induced ER accumulation of the AtALEU precursor that contains a NPIR-containing peptide. Our group had demonstrated that $VSR_{PS-1}$ specifically sorted the aleu-GFP to the yeast vacuole *in vivo*. This result confirmed that $VSR_{PS-1}$ was functional in yeast and it was capable of interacting, like Vps10p, with the yeast trafficking machinery. We believed that this yeast approach was a promising tool to study the functional role of the AtVSRs. Unfortunately, we were unable to demonstrate the ligand binding specificity of the AtVSR in yeast. More surprisingly, we were not able to demonstrate quantitatively the function of $VSR_{PS-1}$ as previously described. Therefore, we further re-evaluated the merits of this *in vivo* yeast approach for studying plant vacuolar sorting interactions. We next demonstrated that $VSR_{PS-1}$ does not reach the PVC and is rapidly degraded in yeast suggesting that yeast is indeed not an efficient tool to study the function of the plant VSRs. These results led us to conclude that the plant vacuolar targeting machinery involved in the protein transport to the LV is not similar enough to the yeast vacuolar targeting machinery. When looking at both machineries, important differences can be found. First, there is no conservation in the specific sorting signals that allow soluble proteins to be correctly targeted to the vacuole. The best characterized yeast sorting signal is the QRLP motif within the propeptide of the vacuolar hydrolase CPY (Valls et al., 1990) which is however not conserved in other vacuolar protein precursors. In plants, a conserved NPIR motif have been found to be essential for the vacuolar targeting to the LV (Holwerda and Rogers, 1993; Nakamura et al., 1993) but again is not conserved in other vacuolar protein precursors that interact with the same receptors. This difference has also been confirmed by several studies where plant vacuolar proteins were targeted to the yeast vacuole, independently of the presence of their plant ss-VSD (e.g. Matsuoka and Nakamura, 1992). The vacuolar sorting receptors of plant and yeast do not share any sequence homology. Their CTs harbor multiples signals that must interact with cytosolic factors for the correct trafficking of the receptor proteins. The CT of $VSR_{PS-1}$ and Vps10p display no sequence homology, except for a shared Tyr motif (Kirsch et al., 1994; Cooper and Stevens, 1996; Dell'Angelica and Payne). This

suggests that the CT of $VSR_{PS-1}$ lack most sorting signals to selectively interact with the yeast trafficking machinery, but it must have other plant-specific sorting signals.

Despite the conservation of many proteins involved in the vesicular budding and fusion, in many cases, the localization and function of plant proteins do not necessarily correspond to the localization and function of their yeast homologues. For example, the *Arabidopsis* AtVPS45 protein was identified by its sequence similarity to yeast Vps45p (Bassham and Raikhel, 1998) which is required for the vacuolar transport via two distinct pathways: the CPY pathway (Cowles et al., 1994; Piper et al., 1994) and the Cvt (cytoplasm-to-vacuole transport) pathway (Abeliovich et al., 1999). Vps45p is a multi-functional protein, interacting with the t-SNARE Pep12p at the PVC in the CPY pathway and with the t-SNARE TLg2p at the TGN in the Cvt pathway. In contrast, AtVPS45 is localized exclusively to the TGN in *Arabidopsis* roots and interacts with two Tlg2p-like proteins, AtSYP41 (AtTLG2a) and AtSYP42 (AtTLG2b). AtVPS45 does not interact with the *Arabidopsis* Pep12p homologue AtSYP21 and cannot be detected at the PVC (Bassham et al., 2000). The function of AtVPS45 thus appears to have diverged from that of its yeast equivalent, although the precise function of AtVPS45 still remains unclear.

Unlike in yeast, some plant proteins can reside on different organelles in different cell types within the same species, e.g. the *Arabidopsis* AtSY22 protein (AtVam3). *AtSYP22* was identified as a cDNA that is able to complement some phenotypes of a yeast *vam3* mutant (Sato et al., 1997). Vam3p is a t-SNARE on the yeast vacuole that functions in multiples transport pathways to this organelle (Wada et al., 1997). The AtSYP22 protein was originally reported to reside on the tonoplast in the shoot apical meristem. However, further examination of the localization of AtSYP22 demonstrated that in roots and leaves, AtSYP22 is found on the PVC where it co-localizes with AtSYP21, and it is not detectable on the tonoplast (Sanderfoot et al., 1999). The tonoplast localization was then assumed to be restricted to specialized cell types in plants, and in most tissues AtSYP22 is probably prevacuolar. It was suggested that the yeast mutant complementation was due in fact to mistargeting of AtSYP22, due to differences in the endomembrane systems and in protein targeting between yeast and plants.

Another observation is that two or more plant genes appear to correspond to a single yeast gene (Sanderfoot and Raikhel, 1999). At least three *Arabidopsis* genes are closely related to the yeast Pep12 gene: AtSYP21 (Bassham et al., 1995), AtSYP22 (t-SNARE at the PVC, Sato et al., 1997) and AtSYP23 (AtPLP) (Zheng, 1999). AtSYP21 resides on the *Arabidopsis* PVC and forms a complex with characteristics of fusion complexes (Bassham and Raikhel, 1999). Despite the ability of AtSYP22 to functionally replace Vam3p in yeast, its sequence is closely related to AtSYP21 and yeast Pep12p and less to Vam3p. Interestingly, despite the sequence conservation, AtSYP22 is not able to complement *pep12* mutant, indicating that AtSYP21 and AtSYP22 do have distinct functions.

One can conclude that plants have a more complex vacuolar system than yeast. Therefore, to study vacuolar sorting events of plants in yeast, we must carefully interpret the data because as already mentioned (1) the localization of some plant proteins associated to the trafficking machinery in yeast do not necessarily correspond to the localization in their native plant species, and (2) plant proteins can reside in different organelles in different cell types within the same species, because of putative differences in the endomembrane structures between cell types.

This thesis is an additional example of the need to carefully study and interpret the function and localization of a plant protein when expressed in yeast. In particular, we present new data on $VSR_{PS-1}$ trafficking in yeast demonstrated by the stability of the receptor in various yeast mutant strains, indicating a clear relationship between trafficking and stability. We also contribute to clarify whether yeast cells could be used to study the functional role of plant VSRs. Therefore, these data could be useful for future general evaluation of either plant vacuolar targeting or plant protein studies using yeast as a host.

Despite our new results, we would like to point out that the *S.cerevisiae* is still a useful model organism to study heterologous protein transport. Yeast has been used to identify various plant proteins involved in the vesicular trafficking (see above). Using yeast, it was possible to identify ER-retention signals of some plant secretory proteins (Kaletta et al., 1998) and to demonstrate the function of some receptor proteins like the AtErd2 receptor (Lee et al., 1993). Yeast could also be used to study ligand-receptor interactions such as the two-hybrid approach which is commonly used to identify a binding partner for a chosen protein. In fact the original aim of this yeast assay was to provide the equivalent of a two-hybrid system for the secretory pathway of plants, in order to study protein-protein interactions that require the special environment provided by the lumen of this endomembrane system. It is clear that the study of receptor-ligand interactions will have to be done with other systems, either *in vitro* or *in planta*, as are on their way in our laboratory. *S.cerevisiae* is also considered a valuable system for identifying proteins involved in endocytosis and for elucidating the mechanisms of internalization and post-internalization events (e.g. the role of ubiquitination, Shaw et al., 2001). Yeast has been extensively used to study the intracellular transport of various heterologous proteins (Kiser et al., 2001; Prinz et al., 2003) and to optimize the production of a variety of heterologous membrane proteins, particularly of medical importance (Cereghino and Cregg, 2000). Finally, it has also been used to investigate several protein quality control mechanisms like the unfolded protein response (UPR, Griffith et al., 2003) and the different degradation pathways (Holkeri and Makarow, 1998).

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# Appendixes

## Appendix 1: LETTER TO THE EDITOR

### The Yeast *Saccharomyces cerevisiae* is Not an Efficient Tool for *in Vivo* Studies of Plant Vacuolar Sorting Receptors. The Plant Cell. Vol. 17, 1-4, May 2005.

Previously, we demonstrated a specific interaction between the vacuolar sorting receptor PS1 ($VSR_{PS-1}$, also named BP-80) and the petunia aleurain vacuolar sorting determinant (VSD) fused to a GFP (aleu-GFP) *in vivo* in the yeast *S. cerevisiae* (Humair et al., 2001). These results also showed that $VSR_{PS-1}$ could partially replace the function of its yeast counterpart Vps10p to mediate the transport of aleu-GFP to the vacuole. In this previous work, $VSR_{PS-1}$ was expressed in a yeast mutant strain lacking the *VPS10* gene and redirected roughly 10 times more aleu-GFP to the vacuole when compared to a negative control. The aleu-GFP also accumulated in the vacuole in a Vps10p-dependent manner (Figure 1A; see also Humair et al., 2001). Vps10p has been shown to interact with and transport to the vacuole misfolded proteins (Hong et al., 1996). Therefore, Vps10p probably mediates the transport of aleu-GFP to the vacuole by interacting with as yet unfolded structures of GFP, which was shown to fold slowly in the secretory pathway (Wooding and Pelham, 1998). $VSR_{PS-1}$-mediated sorting was strictly dependent on the interaction between $VSR_{PS-1}$ and the aleurain VSD since $VSR_{PS-1}$ was unable to transport to the vacuole GFP alone (Sec-GFP) or GFP fused either to the yeast vacuolar targeting signal of carboxypeptidase Y (CPY-GFP) or to another type of VSD from a plant vacuolar protein (GFP-Chi).

By a similar approach, we then tested whether the family of vacuolar sorting receptors identified in *Arabidopsis thaliana* (AtVSR1, 2, 2', and 3 to 6) and showing high homology to $VSR_{PS-1}$, is able to redirect to the vacuole the GFP reporter protein fused to different plant VSDs. The aleu-GFP or the GFP-Chi was expressed alone or together with each putative Arabidopsis VSR in the Δ*vps10* mutant strain. The GFP retained in the yeast vacuole by AtVSRs was then detected by fluorescence microscopy. None of the five tested AtVSRs significantly retains intracellularly either GFP construct (data not shown). But more surprisingly, we were unable to demonstrate the function of $VSR_{PS-1}$ as a sorting receptor in yeast as described before (Humair et al., 2001). When compared to Vps10p, no significant accumulation of aleu-GFP in the vacuole was observed in Δ*vps10* cells expressing $VSR_{PS-1}$ (Figure 1A). How do we explain these new confocal observations? Our previous conclusion of a positive assay was made by comparison with the mutant yeast expressing the GFP reporter alone and was based both (1) on the intensity of the vacuolar signal obtained upon expression of the plant VSR and (2) on the number of cells

showing fluorescent labeling (up to 60%). Despite the fact that the vacuolar signal was often variable in the negative controls and was on average much weaker in Δ*vps10* cells expressing $VSR_{PS-1}$ than in the wild type strain, the retention of aleu-GFP by $VSR_{PS-1}$ was confirmed by immunoblot analysis (Figure 5C of Humair et al., 2001). In the present study, we used different growth conditions and observed GFP fluorescence during the late-exponential phase of cell growth, which gave a more homogeneous vacuolar signal in wild-type cells and is more physiologically relevant for studying protein trafficking (Figure 1A; almost 100% of the cells consistently display a vacuolar GFP signal). It is possible that the growth conditions described in Humair et al. (2001), where the fluorescence detection was performed on cells in the late-stationary phase ($OD_{600}$ between 5 to 10), are more stressful, leading to an accumulation of aleu-GFP in the vacuole in the presence of $VSR_{PS-1}$. Nevertheless, we doubt that growth conditions alone can explain the observed differences because in the previous study we had tested cell growth in the late-exponential phase, and in the present study we tested cells in the late-stationary phase. In both cases, there was no influence of growth conditions on the respective confocal observations (data no shown). It is also noteworthy that we previously observed the efficiency of the assay to be higher in less manipulated cells. This observation suggests that $VSR_{PS-1}$ requires some trafficking components associated with the Vps10p pathway that are quickly down-regulated in the yeast mutant strain. The fact that we used the same strain over the length of this new study may additionally indicate that this effect is not reversible.

Because the fluorescence background of the aleu-GFP reporter observed in the vacuole of the Δ*vps10* mutant was problematic in drawing conclusions, we set up an alternative straightforward approach to demonstrate a functional interaction between the plant VSRs with our different VSD-GFP constructs. In this approach, we took advantage of the fact that most soluble yeast vacuolar proteins are secreted by default in the absent of functional Vps10p. Thus, instead of detecting the GFP that is retained intracellularly by plant VSRs, we determined the amount of secreted GFP binding to a nitrocellulose filter in a colony blot assay (Figure 1B). As expected, a large amount of secreted Sec-GFP and aleu-GFP was detected in the absence of Vps10p. The expression of $VSR_{PS-1}$ in the Δ*vps10* mutant did not lead to a detectable reduction of secreted aleu-GFP compared to cells expressing Sec-GFP or to the control expressing Vps10p. In addition, we also showed that expression of any of the five tested Arabidopsis VSRs (AtVSR 1 to 3, 5, and 6) did not significantly prevent the secretion of either aleu-GFP or GFP-Chi (Figure 1B).

Unfortunately, we have no information on the ratio of secreted/retained aleu-GFP when $VSR_{PS-1}$ is expressed in yeast. We previously estimated that $VSR_{PS-1}$ has the potential to retain ~30% of aleu-GFP when compared to the wild type strain (Figure 5C of Humair et al., 2001). It is likely that the colony blot assay, which is designed to detect massive changes in secretion,

does not detect such a small variation. Therefore, our failure to detect a decreased secretion of aleu-GFP is not in contradiction with the previously reported quantitative data (Humair et al., 2001), but indicates that the efficiency described before is likely to represent the maximum for this yeast assay. To conclude, our original aim to improve and widely use yeast in a simple quantitative assay for *in vivo* studies of plant vacuolar receptors was overly optimistic.

To determine the stability and the localization of $VSR_{PS-1}$ in yeast cells, we next expressed a hemagglutinin (HA) epitope tagged-$VSR_{PS-1}$ ($VSR_{PS-1}$-HA) under the control of the GAL promoter in the Δ*vps10* strain. Cells were grown to early exponential phase at 30°C, and after 24 hours induction in the presence of galactose; cells were treated with cycloheximide (CHX) to block protein synthesis. An equivalent amount of cells was harvested after 0, 15, 30 and 60 minutes of CHX treatment and subjected to Western blot analysis. In contrast to Vps10p-HA, which was not degraded after several hours of CHX treatment, overexpressed $VSR_{PS-1}$-HA is rapidly degraded (half-life <15min) (Figure 2A). A similar rate of degradation was also observed for an untagged version of $VSR_{PS-1}$ using specific monoclonal antibodies against VSRs (data not shown), thus ruling out a negative effect of the HA tag on $VSR_{PS-1}$ stability. Misfolded proteins that leave the endoplasmic reticulum (ER) or mutated proteins that are not recycled from the prevacuolar compartment (PVC) to the trans-Golgi network (TGN) are usually delivered to the vacuole where they are rapidly degraded. This type of degradation is prevented either in vacuolar protease-deficient cells or in mutants blocking protein transport to the vacuole. Indeed, we found that the degradation of $VSR_{PS-1}$-HA was completely blocked in the *sec18-1* (yeast NSF) mutant (Figure 2B), which prevents the fusion of ER-derived vesicles with the *cis*-Golgi, indicating that $VSR_{PS-1}$ enters the secretory pathway and is able to reach the *cis*-Golgi. By contrast, degradation of $VSR_{PS-1}$-HA still occurred in the Δ*vps45* mutant, which blocks transport from the Golgi to the PVC, and in the Δ*pep4* mutant, which reduces the activities of major vacuolar proteases (Figure 2B), meaning that the receptor's degradation and its inability to reach the PVC are linked. Consistent with this idea, we also found that $VSR_{PS-1}$-HA did not sediment with the PVC Pep12p marker (P100) in a differential centrifugation experiment but instead accumulated in larger compartment (P13) including the ER, vacuole and plasma membrane (Figure 2C).

Interestingly, we detected some signal for $VSR_{PS-1}$-HA in the high-speed pellet (P100) containing the Golgi and PVC in spite of the very short half-life of the protein. This might corroborate our previous immunolocalization results in wild-type cells showing a partial colocalization of $VSR_{PS-1}$ with its yeast counterpart Vps10p (Figure 6 of Humair et al., 2001) and thus suggests that a small fraction of $VSR_{PS-1}$ is able to reach the PVC and to be active.

The lack of massive retention of aleu-GFP and the rapid degradation of $VSR_{PS-1}$ indicate that most of the expressed VSRs are not properly sorted in the yeast cell, thus preventing an appropriate interaction with ligands. Vsp10p mediates the transport of soluble vacuolar proteins by

cycling between the TGN and the PVC (Cooper and Stevens, 1996). The cytoplasmic tail domain of Vps10p plays a critical role in this transport cycle since it harbors multiple signals that are necessary for the sorting and the stability of the receptor. This was demonstrated by the finding that Vps10p mutants lacking the cytoplasmic tail domain or with mutated residues travel to the PVC before being rapidly degraded in the vacuole (Cooper and Stevens, 1996). The cytosolic tail domain of $VSR_{PS-1}$ also appears important in recycling to the TGN because a truncated form lacking the cytosolic domain is substantially less stable than the intact receptor in tobacco protoplasts (Jiang and Rogers, 1998). However, the cytosolic domains of $VSR_{PS-1}$ and Vps10p display no sequence homology, except for a shared Tyr motif, and differ in length (38 versus 164 residues). This suggests that the cytosolic domain of $VSR_{PS-1}$ lacks most sorting signals to selectively interact with the yeast trafficking machinery and that $VSR_{PS-1}$ is unable to cycle efficiently between the TGN and the PVC. Because the localization of certain proteins may also depend on the length and the amino acid composition of their transmembrane domain (TMD) (Sato et al., 1996; Rayner and Pelham, 1997), the mislocalization of $VSR_{PS-1}$ could be due either to its longer TMD (23 versus 17 residues for Vps10p) or to its sequence. Thus, to improve the stability and sorting of $VSR_{PS-1}$, we constructed a hybrid protein in which the $VSR_{PS-1}$ luminal domain was fused to the Vps10p-HA TMD and cytosolic domains. Although this hybrid protein was less rapidly degraded than $VSR_{PS-1}$, it did not redirect the aleu-GFP to the vacuole as judged by fluorescence and still sedimented in lower density fractions than Golgi and endosomal membranes (data not shown). Therefore, it is possible that the luminal domain of $VSR_{PS-1}$ is responsible for its stability. This would be in agreement with previous observations showing that luminal domains of membrane proteins can affect their stability and localization. For instance, various deletions in the luminal domain of Vps10p result in either instability or mislocalization (Jorgensen et al., 1999). In addition, increasing evidence indicates that proteins with minor conformational defects that transit through the Golgi are retrieved to the ER to be either properly re-folded or degraded by proteasomes (Caldwell et al., 2001; Vashist et al., 2001). Therefore, it is possible that the majority of $VSR_{PS-1}$ does not pass the quality control in the *cis*-Golgi compartment, and is transported back to the ER to be dislocated to the cytosol and then degraded by the proteasomes.

Our original work aimed at showing an *in vivo* interaction between $VSR_{PS-1}$ and a vacuolar protein precursor, which could not yet be shown in plants. Meanwhile, it has recently been reported that a homozygous “knock-out” of AtVSR1 causes mutant plants to missort a subset of the seed storage proteins to the cell wall, without effect on other tissues (Shimada et al., 2003). A soluble, ER-retained form of the pumpkin VSR PV72 specifically causes the accumulation in the ER of a vacuolar protein precursor (Watanabe et al., 2004). These two results indicate that VSRs can indeed act in planta as sorting receptors.

In summary, we showed that the majority of $VSR_{PS-1}$-HA expressed in the yeast Δ*vps10* mutant strain is rapidly degraded, most likely because of inefficient transport and/or retention to the PVC. This demonstrates that yeast is not well suited for study of the *in vivo* function of plant VSRs and supports the idea that "plant cells are not just green yeast" (Bassham and Raikhel, 2000b).

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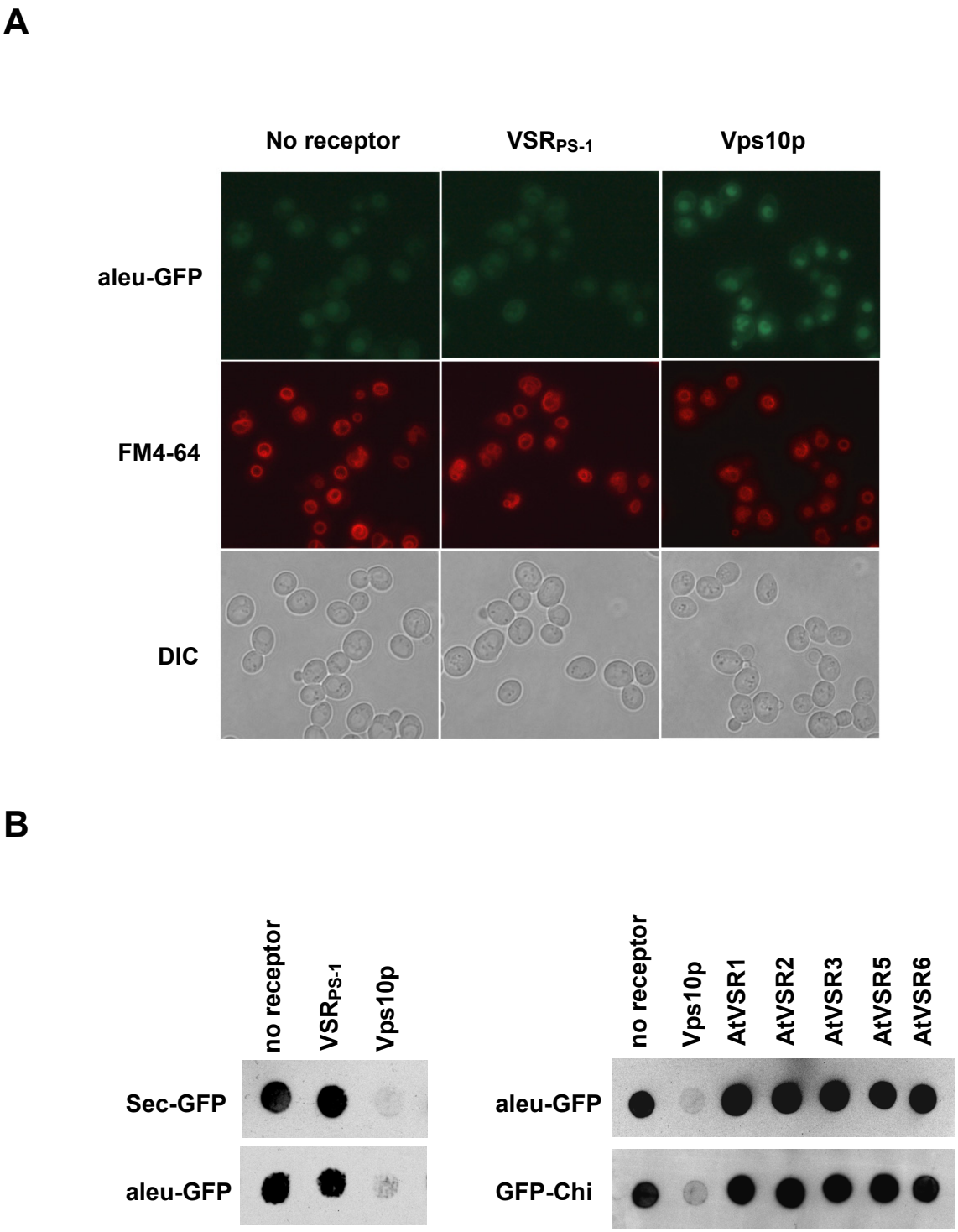


**Figure 1. $VSR_{PS-1}$ does not cause significant accumulation of aleu-GFP in the vacuole. (A)** The Δ*vps10* mutant containing the aleu-GFP construct was transformed with *VPS10* or $VSR_{PS-1}$ under the control of a GAL promoter. Cells were visualized by fluorescence microscopy during the exponential growth phase, after 24 hours of induction in galactose medium at 30°C. Pictures of GFP fluorescence, of FM4-64 staining vacuolar membranes and of intact cells by differential interference contrast (DIC) are

shown. **(B)** Sec-GFP, aleu-GFP or GFP-Chi fusion proteins were co-expressed with a plant VSR or Vps10p in the Δ*vps10* mutant using a GAL promoter, as indicated. Equal amounts of each transformant were spotted on inducing galactose medium plates and covered with a nitrocellulose filter. After 24 hours at 30°C, the filters were removed and the secreted GFP constructs were detected by immunostaining.

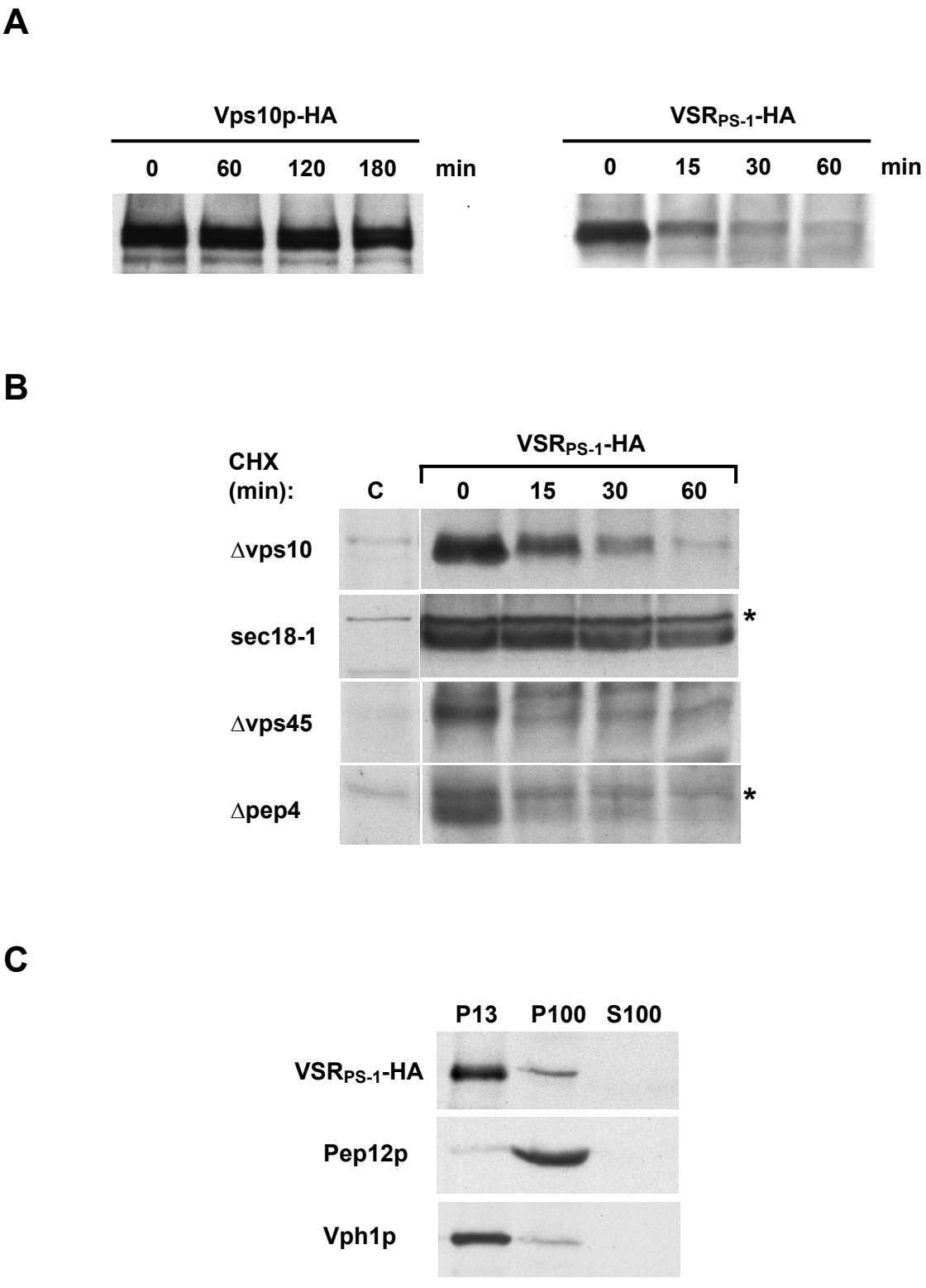


**Figure 2. $VSR_{PS-1}$ is rapidly degraded in yeast cells, does not travel through the PVC, and is not associated with Golgi or endosomal compartments. (A)** The HA-tagged $VSR_{PS-1}$ and HA-tagged Vps10p were expressed in the Δ*vps10* mutant. After 24 hours of induction, protein synthesis was blocked by the addition of cycloheximide (CHX). At the indicated times, cells were harvested, whole cell extracts prepared and subjected to immunoblot analysis. Proteins were detected by monoclonal HA antibodies. **B)**

The degradation of $VSR_{PS-1}$-HA is not prevented in mutants blocking the biosynthetic transport to the vacuole and in vacuolar protease deficient cells. $VSR_{PS-1}$-HA was expressed in the Δ*vps10*, *sec18-1*$^{ts}$, Δ*vsp45* and Δ*pep4* strains. Protein expression was induced at 30°C or at 37°C for the *sec18-1* mutant; cells were then treated with cycloheximide (CHX) and analyzed as described in the experimental procedure. C is the control of mutant cells transformed with the vector alone; (*) indicates the presence of non specific bands. **(C)** Subcellular localization of the plant receptor $VSR_{PS-1}$-HA in yeast cells. Δ*vps10* spheroplasts expressing $VSR_{PS-1}$-HA were gently lysed by osmotic shock. Subcellular fractionation was then performed by differential centrifugation to yield a low speed pellet (P13), a high speed pellet (P100) and a supernatant (S100). The amount of $VSR_{PS-1}$-HA, Pep12p (a PVC marker) and Vph1p (a vacuolar marker) in each of the fractions was assessed by immunoblotting.

## Supplemental information (Data provide online)

### Supplemental experimental procedure

#### *Materials, strains and growth conditions*

Yeast cells were grown in rich (YPD) or synthetic media (SD) with appropriate supplements (Sherman, 1991) at the indicated temperatures. Protein expression under the GAL promoter was induced in synthetic medium containing 2% galactose and 1% raffinose (SGal medium). Yeast transformation was accomplished using standard methods (Ausubel et al., 1987-1995). The *S. cerevisiae* strains used in this study are EMY3 (*Matα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 vps10Δ1::HIS3*) (Marcusson et al., 1994), RSY372 (*Matα leu2-3,112 ura3-52 sec 18-1)* (R. Schekman; lab collection*),* CCY120 (*Matα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 vps45Δ2::HIS3*) (Burd et al., 1997) and EHY191 (*Mat**a** leu2-3,112 ura3-1 his3-11 trp1-Δ ade2-1 pep4Δ::TRP1 TPI::SUC2::HIS3*) (Harsay and Schekman, 2002).

*Escherichia coli* strain XL1-Blue was used to amplify plasmid DNA. Plasmid isolation from transformed bacteria and recombinant DNA manipulations were done using standard procedures (Sambrook et al., 1989). Enzymes for the manipulation of DNA were purchased from New England Biolabs (Beverly, MA). Zymolyase and CHX were purchased from Seikagaku Corporation (Tokyo) and Sigma-Aldrich Co (St. Louis, MO), respectively. Mouse anti-Pep12p (1:5,000), mouse anti-Vph1p (1:5,000) and rabbit anti-GFP (1:10,000) antibodies were obtained from Molecular Probes Inc. (Eugene, OR). Anti-VSR monoclonal antibodies (17F9; (Cao et al., 2000)) were kindly provided by John C. Rogers (Pullman, WA). Mouse 12CA5 anti-hemagglutinin (HA) antibodies were purchased from Berkeley Antibodies (Richmond, CA).

#### *Plasmids and DNA cloning*

The construction of pYWG1-Sec-GFP, pYWG1-aleu-GFP, pYWG1-GFP-Chi and pGAL-BglII-VSR-PS1 plasmids has been previously described (Humair et al., 2001). The pRS316-VPS10-HA plasmid was described in Deloche et al. (2001). The influenza HA epitope-tagged VSR-PS1 allele was constructed by first inserting a NotI site by PCR just upstream of the VSR-PS1 stop codon in the pGAL-BglII-VSR-PS1 plasmid. A NotI fragment containing three copies of the HA epitope (YPYDVPDYA) was then ligated into the NotI site to give pGal ΔBglII-VSR-PS1-HA.

The five *A. thaliana* VSRs (AtVSR 1,2,3,5,6) were cloned under the GAL promoter. The cDNA containing the open reading frame (ORF) of AtVSR1 with 242 base pairs (bp) upstream and 172 bp downstream from pBluescript Z38123 (Paris et al, 1997) was subcloned into the

pGAL-BglII vector. The cDNA for AtVSR2 (ORF with 132 bp downstream) was amplified by PCR from the pSPORT 1 MJ447 plasmid (Paris et al., 1997), and subsequently cloned into the pGAL-BglIII vector. The cDNAs of AtVSR3 (ORF with 60 bp upstream and 18 bp downstream), AtVSR5 (ORF with 17 bp upstream and 7 bp downstream) and AtVSR6 (ORF with 14 bp upstream and 11 bp downstream) (Ecotype Columbia) were amplified by RT-PCR (Qiagen OneStep RT-PCR Kit, Qiagen) and cloned into either the pBluescript II SK(+/-) (Stratagene) or pGEM-T Easy vector. Subsequently, the three amplified fragments were subcloned into the pGal ΔBglII vector. All PCR products were checked by sequencing.

***Fluorescence Microscopy***

Transformed cells were grown to exponential phase ($OD_{600}$ of 0.7) at 30°C in SD medium. Cells were washed and diluted to an $OD_{600}$ of 0.35 in SGal medium. Cells then were grown for 24 hours to an $OD_{600}$ of 1-1.5. The yeast vacuolar membrane was then stained using the lipophilic styryl dye FM4-64 (*N*-[3-triethylammoniumpropyl]-4-[*p*-diethylaminophenylhexatrienyl] pyridinium dibromide (Molecular Probes) (Humair et al., 2001). Cells were visualized using a Leica confocal laser microscope equipped with a Leica TCS 4 operating system.

***GFP Colony Blot Assay***

Cells containing the indicated receptor and GFP constructs were grown to exponential phase ($OD_{600}$ of 0.7) at 30°C in SD medium. Cells equivalent to 1 $OD_{600}$ unit were collected, washed twice with the SGal medium and subsequently spotted (4 µl) on SGal plates. The plates were overlaid with nitrocellulose filters (Schleicher and Schuell, Dassel Germany) and incubated at 30°C for 18-24 h. The nitrocellulose membranes were then removed and subjected to immunostaining with anti-GFP antibodies (1:5,000) as described in Roberts et al. (1991).

***Cycloheximide treatment and whole cell extracts***

Transformed cells were grown to exponential phase in SGal medium at 30°C as described above. CHX (1 µg /ml) was added to cultures at an $OD_{600}$ of 1. At the indicated times, 3 $OD_{600}$ units of cells were harvested and subjected to Post-Alkaline Protein Extraction (Kushnirov, 2000). Samples were then subjected to immunoblot and proteins were detected with monoclonal mouse 12CA5 anti-hemagglutinin (HA) antibody and using the ECL Western Blotting Analysis System (Amersham Biosciences). For the *sec18-1ts* mutant, cells were grown at 22°C and then shifted to 37°C for 30 minutes before the addition of CHX. For the use of the 17F9 monoclonal antibody, recognizing only the native $VSR_{PS-1}$ protein, cells were lysed by a glass bead procedure (modified from Romanos et al., 1992). Briefly, cells equivalent to 3 $OD_{600}$ units

were transferred to Eppendorf tubes and resuspended in 60 µl of 50 mM Tris-HCL pH 7.5, 10 mM EDTA, 1 mM PMSF and protease inhibitor cocktail (Roche). Then, a two-thirds volume of acid washed glass beads (0.45 - 0.5 mm, Sigma) was added and cells were lysed by vortexing four times for 90 seconds at full speed. Between each cycle, cells were frozen in liquid nitrogen and thawed at 40°C. Lysates were subsequently centrifuged for 1 min at 14,000 rpm in an Eppendorf microfuge, and supernatant mixed with 1 volume of Laemmli 2X buffer without ß- mercaptoethanol (Laemmli, 1970). Samples were subjected to immunoblot using the 17F9 monoclonal antibodies.

***Cell fractionation by differential centrifugation***

Cells were fractionated by differential centrifugation after osmotic lysis (Horazdovsky and Emr, 1993). Cells were grown to exponential phase ($OD_{600}$ of 0.7) at 30°C in SD-URA medium and induced as described above. Cells equivalent to 10 $OD_{600}$ units were harvested and converted to spheroplasts by treatment with zymolyase 20T (20 µg/OD, Seikagaku Corporation) in 1 ml of 0.8 M sorbitol, 10 mM Tris pH 7.5, 1 mM DTT, 20 mM $NaN_3$ at 30°C for 45 minutes. Spheroplasts were washed once in 0.5 ml of ice cold MES/0.8 M sorbitol buffer (0.1 M MES, pH 6.5, 0.8 M sorbitol, 0.5 m$MgCl_2$, 1 mM $NaN_3$, 1 mM EGTA, 0.2 mM DTT, 1 mM PMSF) and lysed in 0.5 ml of MES/0.25 M sorbitol Buffer containing a protease inhibitor cocktail (Roche). Whole cell extracts were subjected to centrifugation at 13,000 X g for 10 min to yield a P13 (pellet). The resulting supernatant fraction was subjected to centrifugation at 100,000 X g for 60 min, yielding P100 (pellet) and S100 (supernatant). Proteins were precipitated from the S100 with the use of 10 % trichloroacetic acid (TCA). Precipitated proteins, P13 and P100 pellets were resuspended in 60 µl of Laemmli buffer (Laemmli, 1970), and 10 µl each were subjected to immunoblot analysis.

## Appendix 2. Sequences of the VSRs cloned in this study

### 1) Sequence of $VSR_{PS-1}$ (cDNA AC: U79958)

```
   1 GAATTCGGCA CGAGAAAAAA ATGAAGTGTT GGAGATTGTC GGCGATTCTG TTTCTAGGGT
                         M  K  C  W   R  L  S   A  I  L   F  L  G  F

  61 TTATGTTGAC TAGCTTGTCA ACGGCGAGAT TCGTGGTGGA GAAGAACAGT TTGTCGGTCA
     M  L  T   S  L  S   T  A  R  F   V  V  E   K  N  S   L  S  V  T
                                     ------>  VSR_PS-1 NT

 121 CTTCGCCGGA GAAAATCAAA GGCAAGCACG ACAGTGCCAT CGGTAACTTC GGAATACCTC
     S  P  E   K  I  K   G  K  H  D   S  A  I   G  N  F   G  I  P  Q

 181 AATACGGTGG TAGCATGGCC GGAAACGTTG TTTACCCTAA AGATAATAGT AAGGGTTGTA
     Y  G  G   S  M  A   G  N  V  V   Y  P  K   D  N  S   K  G  C  K

 241 AAGACTTCGA TTCATCCTTC AAATCACGCC CTGGTGCTCT TCCCACTATC CTTTTGCTCG
     D  F  D   S  S  F   K  S  R  P   G  A  L   P  T  I   L  L  L  D

 301 ATCGTGGAAG TTGCTTCTTT GCTTTGAAGG TTTGGAATGC TCAGAAGGCT GGAGCTTCAG
     R  G  S   C  F  F   A  L  K  V   W  N  A   Q  K  A   G  A  S  A

 361 CGGTTCTTGT TGCGGATGAT ATAGAGGAAC CGTTGATTAC TATGGATACG CCGGAAGAGG
     V  L  V   A  D  D   I  E  E  P   L  I  T   M  D  T   P  E  E  D

 421 ATGTATCTTC TGCTAAGTAC ATTGAGAATA TTACAATACC TTCTGCTCTT ATTGGAAAGA
     V  S  S   A  K  Y   I  E  N  I   T  I  P   S  A  L   I  G  K  S

 481 GTTTTGGTGA GAAGTTGAAG GATGCTATCA GTGGTGGGGA TATGGTTAAT GTTAATCTTG
     F  G  E   K  L  K   D  A  I  S   G  G  D   M  V  N   V  N  L  D

 541 ACTGGAGAGA GGCTGTTCCT CACCCTGATG ATCGTGTTGA GTATGAGTTG TGGACAAATA
     W  R  E   A  V  P   H  P  D  D   R  V  E   Y  E  L   W  T  N  S

 601 GTAATGATGA GTGTGGTGTT AAATGTGATA TGTTGATTGA GTTTTTGAAG GATTTTAAGG
     N  D  E   C  G  V   K  C  D  M   L  I  E   F  L  K   D  F  K  G

 661 GTGCGGCGCA GATTTTAGAG AAAGGAGGTT ATACTCAGTT TACACCTCAT TATATAACTT
     A  A  Q   I  L  E   K  G  G  Y   T  Q  F   T  P  H   Y  I  T  W

 721 GGTATTGTCC TCATGCATTT ACGTTGAGTA AGCAGTGTAA GTCTCAATGT ATAAACCATG
     Y  C  P   H  A  F   T  L  S  K   Q  C  K   S  Q  C   I  N  H  G

 781 GAAGATATTG CGCTCCGGAT CCTGAGCAGG ACTTCAACAC AGGTTATGAT GGAAAAGATG
     R  Y  C   A  P  D   P  E  Q  D   F  N  T   G  Y  D   G  K  D  V

 841 TGGTTGTTGA AAATTTAAGG CAGCTATGTG TTTTCAAGGT GGCAAAAGAA ACAGAAAAGT
     V  V  E   N  L  R   Q  L  C  V   F  K  V   A  K  E   T  E  K  S

 901 CTTGGGTGTG GTGGGACTAT GTTACTGATT TTCAAATTAG ATGCCCGATG AAGGAGAAAA
     W  V  W   W  D  Y   V  T  D  F   Q  I  R   C  P  M   K  E  K  K

 961 AGTACAATAA GGAATGTGCA AATTCCGTCA TTAAATCTCT GGGTCTTGAC GTCGAAAAGA
     Y  N  K   E  C  A   N  S  V  I   K  S  L   G  L  D   V  E  K  I

1021 TTGACAAGTG CATGGGAGAT CCGAATGCTG ATACTGAAAA TTCCATTTTA AAAGAAGAGC
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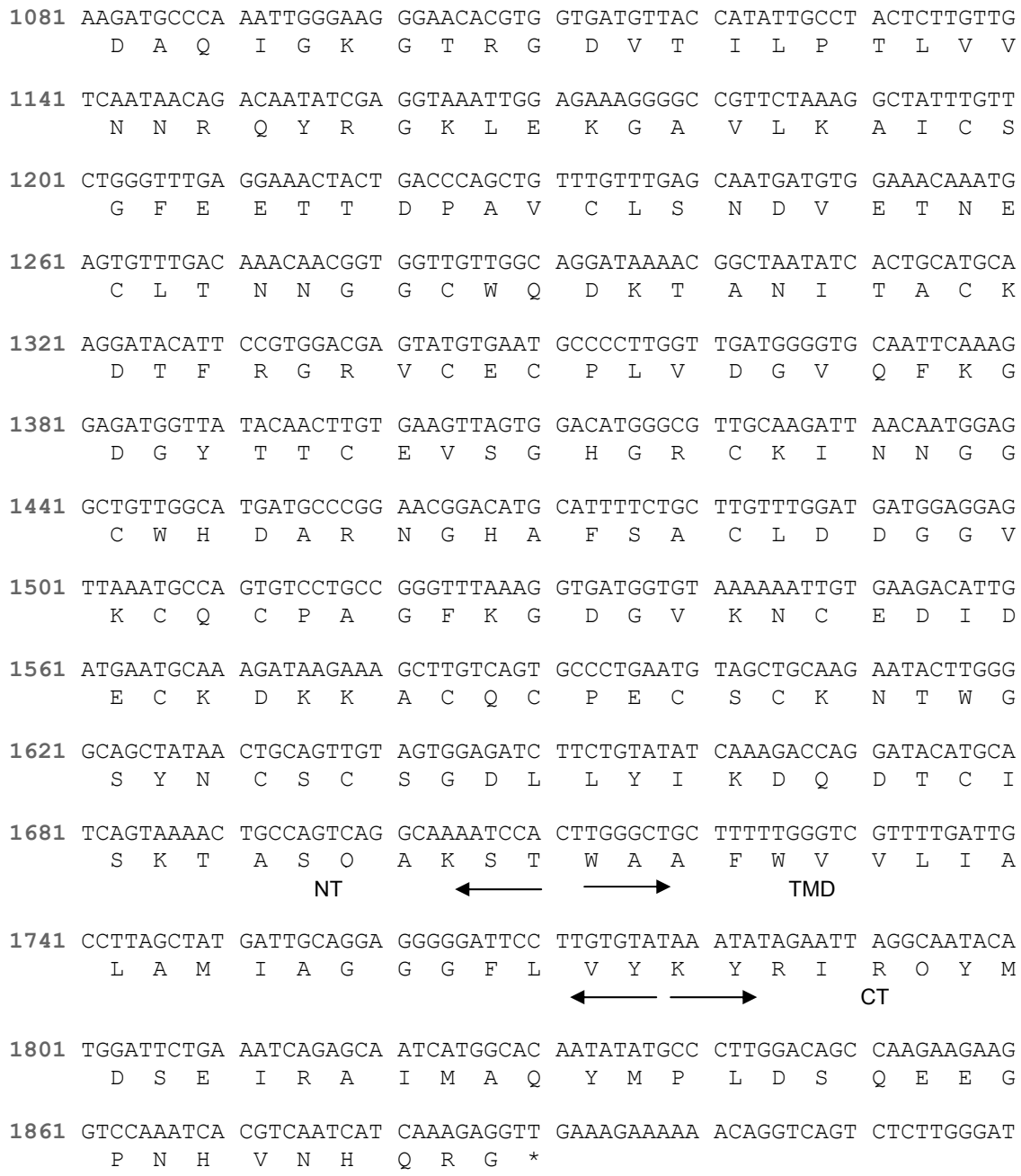


**Note:** The arrows define the beginning and ending of the N-luminal domain (NT), transmembrane domain (TMD) and cytosolic tail domain (CT) of $VSR_{PS-1}$.

## 2) Sequence of AtVSR1 (cDNA AC: ATU79959= Z38123)

```
   1 GATTGGGTAT TAAGTTGTAA AGGAAGGAAC TTGGAATCAT GAAGCTTGGG CTTTTCACTC
                                          M   K   L   G   L   F   T   L

  61 TCTCGTTTCT TCTGATCTTG AATCTAGCAA TGGGTAGATT CGTTGTTGAG AAGAACAATC
       S   F   L   L   I   L   N   L   A   M   G   R   F   V   V   E   K   N   N   L

 121 TCAAAGTTAC ATCACCTGAT TCGATCAAAG GTATTTACGA ATGTGCCATT GGTAATTTCG
       K   V   T   S   P   D   S   I   K   G   I   Y   E   C   A   I   G   N   F   G

 181 GAGTTCCTCA ATACGGTGGT ACTTTAGTCG GCACCGTCGT CTATCCTAAA TCCAATCAGA
       V   P   Q   Y   G   G   T   L   V   G   T   V   V   Y   P   K   S   N   Q   K

 241 AAGCTTGTAA AAGCTACTCC GATTTCGATA TCTCCTTCAA ATCCAAACCT GGACGATTAC
       A   C   K   S   Y   S   D   F   D   I   S   F   K   S   K   P   G   R   L   P

 301 CAACTTTTGT CCTTATCGAT CGTGGAGATT GTTACTTCAC TTTGAAAGCA TGGATAGCTC
       T   F   V   L   I   D   R   G   D   C   Y   F   T   L   K   A   W   I   A   Q

 361 AACAAGCTGG AGCAGCAGCG ATACTTGTAG CTGATAGTAA AGCTGAGCCA TTGATTACAA
       Q   A   G   A   A   A   I   L   V   A   D   S   K   A   E   P   L   I   T   M

 421 TGGATACACC TGAAGAAGAT AAATCTGATG CGGATTATCT TCAGAACATT ACCATTCCTT
       D   T   P   E   E   D   K   S   D   A   D   Y   L   Q   N   I   T   I   P   S

 481 CTGCTCTCAT TACTAAAACA TTGGGAGACA GTATAAAGTC TGCTCTTTCC GGTGGTGATA
       A   L   I   T   K   T   L   G   D   S   I   K   S   A   L   S   G   G   D   M

 541 TGGTTAACAT GAAGCTAGAT TGGACTGAGT CGGTTCCACA TCCTGATGAG CGAGTAGAGT
       V   N   M   K   L   D   W   T   E   S   V   P   H   P   D   E   R   V   E   Y

 601 ATGAACTGTG GACTAATAGC AATGACGAGT GTGGGAAGAA GTGTGATACT CAGATTGAGT
       E   L   W   T   N   S   N   D   E   C   G   K   K   C   D   T   Q   I   E   F

 661 TTCTCAAGAA TTTTAAAGGA GCTGCTCAGA TTCTTGAGAA AGGTGGGCAT ACTCAGTTCA
       L   K   N   F   K   G   A   A   Q   I   L   E   K   G   G   H   T   Q   F   T

 721 CGCCACATTA TATTACTTGG TACTGTCCTG AAGCGTTTAC GTTGAGTAAA CAGTGTAAGT
       P   H   Y   I   T   W   Y   C   P   E   A   F   T   L   S   K   Q   C   K   S

 781 CTCAGTGCAT CAACCATGGA AGGTATTGTG CGCCTGATCC TGAGCAGGAT TTTACGAAAG
       Q   C   I   N   H   G   R   Y   C   A   P   D   P   E   Q   D   F   T   K   G

 841 GGTATGATGG AAAGGATGTT GTTGTTCAGA ATCTACGTCA GGCTTGTGTC TACAGAGTGA
       Y   D   G   K   D   V   V   V   Q   N   L   R   Q   A   C   V   Y   R   V   M

 901 TGAATGATAC CGGTAAGCCG TGGGTCTGGT GGGACTATGT GACTGACTTT GCTATCCGGT
       N   D   T   G   K   P   W   V   W   W   D   Y   V   T   D   F   A   I   R   C

 961 GTCCAATGAA GGAGAAGAAG TACACCAAGG AATGCGCAGA TGGAATTATT AAGTCCCTTG
       P   M   K   E   K   K   Y   T   K   E   C   A   D   G   I   I   K   S   L   G

1021 GCATTGATCT CAAAAAGGTG GACAAGTGTA TCGGAGACCC TGAAGCAGAT GTGGAGAACC
       I   D   L   K   K   V   D   K   C   I   G   D   P   E   A   D   V   E   N   P

1081 CAGTTCTTAA AGCAGAGCAG GAGTCACAGA TAGGCAAAGG TTCCCGTGGA GACGTGACTA
       V   L   K   A   E   Q   E   S   Q   I   G   K   G   S   R   G   D   V   T   I
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       L  P  A   L  V  V   N  N  R  Q   Y  R  G   K  L  E   K  G  A  V

1201 TGCTTAAGGC TATGTGTTCG GGTTTTCAAG AGTCAACGGA ACCAGCTATC TGTCTTACTG
       L  K  A   M  C  S   G  F  Q  E   S  T  E   P  A  I   C  L  T  E

1261 AAGATTTGGA AACTAATGAA TGTTTGGAAA ACAACGGTGG ATGCTGGCAA GACAAAGCTG
       D  L  E   T  N  E   C  L  E  N   N  G  G   C  W  Q   D  K  A  A

1321 CCAACATTAC TGCATGCAGG GATACTTTTA GGGGAAGATT GTGTGAGTGC CCTACTGTTC
       N  I  T   A  C  R   D  T  F  R   G  R  L   C  E  C   P  T  V  Q

1381 AAGGTGTTAA ATTCGTTGGT GACGGTTACA CTCACTGCAA AGCCTCTGGA GCTTTGCATT
       G  V  K   F  V  G   D  G  Y  T   H  C  K   A  S  G   A  L  H  C

1441 GTGGTATCAA CAATGGAGGA TGCTGGAGAG AATCCCGAGG TGGCTTCACT TACTCTGCTT
       G  I  N   N  G  G   C  W  R  E   S  R  G   G  F  T   Y  S  A  C

1501 GCGTAGATGA TCATTCAAAG GATTGCAAAT GCCCACTTGG GTTCAAGGGC GATGGAGTGA
      V  D  D   H  S  K   D  C  K  C   P  L  G   F  K  G   D  G  V  K

1561 AGAACTGTGA AGATGTGGAC GAGTGCAAAG AAAAAACGGT GTGCCAGTGC CCAGAGTGTA
       N  C  E   D  V  D   E  C  K  E   K  T  V   C  Q  C   P  E  C  K

1621 AATGTAAAAA CACTTGGGGA AGTTATGAAT GCAGCTGCAG CAACGGTTTG CTTTACATGC
       C  K  N   T  W  G   S  Y  E  C   S  C  S   N  G  L   L  Y  M  R

1681 GTGAGCACGA CACTTGCATA GGTTCAGGCA AAGTTGGAAC CACAAAACTC AGCTGGAGCT
       E  H  D   T  C  I   G  S  G  K   V  G  T   T  K  L   S  W  S  F

1741 TTTTGTGGAT CCTTATAATC GGGGTGGGTG TTGCAGGTCT TTCTGGATAT GCAGTCTACA
       L  W  I   L  I  I   G  V  G  V   A  G  L   S  G  Y   A  V  Y  K

1801 AATACAGAAT CAGGAGTTAC ATGGATGCGG AAATTAGAGG GATCATGGCA CAGTACATGC
       Y  R  I   R  S  Y   M  D  A  E   I  R  G   I  M  A   Q  Y  M  P

1861 CATTGGAAAG TCAACCACCC AACACAAGTG GTCACCATAT GGATATATGA GTGAAGTTAT
       L  E  S   Q  P  P   N  T  S  G   H  H  M   D  I  *
```

### 3) Sequence of AtVSR2 (cDNA ATU9960=MJ447)

```
   1 TCCTTTGAAT TTGGTGCGGA GATCTCGAAG ATGAAGCAGC TTCTGTGTTA TCTTCCATGG
                                       M  K  Q  L   L  C  Y   L  P  W

  61 CTGCTTCTTC TCTCTCTTGT GGTTTCCCCT TTTAGCGAGG CTAGATTCGT TGTGGAGAAG
     L  L  L  L   S  L  V   V  S  P   F  S  E  A   R  F  V   V  E  K

 121 AATAGTTTGT CGGTGACGTC GCCGGAGAGT ATAAAAGGAA CACATGATAG TGCAATTGGT
     N  S  L  S   V  T  S   P  E  S   I  K  G  T   H  D  S   A  I  G

 181 AACTTCGGGA TTCCTCAATA CGGTGGAAGT ATGGCTGGTA CGGTGGTTTA TCCGAAAGAG
     N  F  G  I   P  Q  Y   G  G  S   M  A  G  T   V  V  Y   P  K  E

 241 AATCAGAAAT CGTGTAAGGA ATTTAGCGAT TTCTCGATTT CGTTCAAGTC TCAGCCTGGT
     N  Q  K  S   C  K  E   F  S  D   F  S  I  S   F  K  S   Q  P  G

 301 GCTTTACCTA CTTTCCTCTT AGTTGATCGT GGAGATTGTT TCTTCGCTTT GAAGGTATGG
     A  L  P  T   F  L  L   V  D  R   G  D  C  F   F  A  L   K  V  W
```

```
361 AACGCACAGA AAGCAGGTGC TTCTGCTGTT CTTGTGGCTG ATAATGTTGA TGAGCCTTTG
    N  A  Q  K   A  G  A   S  A  V   L  V  A  D   N  V  D   E  P  L

421 ATTACAATGG ATACACCTGA AGAAGATGTT TCTTCTGCAA AGTATATCGA GAATATTACT
    I  T  M  D   T  P  E   E  D  V   S  S  A  K   Y  I  E   N  I  T

481 ATACCTTCTG CTCTTGTTAC TAAAGGTTTT GGTGAAAAGC TGAAGCAAGC TATTAGTGGA
    I  P  S  A   L  V  T   K  G  F   G  E  K  L   K  Q  A   I  S  G

541 GGTGATATGG TTAACTTGAA TCTTGACTGG AGAGAGGCTG TTCCACATCC TGATGACCGT
    G  D  M  V   N  L  N   L  D  W   R  E  A  V   P  H  P   D  D  R

601 GTTGAGTATG AGTTGTGGAC TAATAGTAAT GATGAATGTG GGGTTAAGTG TGATATGTTG
    V  E  Y  E   L  W  T   N  S  N   D  E  C  G   V  K  C   D  M  L

661 ATGGAGTTTG TGAAGGATTT TAAGGGAGCG GCGCAGATTC TTGAGAAAGG CGGTTTTACG
    M  E  F  V   K  D  F   K  G  A   A  Q  I  L   E  K  G   G  F  T

721 CAGTTTAGGC CTCATTATAT TACTTGGTAT TGTCCTCATG CTTTCACGTT GAGTCGACAG
    Q  F  R  P   H  Y  I   T  W  Y   C  P  H  A   F  T  L   S  R  Q

781 TGTAAGTCTC AGTGTATCAA TAAAGGAAGG TACTGTGCTC CTGATCCAGA GCAGGACTTT
    C  K  S  Q   C  I  N   K  G  R   Y  C  A  P   D  P  E   Q  D  F

841 AGCTCGGGAT ACGATGGAAA AGACGTGGTT GTGGAAAATT TGAGACAGCT TTGTGTTTAC
    S  S  G  Y   D  G  K   D  V  V   V  E  N  L   R  Q  L   C  V  Y

901 AAGGTGGCGA ATGAAACCGG CAAACCTTGG GTCTGGTGGG ATTATGTTAC TGATTTCCAG
    K  V  A  N   E  T  G   K  P  W   V  W  W  D   Y  V  T   D  F  Q

961 ATCAGATGTC CAATGAAGGA GAAAAAATAC AACAAAGATT GTGCTGAGTC TGTAATCAAA
    I  R  C  P   M  K  E   K  K  Y   N  K  D  C   A  E  S   V  I  K

1021 TCTCTTGGAA TCGATAGCAG AAAAATTGAC AAGTGTATGG GAGACCCTGA TGCTGACTTG
     S  L  G  I   D  S  R   K  I  D   K  C  M  G   D  P  D   A  D  L

1081 GACAATCCAG TTTTAAAGGA AGAACAAGAT GCTCAAGTTG GCAAGGGTAC AAGGGGTGAT
     D  N  P  V   L  K  E   E  Q  D   A  Q  V  G   K  G  T   R  G  D

1141 GTTACCATAT TGCCTACCTT AGTTGTCAAC AACAGACAGT ACCGAGGCAA GTTGGAGAAG
     V  T  I  L   P  T  L   V  V  N   N  R  Q  Y   R  G  K   L  E  K

1201 AGTGCAGTAC TCAAGGCTCT ATGCTCTGGT TTTGAGGAGT CAACTGAACC AGCTATATGC
     S  A  V  L   K  A  L   C  S  G   F  E  E  S   T  E  P   A  I  C

1261 CTCAGCACAG ATATGGAGAC AAACGAGTGC TTAGATAACA ATGGCGGTTG TTGGCAAGAT
     L  S  T  D   M  E  T   N  E  C   L  D  N  N   G  G  C   W  Q  D

1321 AAATCAGCCA ACATAACTGC TTGCAAGGAT ACGTTTCGTG GAAAAGTATG CGTGTGTCCT
     K  S  A  N   I  T  A   C  K  D   T  F  R  G   K  V  C   V  C  P

1381 ATAGTTGATG GTGTGCGATT CAAAGGAGAT GGTTACAGCC ACTGTGAGCC AAGCGGGCCA
     I  V  D  G   V  R  F   K  G  D   G  Y  S  H   C  E  P   S  G  P

1441 GGGAGATGTA CAATCAACAA TGGAGGTTGT TGGCATGAAG AGAGAGATGG ACATGCGTTC
     G  R  C  T   I  N  N   G  G  C   W  H  E  E   R  D  G   H  A  F

1501 TCTGCTTGTG TGGACAAGGA CAGTGTGAAA TGCGAGTGTC CTCCAGGATT TAAAGGAGAC
     S  A  C  V   D  K  D   S  V  K   C  E  C  P   P  G  F   K  G  D
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1561 GGTGTTAAGA AATGTGAAGA CATCAATGAG TGCAAAGAGA AGAAAGCATG TCAGTGCCCG
     G  V  K  K   C  E  D   I  N  E   C  K  E  K   K  A  C   Q  C  P

1621 GAATGTAGCT GTAAGAACAC CTGGGGAAGC TATGAGTGCT CTTGTAGCGG GGACCTTCTC
     E  C  S  C   K  N  T   W  G  S   Y  E  C  S   C  S  G   D  L  L

1681 TACATGAGAG ACCATGACAC TTGCATCAGC AAGACGGGTT CACAAGTGAA ATCAGCGTGG
     Y  M  R  D   H  D  T   C  I  S   K  T  G  S   Q  V  K   S  A  W

1741 GCGGGCGTTT GGCTTATAAT GTTATCATTG GGACTTGCAG CTGCTGGTGC ATACCTCGTT
     A  G  V  W   L  I  M   L  S  L   G  L  A  A   A  G  A   Y  L  V

1801 TACAAATATA GATTGAGGCA ATACATGGAC TCAGAGATCA GAGCCATAAT GGCACAGTAC
     Y  K  Y  R   L  R  Q   Y  M  D   S  E  I  R   A  I  M   A  Q  Y

1861 ATGCCACTGG ACAGCCAACC CGAGGTCCCG AACCACACGA ATGATGAACG TGCCTAAGAG
     M  P  L  D   S  Q  P   E  V  P   N  H  T  N   D  E  R   A  *
```

## 4) Sequence of AtVSR3 (AC: AT F18F4.210)

```
   1 TTGGTGGATT CTTCAGAGAT GGGTTTAGTC AACGGGAGAG CTTCGTTGAC CTTTCTCCTC
                        M   G  L  V   N  G  R  A   S  L  T   F  L  L

  61 GCGGCGTTGA CCATCATCGC TATGGTCGTC GAGGCTAGGT TTGTGGTGGA GAAAGAAAGC
     A  A  L  T   I  I  A   M  V  V   E  A  R  F   V  V  E   K  E  S

 121 ATAAGCGTGC TGAATCCAGA GGAGATGAGG TCGAAGCACG ACGGCTCGAT AGCCAATTTC
     I  S  V  L   N  P  E   E  M  R   S  K  H  D   G  S  I   A  N  F

 181 GGTTTACCCG ATTACGGTGG GTTTTTAATC GGGTCAGTGG TTTATCCGGA TAGTAAAACC
     G  L  P  D   Y  G  G   F  L  I   G  S  V  V   Y  P  D   S  K  T

 241 GATGGATGCT CTGCTTTTGG TAAAACCTTC AAGCCCAAGT TTCCTCGTCC CACTATTCTG
     D  G  C  S   A  F  G   K  T  F   K  P  K  F   P  R  P   T  I  L

 301 CTTCTTGATC GTGGAGGTTG CTACTTTGCC TTAAAAGCGT GGCACGCGCA GCAAGCAGGC
     L  L  D  R   G  G  C   Y  F  A   L  K  A  W   H  A  Q   Q  A  G

 361 GCGGCTGCAG TTCTTGTGGC GGATAATGTA GACGAGCCAT TGTTGACAAT GGATTCACCA
     A  A  A  V   L  V  A   D  N  V   D  E  P  L   L  T  M   D  S  P

 421 GAGGAGAGCA AAGATGCGGA TGGTTTCATA GAGAAGCTAA CAATCCCATC GGTGTTAATC
     E  E  S  K   D  A  D   G  F  I   E  K  L  T   I  P  S   V  L  I

 481 GATAAATCAT TTGGAGATGA CTTAAGACAA GGGTTTCAGA AAGGGAAAAA CATAGTTATA
     D  K  S  F   G  D  D   L  R  Q   G  F  Q  K   G  K  N   I  V  I

 541 AAACTAGATT GGAGAGAGTC TGTGCCTCAT CCTGATAAGA GAGTAGAATA TGAGCTGTGG
     K  L  D  W   R  E  S   V  P  H   P  D  K  R   V  E  Y   E  L  W

 601 ACTAATAGCA ATGATGAGTG TGGTGCACGG TGTGATGAAC AGATGGACTT TGTCAAGAAC
     T  N  S  N   D  E  C   G  A  R   C  D  E  Q   M  D  F   V  K  N

 661 TTTAAAGGTC ATGCTCAGAT ACTCGAAAAA GGCGGTTATA CCGCGTTTAC GCCGCATTAT
     F  K  G  H   A  Q  I   L  E  K   G  G  Y  T   A  F  T   P  H  Y

 721 ATTACTTGGT TTTGCCCTTT TCAGTTTATA AACAGTCCAC ATTGTAAGTC TCAGTGTATA
     I  T  W  F   C  P  F   Q  F  I   N  S  P  H   C  K  S   Q  C  I
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 781 AACCATGGGA GGTATTGTGC TCCTGACCCT GAGGATAATT TCAGAGAAGG GTATGAAGGG
     N  H  G  R  Y  C  A  P  D  P  E  D  N  F  R  E  G  Y  E  G

 841 AAAGATGTTG TGCTTGAGAA TCTGAGACAG CTTTGTGTGC ATAGAGTTGC GAATGAGAGT
     K  D  V  V  L  E  N  L  R  Q  L  C  V  H  R  V  A  N  E  S

 901 AGCAGGCCTT GGGTTTGGTG GGATTATGTT ACCGATTTTC ATTCTCGATG TTCGATGAAG
     S  R  P  W  V  W  W  D  Y  V  T  D  F  H  S  R  C  S  M  K

 961 GAGAAGAAAT ACAGCATAGA TTGTGCTGAG AGTGTCATCA AATCTCTGAA TTTACCTATT
     E  K  K  Y  S  I  D  C  A  E  S  V  I  K  S  L  N  L  P  I

1021 GAGAAGATCA AGAAATGCAT TGGTGATCCT GAGGCTGATA CAGAGAACCA AGTTCTGAGA
     E  K  I  K  K  C  I  G  D  P  E  A  D  T  E  N  Q  V  L  R

1081 ACTGAGCAAG TATCTCAGAT TGGCCGAGGA AACCGGGGAG ATGTTACGAT ATTGCCAACA
     T  E  Q  V  S  Q  I  G  R  G  N  R  G  D  V  T  I  L  P  T

1141 TTAGTCATCA ATAACGCTCA ATATCGAGGG AGATTGGAGA GAACCGCGGT TTTAAAGGCG
     L  V  I  N  N  A  Q  Y  R  G  R  L  E  R  T  A  V  L  K  A

1201 ATATGCGCTG GTTTTAATGA AACATCGGAG CCTGCCATTT GCTTAAACAC AGGTCTAGAG
    I  C  A  G  F  N  E  T  S  E  P  A  I  C  L  N  T  G  L  E

1261 ACAAATGAGT GCCTTGAAAA CAATGGTGGT TGCTGGCAGG ATACAAAAGC AAACATCACT
     T  N  E  C  L  E  N  N  G  G  C  W  Q  D  T  K  A  N  I  T

1321 GCTTGTCAAG ACACATTCAG AGGAAGACTC TGCGAGTGTC CGGTTGTAAA AGGTGTTCAA
     A  C  Q  D  T  F  R  G  R  L  C  E  C  P  V  V  K  G  V  Q

1381 TATAAAGGAG ACGGGTACAC TTCATGTACA CCTTATGGGC CTGCGAGGTG TACTATGAAC
     Y  K  G  D  G  Y  T  S  C  T  P  Y  G  P  A  R  C  T  M  N

1441 AATGGAGGTT GCTGGTCTGA CACAAGGAAC GGCTTAACTT TCTCTGCTTG CTCAGACTCT
     N  G  G  C  W  S  D  T  R  N  G  L  T  F  S  A  C  S  D  S

1501 GTATCTACTG GCTGCAAATG TCCTGAAGGT TTCCAAGGCG ACGGTTTGAC GTGTGAAGAT
     V  S  T  G  C  K  C  P  E  G  F  Q  G  D  G  L  T  C  E  D

1561 ATTAACGAAT GTAAAGAGCG TTCGGTATGT CAATGTAGCG GTTGCAGATG CAAGAACTCA
     I  N  E  C  K  E  R  S  V  C  Q  C  S  G  C  R  C  K  N  S

1621 TGGGGTGGAT ACAAATGCAG CTGTTCTGGT GACCGGCTTT ACATAAACGA TCAAGATACT
     W  G  G  Y  K  C  S  C  S  G  D  R  L  Y  I  N  D  Q  D  T

1681 TGTATAGAGA GATATGGATC CAAAACGGCA TGGTGGCTCA CATTCTTGAT ACTGGCTATC
     C  I  E  R  Y  G  S  K  T  A  W  W  L  T  F  L  I  L  A  I

1741 GTTGCAGTAG CCGGTTTAGC TGGTTATATA TTCTACAAAT ACCGGTTCAG GTCTTACATG
     V  A  V  A  G  L  A  G  Y  I  F  Y  K  Y  R  F  R  S  Y  M

1801 GACTCAGAGA TTATGACGAT CATGTCACAG TATATGCCAC TTGAGAGCCA AAGAGCTCGT
     D  S  E  I  M  T  I  M  S  Q  Y  M  P  L  E  S  Q  R  A  R

1861 GAAGTTCCAT CAGAAGCCGA GCCTTTTACA CTCTAATAGT ATATGACCAA AGTAACCAAA
     E  V  P  S  E  A  E  P  F  T  L  *

1921 ACTGTCATACACAAAGAAATGTTTGAGATCAATTAA
```

## 5) Sequence of AtVSR5 (AC: F19I3.17)

```
   1 GAAAAGAATG TCTCCGAGCA ATAAAGGAAC CGTCTTGGCT CTGATTCTAG CGTTGACCAT
           M   S  P  S  N   K  G  T   V  L  A   L  I  L  A   L  T  M

  61 GGTGGTGGTC AACGGGTTTT CATCGAGATT CTTCGTGGAG AAAAGCAGCT TGACGGTCCT
     V  V  V   N  G  F  S   S  R  F   F  V  E   K  S  S  L   T  V  L

 121 TAACTCATGG GAAATGGGAG CTAAGCACGA CGCGGCCATA GCAAACTTTG GTCTCCCAAA
     N  S  W   E  M  G  A   K  H  D   A  A  I   A  N  F  G   L  P  K

 181 GTACGGCGGT TTCATGATCG GCTCTGTGGT CTACGCAGGC CAAGACGCTT ACGGATGCAA
     Y  G  G   F  M  I  G   S  V  V   Y  A  G   Q  D  A  Y   G  C  N

 241 CTCTTTCAAC AAAACCTTCA ATACCAAGTC TCCTTATCCC AAAATTCTCC TCATTGATCG
     S  F  N   K  T  F  N   T  K  S   P  Y  P   K  I  L  L   I  D  R

 301 TGGAGTGTGT AACTTTGCTT TGAAGATATG GAACGGACAA CAATCCGGCG CAGCGGCTGT
     G  V  C   N  F  A  L   K  I  W   N  G  Q   Q  S  G  A   A  A  V

 361 TCTTTTAGCA GATAACATTG TTGAGCCATT GATAACAATG GATACACCCC AAGATGAAGA
     L  L  A   D  N  I  V   E  P  L   I  T  M   D  T  P  Q   D  E  D

 421 TCCTGACTTT ATAGACAAAG TCAAGATCCC ATCAGCTTTA ATCCTTCGCT CTTTCGGTGA
     P  D  F   I  D  K  V   K  I  P   S  A  L   I  L  R  S   F  G  D

 481 TAGCCTCAAG AAAGCTCTTA AAAGAGGTGA GGAGGTAATC TTGAAGATGG ATTGGAGTGA
     S  L  K   K  A  L  K   R  G  E   E  V  I   L  K  M  D   W  S  E

 541 GTCTATACCA AACCCTGATG AGAGAGTTGA GTATGAGCTA TGGGCTAATA CTAATGATGA
     S  I  P   N  P  D  E   R  V  E   Y  E  L   W  A  N  T   N  D  E

 601 ATGTGGTGTA CACTGCGATA AACAGATAGA TTTCATTAAG AACTTTAAGG GAATGGCTCA
     C  G  V   H  C  D  K   Q  I  D   F  I  K   N  F  K  G   M  A  Q

 661 GATTCTTGAA AAAGGCGGTT ATACTTTGTT CAGACCTCAC TACATTTCTT GGGTTTGTCC
     I  L  E   K  G  G  Y   T  L  F   R  P  H   Y  I  S  W   V  C  P

 721 CAAAGAGCTT CTACTTAGCA AGCAGTGTAG GACTCAGTGT ATAAACCAAG GGAGGTATTG
     K  E  L   L  L  S  K   Q  C  R   T  Q  C   I  N  Q  G   R  Y  C

 781 TGCTCTTGAT ACTAAGCAAG AATTTGAAGA TGGATATAAT GGGAAAGACG TCGTTTATGA
     A  L  D   T  K  Q  E   F  E  D   G  Y  N   G  K  D  V   V  Y  E

 841 GAATCTGAGG CAGTTATGTG TTCATAAAGT AGCTAAGGAG AAGAACACTT CTTGGGTTTG
     N  L  R   Q  L  C  V   H  K  V   A  K  E   K  N  T  S   W  V  W

 901 GTGGGACTAT GTGACAGATT TTAACATCAG GTGTTCTATG AAGGAGAAGA AATACAGCAG
     W  D  Y   V  T  D  F   N  I  R   C  S  M   K  E  K  K   Y  S  R

 961 AGAATGTGCA GAGACTATTG TGGAATCTCT CGGGCTGTCT CTTGAGAAGA TCAAGAAATG
     E  C  A   E  T  I  V   E  S  L   G  L  S   L  E  K  I   K  K  C

1021 CATTGGTGAT CCTGATGCTG ATGTAGAGAA TGAAGTTCTA AAAGCCGAGG AAGCTTTTCA
     I  G  D   P  D  A  D   V  E  N   E  V  L   K  A  E  E   A  F  Q

1081 GTTAGGCCAA GAGAATCGTG GCATTGTTAC AATCTTTCCT ACATTAATGA TCAACAATGC
     L  G  Q   E  N  R  G   I  V  T   I  F  P   T  L  M  I   N  N  A
```

```
1141 TCAATATCGC GGTAAACTGG AGAGAACCGC GGTGCTGAAG GCTATATGTT CAGGATTCAA
       Q  Y  R   G  K  L  E   R  T  A   V  L  K   A  I  C  S   G  F  K

1201 GGAAAGAACA GAACCGTCAA TATGTTTGAA TTCAGATATA GAGACCAATG AATGTCTTAT
       E  R  T   E  P  S  I   C  L  N   S  D  I   E  T  N  E   C  L  I

1261 AGAAAATGGA GGATGTTGGC AAGACAAAAG ATCCAATGTA ACTGCTTGCA AGGACACATT
       E  N  G   G  C  W  Q   D  K  R   S  N  V   T  A  C  K   D  T  F

1321 TAGGGGAAGA GTATGTGAGT GCCCGGTTGT CGATGGTGTT CAATATAAAG GAGATGGTTA
       R  G  R   V  C  E  C   P  V  V   D  G  V   Q  Y  K  G   D  G  Y

1381 TACCTCCTGC AAACCTTATG GACCTGCGAG ATGTTCAATG AACAATGGAG ACTGCTGGTC
       T  S  C   K  P  Y  G   P  A  R   C  S  M   N  N  G  D   C  W  S

1441 TGAAACCAGA AAGGGTCTAA CTTTCTCTTC TTGTTCAGAC TCAGAGACAT CAGGATGTCG
       E  T  R   K  G  L  T   F  S  S   C  S  D   S  E  T  S   G  C  R

1501 TTGCCCTCTA GGTTTCCTTG GAGATGGTCT AAAATGTGAA GACATTGATG AATGCAAAGA
       C  P  L   G  F  L  G   D  G  L   K  C  E   D  I  D  E   C  K  E

1561 GAAATCAGCT TGTAAATGTG ATGGCTGCAA ATGCAAGAAC AATTGGGGAG GATATGAATG
       K  S  A   C  K  C  D   G  C  K   C  K  N   N  W  G  G   Y  E  C

1621 CAAATGTTCT AACAATAGTA TCTACATGAA AGAAGAGGAC ACTTGTATCG AGAGAAGAAG
       K  C  S   N  N  S  I   Y  M  K   E  E  D   T  C  I  E   R  R  S

1681 TGGATCAAGA AGCAGAGGGT TGTTCACAAT TGTGGTTCTA ACCGCCATCG CGGGTATCTC
       G  S  R   S  R  G  L   F  T  I   V  V  L   T  A  I  A   G  I  S

1741 TTTAGGTGCT TATATATTCT ACAAGTACCA TCTTCAGTCA TACATGGATT CAGAGATCGT
       L  G  A   Y  I  F  Y   K  Y  H   L  Q  S   Y  M  D  S   E  I  V

1801 GTCCATTATG TCTCAGTACA TACCACTCGA TAGCCAAAGC ATTAACCAAG ACTCTTTTAA
       S  I  M   S  Q  Y  I   P  L  D   S  Q  S   I  N  Q  D   S  F  K

1861 GTAAATATAT AGGAACTCTT GTCATCTCAA
       *
```

## 6) Sequence of AtVSR6 (AC: F17F8.5)

```
   1 GATCAGAAAC AATGTCTTTG ATTCATAAAG GAGCCACCTT GGCTCTGTTT CTAGCGTTGA
                 M  S  L   I  H  K  G   A  T  L   A  L  F   L  A  L  T

  61 CTATGGTGGT CAACGGAGTT TTCGGGAGAT TCATCGTTGA GAAGAGTAGC GTGACGATTC
       M  V  V   N  G  V   F  G  R  F   I  V  E   K  S  S   V  T  I  L

 121 TAAACCCTTT GGCAATGCGG TCTAAGCACG ACGCAGCCAT TGCTAACTTC GGTGTTCCTA
       N  P  L   A  M  R   S  K  H  D   A  A  I   A  N  F   G  V  P  N

 181 ACTACGGTGG TTACATGATC GGCTCCGTCG TTTACGCCGG TCAAGGAGCT TATGGATGTG
       Y  G  G   Y  M  I   G  S  V  V   Y  A  G   Q  G  A   Y  G  C  D

 241 ACTCTTTTGA CAAAACTTTC AAACCCAAAT TCCCTCGTCC TACCATTTTG ATCATCGATC
       S  F  D   K  T  F   K  P  K  F   P  R  P   T  I  L   I  I  D  R

 301 GTGGAGAGTG TTACTTTGCA TTAAAGGTAT GGAACGGTCA ACAATCCGGT GTAGCAGCAG
       G  E  C   Y  F  A   L  K  V  W   N  G  Q   Q  S  G   V  A  A  V
```

```
 361 TTTTAGTAGC TGATAACGTC GATGAGCCAT TGATAACAAT GGATTCACCT GAGGAATCCA
       L  V  A    D  N  V    D  E  P  L    I  T  M    D  S  P    E  E  S  K

 421 AAGAAGCTGA TGACTTTATA GAGAAACTCA ACATTCCATC GGCTTTAATA GACTTTTCTT
       E  A  D    D  F  I    E  K  L  N    I  P  S    A  L  I    D  F  S  F

 481 TCGCCAATAC TCTCAAGCAA GCTCTTAAGA AAGGTGAGGA AGTAGTCCTG AAGATAGACT
       A  N  T    L  K  Q    A  L  K  K    G  E  E    V  V  L    K  I  D  W

 541 GGAGTGAGTC ATTACCTCAT CCGGATGAGA GAGTTGAGTA TGAGCTATGG ACTAACACGA
       S  E  S    L  P  H    P  D  E  R    V  E  Y    E  L  W    T  N  T  N

 601 ACGATGAGTG TGGTGCACGG TGTGATGAGC AGATGAATTT CGTAAAAAAC TTCAAAGGAC
       D  E  C    G  A  R    C  D  E  Q    M  N  F    V  K  N    F  K  G  H

 661 ATGCGCAGAT TCTTGAAAAA GGAGGATACT CTTTGTTCAC ACCTCATTAC ATTACATGGT
       A  Q  I    L  E  K    G  G  Y  S    L  F  T    P  H  Y    I  T  W  F

 721 TTTGTCCTAA AGATTATGTT TCTAGCAATC AATGTAAGTC TCAGTGTATA AACCAAGGGA
       C  P  K    D  Y  V    S  S  N  Q    C  K  S    Q  C  I    N  Q  G  R

 781 GGTATTGTGC TCCTGACCCT GAACAAGACT TTGGTGATGG ATACGATGGT AAAGACATTG
       Y  C  A    P  D  P    E  Q  D  F    G  D  G    Y  D  G    K  D  I  V

 841 TCTTCGAGAA CTTGAGACAG TTGTGTGTTC ATAAAGTAGC TAAAGAGAAT AACCGGTCTT
       F  E  N    L  R  Q    L  C  V  H    K  V  A    K  E  N    N  R  S  W

 901 GGGTTTGGTG GGACTATGTG ACTGATTTTC ACATTAGATG TTCAATGAAG GAGAAGAAGT
       V  W  W    D  Y  V    T  D  F  H    I  R  C    S  M  K    E  K  K  Y

 961 ATAGCAAAGA ATGTGCAGAG AGAGTTGTTG AATCTCTAGG TTTGCCACTT GACAAGATCA
       S  K  E    C  A  E    R  V  V  E    S  L  G    L  P  L    D  K  I  K

1021 AGAAATGTAT TGGTGATCCT GATGCTAATG TGGAGAATGA AGTTTTGAAA GCTGAGCAAG
       K  C  I    G  D  P    D  A  N  V    E  N  E    V  L  K    A  E  Q  A

1081 CACTTCAGGT AGGACAAGGT GACCGCGGAG ATGTCACAAT CTTGCCAACA TTGATCGTCA
       L  Q  V    G  Q  G    D  R  G  D    V  T  I    L  P  T    L  I  V  N

1141 ACAATGCTCA ATACCGCGGT AAACTTGAGA GAAATGCAGT ACTTAAGGCT ATATGTTCTG
       N  A  Q    Y  R  G    K  L  E  R    N  A  V    L  K  A    I  C  S  G

1201 GATTCAAGGA AAGAACCGAA CCCGGGATCT GTCTAAGTGG AGATATTGAA ACAAATGAAT
       F  K  E    R  T  E    P  G  I  C    L  S  G    D  I  E    T  N  E  C

1261 GTCTCGAAGC AAATGGAGGG TGTTGGGAGG ACAAGAAGTC CAATGTAACA GCTTGCAAGG
       L  E  A    N  G  G    C  W  E  D    K  K  S    N  V  T    A  C  K  D

1321 ACACATTTAG AGGAAGAGTC TGTGAATGCC CTGTTGTGAA TGGTGTACAG TATAAAGGAG
       T  F  R    G  R  V    C  E  C  P    V  V  N    G  V  Q    Y  K  G  D

1381 ATGGATATAC ATCATGTGAA CCTTATGGCC CTGCAAGATG CTCGATTAAC CAAGGAGGTT
       G  Y  T    S  C  E    P  Y  G  P    A  R  C    S  I  N    Q  G  G  C

1441 GCTGGTCTGA AACCAAAAAG GGCTTAACTT TCTCGGCTTG CTCGAACTTG GAGACATCGG
       W  S  E    T  K  K    G  L  T  F    S  A  C    S  N  L    E  T  S  G

1501 GATGTCGCTG CCCTCCAGGG TTTAAAGGAG ATGGTCTTAA ATGTGAAGAC ATTGATGAGT
       C  R  C    P  P  G    F  K  G  D    G  L  K    C  E  D    I  D  E  C
```

```
1561 GTAAGGAGCA ATCAGCTTGT CAATGTGATG GATGCAACTG TAAGAACAAA TGGGGAGGCT
       K  E  Q   S  A  C   Q  C  D  G   C  N  C   K  N  K   W  G  G  F

1621 TTGAATGCAA ATGCTCTGGA AATCGTCTCT ACATGAAAGA ACAAGACACT TGTATTGAGA
       E  C  K   C  S  G   N  R  L  Y   M  K  E   Q  D  T   C  I  E  R

1681 GAAGCGGATC AAGAATCGGA TGGTTCCCTA CATTTGTGAT TCTAGCTGCA GTTGCAAGCA
       S  G  S   R  I  G   W  F  P  T   F  V  I   L  A  A   V  A  S  I

1741 TATGTGTAGG TGGTTACGTA TTCTACAAGT ATCGTCTCAG GTCTTATATG GATTCAGAAA
       C  V  G   G  Y  V   F  Y  K  Y   R  L  R   S  Y  M   D  S  E  I

1801 TCATGGCGAT TATGTCTCAG TACATGCCAT TAGAGAGCCA AAACACAACC GATCCAATGA
       M  A  I   M  S  Q   Y  M  P  L   E  S  Q   N  T  T   D  P  M  T

1861 CTGGTGAATC TCAACACCAA CAGCTGAGAT TAACTTCTGC AGCCTAATTT TATAAATCTT
       G  E  S   Q  H  Q   Q  L  R  L   T  S  A   A  *
```

## Appendix 3. Sequence of $VSR_{PS-1}$-Vps10p-HA

```
  1 ATGAAGTGTT GGAGATTGTC GGCGATTCTG TTTCTAGGGT TTATGTTGAC TAGCTTGTCA
    M  K  C  W   R  L  S   A  I  L   F  L  G  F   M  L  T   S  L  S

 61 ACGGCGAGAT TCGTGGTGGA GAAGAACAGT TTGTCGGTCA CTTCGCCGGA GAAAATCAAA
    T  A  R  F   V  V  E   K  N  S   L  S  V  T   S  P  E   K  I  K
          ──────►  VSR_PS-1 NT

121 GGCAAGCACG ACAGTGCCAT CGGTAACTTC GGAATACCTC AATACGGTGG TAGCATGGCC
    G  K  H  D   S  A  I   G  N  F   G  I  P  Q   Y  G  G   S  M  A

181 GGAAACGTTG TTTACCCTAA AGATAATAGT AAGGGTTGTA AAGACTTCGA TTCATCCTTC
    G  N  V  V   Y  P  K   D  N  S   K  G  C  K   D  F  D   S  S  F

241 AAATCACGCC CTGGTGCTCT TCCCACTATC CTTTTGCTCG ATCGTGGAAG TTGCTTCTTT
    K  S  R  P   G  A  L   P  T  I   L  L  L  D   R  G  S   C  F  F

301 GCTTTGAAGG TTTGGAATGC TCAGAAGGCT GGAGCTTCAG CGGTTCTTGT TGCGGATGAT
    A  L  K  V   W  N  A   Q  K  A   G  A  S  A   V  L  V   A  D  D

361 ATAGAGGAAC CGTTGATTAC TATGGATACG CCGGAAGAGG ATGTATCTTC TGCTAAGTAC
    I  E  E  P   L  I  T   M  D  T   P  E  E  D   V  S  S   A  K  Y

421 ATTGAGAATA TTACAATACC TTCTGCTCTT ATTGGAAAGA GTTTTGGTGA GAAGTTGAAG
    I  E  N  I   T  I  P   S  A  L   I  G  K  S   F  G  E   K  L  K

481 GATGCTATCA GTGGTGGGGA TATGGTTAAT GTTAATCTTG ACTGGAGAGA GGCTGTTCCT
    D  A  I  S   G  G  D   M  V  N   V  N  L  D   W  R  E   A  V  P

541 CACCCTGATG ATCGTGTTGA GTATGAGTTG TGGACAAATA GTAATGATGA GTGTGGTGTT
    H  P  D  D   R  V  E   Y  E  L   W  T  N  S   N  D  E   C  G  V

601 AAATGTGATA TGTTGATTGA GTTTTTGAAG GATTTTAAGG GTGCGGCGCA GATTTTAGAG
    K  C  D  M   L  I  E   F  L  K   D  F  K  G   A  A  Q   I  L  E

661 AAAGGAGGTT ATACTCAGTT TACACCTCAT TATATAACTT GGTATTGTCC TCATGCATTT
    K  G  G  Y   T  Q  F   T  P  H   Y  I  T  W   Y  C  P   H  A  F

721 ACGTTGAGTA AGCAGTGTAA GTCTCAATGT ATAAACCATG GAAGATATTG CGCTCCGGAT
    T  L  S  K   Q  C  K   S  Q  C   I  N  H  G   R  Y  C   A  P  D
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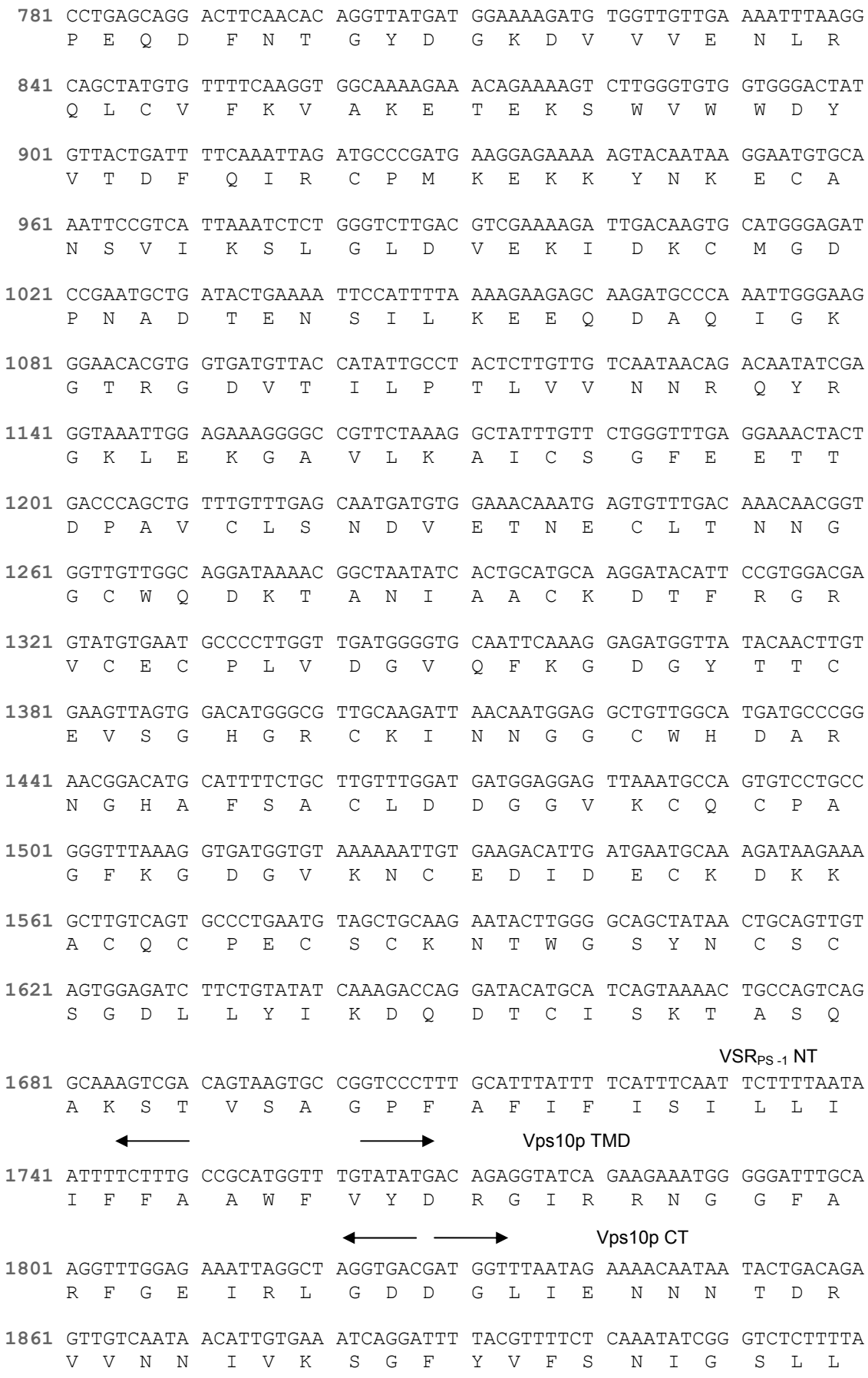
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P E Q D F N T G Y D G K D V V V E N L R
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Q L C V F K V A K E T E K S W V W W D Y
901 GTTACTGATT TTCAAATTAG ATGCCCGATG AAGGAGAAAA AGTACAATAA GGAATGTGCA
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961 AATTCCGTCA TTAAATCTCT GGGTCTTGAC GTCGAAAAGA TTGACAAGTG CATGGGAGAT
N S V I K S L G L D V E K I D K C M G D
1021 CCGAATGCTG ATACTGAAAA TTCCATTTTA AAAGAAGAGC AAGATGCCCA AATTGGGAAG
P N A D T E N S I L K E E Q D A Q I G K
1081 GGAACACGTG GTGATGTTAC CATATTGCCT ACTCTTGTTG TCAATAACAG ACAATATCGA
G T R G D V T I L P T L V V N N R Q Y R
1141 GGTAAATTGG AGAAAGGGGC CGTTCTAAAG GCTATTTGTT CTGGGTTTGA GGAAACTACT
G K L E K G A V L K A I C S G F E E T T
1201 GACCCAGCTG TTTGTTTGAG CAATGATGTG GAAACAAATG AGTGTTTGAC AAACAACGGT
D P A V C L S N D V E T N E C L T N N G
1261 GGTTGTTGGC AGGATAAAAC GGCTAATATC ACTGCATGCA AGGATACATT CCGTGGACGA
G C W Q D K T A N I A A C K D T F R G R
1321 GTATGTGAAT GCCCCTTGGT TGATGGGGTG CAATTCAAAG GAGATGGTTA TACAACTTGT
V C E C P L V D G V Q F K G D G Y T T C
1381 GAAGTTAGTG GACATGGGCG TTGCAAGATT AACAATGGAG GCTGTTGGCA TGATGCCCGG
E V S G H G R C K I N N G G C W H D A R
1441 AACGGACATG CATTTTCTGC TTGTTTGGAT GATGGAGGAG TTAAATGCCA GTGTCCTGCC
N G H A F S A C L D D G G V K C Q C P A
1501 GGGTTTAAAG GTGATGGTGT AAAAAATTGT GAAGACATTG ATGAATGCAA AGATAAGAAA
G F K G D G V K N C E D I D E C K D K K
1561 GCTTGTCAGT GCCCTGAATG TAGCTGCAAG AATACTTGGG GCAGCTATAA CTGCAGTTGT
A C Q C P E C S C K N T W G S Y N C S C
1621 AGTGGAGATC TTCTGTATAT CAAAGACCAG GATACATGCA TCAGTAAAAC TGCCAGTCAG
S G D L L Y I K D Q D T C I S K T A S Q
VSRPS -1 NT
1681 GCAAAGTCGA CAGTAAGTGC CGGTCCCTTT GCATTTATTT TCATTTCAAT TCTTTTAATA
A K S T V S A G P F A F I F I S I L L I
Vps10p TMD
1741 ATTTTCTTTG CCGCATGGTT TGTATATGAC AGAGGTATCA GAAGAAATGG GGGATTTGCA
I F F A A W F V Y D R G I R R N G G F A
Vps10p CT
1801 AGGTTTGGAG AAATTAGGCT AGGTGACGAT GGTTTAATAG AAAACAATAA TACTGACAGA
R F G E I R L G D D G L I E N N N T D R
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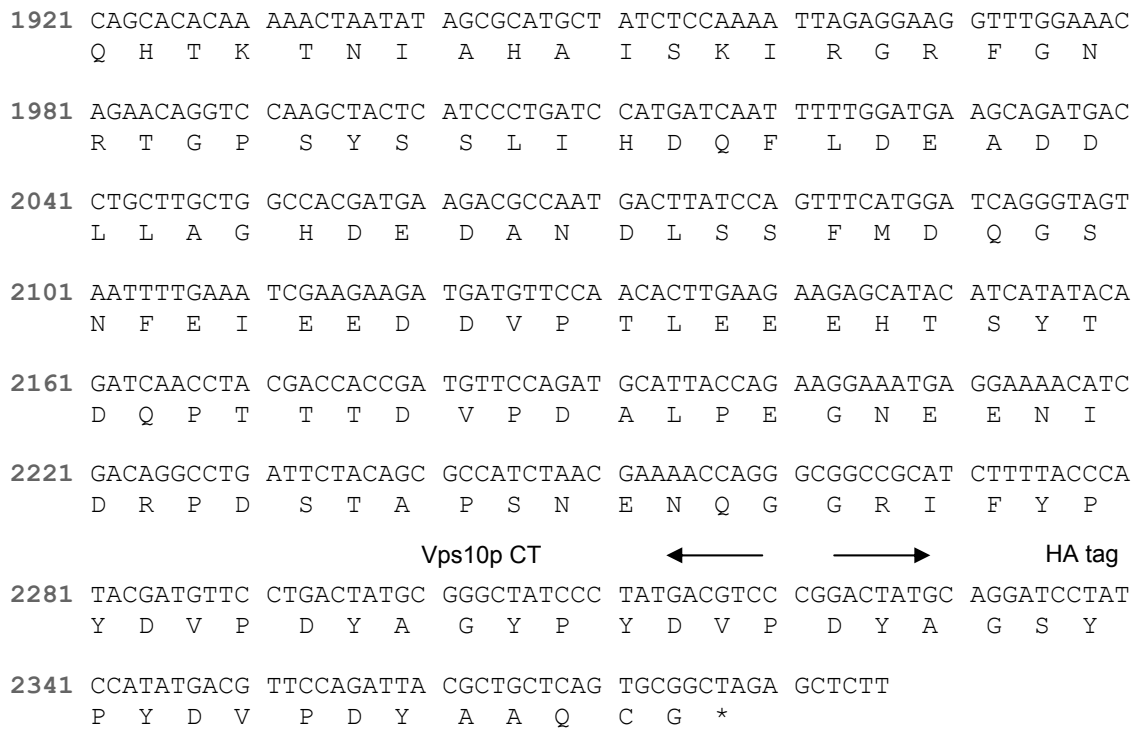


**Note :** The arrows define the beginning and ending of the N-luminal domain (NT), transmembrane domain (TMD) and cytosolic tail domain (CT) of $VSR_{PS-1}$-Vps10p and the beginning of the HA tag.