Functional study of vacuolar sorting receptors in transgenic *Arabidopsis thaliana* plants

Abbreviations

BSA: bovine serum albumin
bp: base pair

BP-80: binding protein of 80 KDa

CCV: clathrin coated vesicles

Ct-VSD: C-terminal vacuolar sorting determinant

CT: cytosolic tail domain

CTPP: C-terminal propeptide

DIP: dark induced protein

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DNase: deoxyribonuclease

$dNTP_S$: deoxyribonucleic acid triphosphate

dsRNA: double stranded RNA

DV: Dense vesicle

EGF: epidermal growth factor

ER: endoplasmic reticulum

EDTA: ethylene diamine tetra acetic acid

GA: Golgi apparatus

GFP: green fluorescent protein

LV: lytic vacuole

NTPP: N-terminal propeptide

PAC: precursor-accumulating

PBS: phosphate buffer saline

PCR: polymerase chain reaction

PM: plasma membrane

PSV: protein storage vacuole

PVC: prevacuolar compartment

RNA: ribonucleic acids

RNase: ribonuclease

rpm: rotation per minute

RT: room temperature

SNARE: Soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors

SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis

SRP: signal recognition particle

ss-VSD: sequence-specific vacuolar sorting determinant

TEMED: N,N,N’,N’-tetramethyl ethylene diamine

TGN: tran Golgi network

TIP: tonoplastic intrinsic protein

VSD: vacuolar sorting determinant

VSV: Vegetative storage vacuole

VSR: vacuolar sorting receptor

Chapter 1

General introduction

Organelles are necessary for cells to stay alive and help cells in synthesis of complex molecules involved in the diversity, in the specific exchange with their environment and help in signalling. Each organelle has its own function. Cells of eukaryotic organisms have similar general organizations. However, there are some differences in different species at the level of the number, the size and the structure of their organelles. Animal cells possess a single centralized Golgi apparatus while in yeast and in plants the Golgi apparatus is dispersed in single cisternal compartments in the cytoplasm. Plant cells are characterized by a pectocellulosic cell wall and have vacuoles often representing more than 70% of the volume of the cell. These differences depend on the cell type, and some organelles such as Golgi apparatus, endoplasmic reticulum, peroxisomes, mitochondria, or vacuoles are dynamic and undergo a variety of changes in response to cellular needs: protein transport, physiological adaptations and the cell cycle (Malhotra and Yaffe 2005). Specialized organelles present in a cell type fill each well defined functions. These organelles allow the compartmentalization of various biochemical processes within the cell. In the secretory system, the number the shape and the size of an organelle seem to be linked to the plasticity of transport carriers of the secretory pathway (Levine and Rabouille 2005; Munro 2005). The principal organelles are the nucleus, mitochondria, the endoplasmic reticulum (ER), the Golgi apparatus, chloroplasts, vacuoles/lysosomes, endosomes and peroxysomes/glyoxysomes. The nucleus contains the genome of the cell; it is responsible for the synthesis of the DNA and RNA. The mitochondria are responsible for cellular breathing via oxidative phosphorylation. The peroxysomes contain enzymes implicated in the degradation of the fatty acids. ER is the major site at which membrane lipids are synthesized in eukaryotes. The chloroplasts are the sites of photosynthesis in plants. However, the synthesis of the majority of proteins of a eukaryotic cell occurs in the cytosol, and it is from there that proteins must then migrate to reach their final destination. These proteins thus contain the information necessary to be transported to the correct target compartment. Several organelles of the eukaryote cell are grouped in a

system called the secretory pathway. The Golgi apparatus is used for secretion of proteins and their post- translation modifications such as N- linked oligosaccharide modifications, protein O-linked glycosylation on threonine and serine residues. In plant cells, the cell wall components hemicelluloses and pectins are synthesized in the Golgi. The lysosomes in mammalian cells or lytic vacuoles (LV) in plants are acidic vesicles containing several hydrolases (proteases, lipases, phosphatases etc.) implicated in the catabolism of a great number of molecules of endogenous or exogenous origin. These hydrolases degrade complex molecules in simple elements which can then be recycled by the cell. In plants a second type of vacuole was identified which is involved in the storage of proteins and especially in seeds and is called protein storage vacuole (PSV). In this thesis work, we will be interested in protein sorting in the secretory pathway and more particularly in sorting to different vacuoles, involving different vacuolar sorting receptors

The secretory pathway.

The secretory pathway of a eukaryotic (plant) cell is schematized in figure 1. Organelles implicated in this system are: the endoplasmic reticulum (ER), the Golgi apparatus, vacuoles, and intermediate compartments as the endosomal/prevacuolar compartments f and the plasma membrane. Proteins are synthesized in the rough RER, transported by vesicles to the Golgi where they are matured, are then packaged and are distributed to the plasma membrane or to vacuoles (Figure **1).** The proteins on their way to secretion or sorting to vacuoles will thus have to pass several organelles of the central vacuolar system.

Proteins are transported from ER to other compartments usually via Golgi complex.

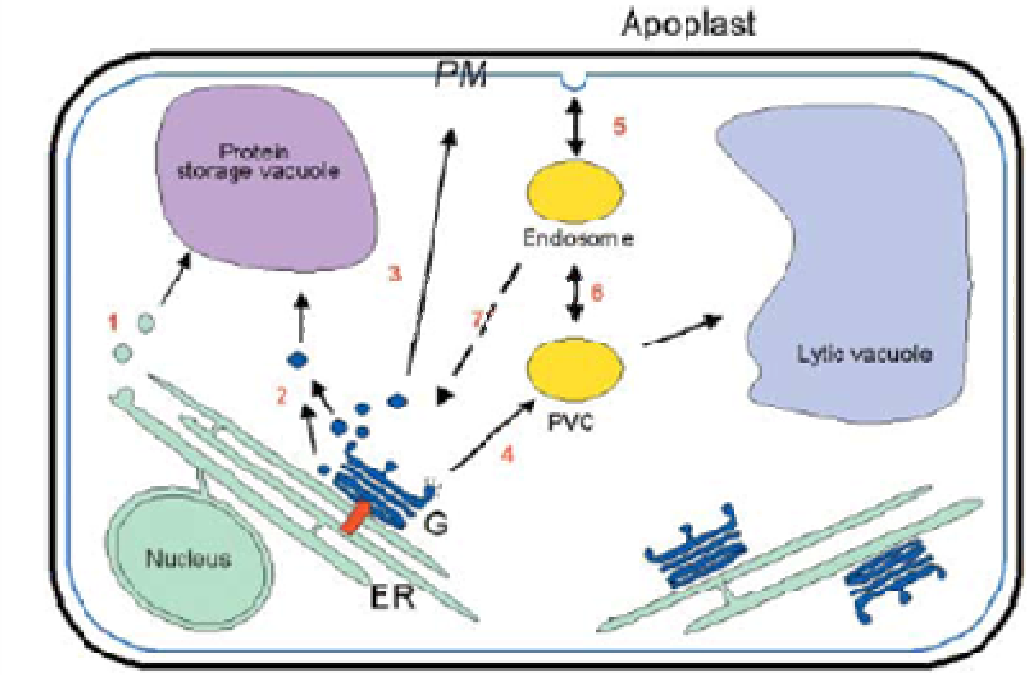


Figure 1 : Scheme of the secretory pathway in a plant cell.(1) Storage protein bypassing the Golgi. (2) Protein transport from the Golgi to the PSV. (3) Secretory protein transport from Golgi to plasma membrane (PM). 4) Protein transport to the lytic vacuole via PVC. (5) and (6), endocytosis of membrane and extracellular material via a putative endosome and to the PVC or (7) to the Golgi. Taken from Hawes (2005)

The transport of proteins from ER to other compartments inside the cell is vesicle dependent.

The Endoplasmic Reticulum

The ER is the first compartment of the secretory pathway and is constituted of interconnected saccules and tubules. It is a multi-functional organelle and is involved in protein synthesis, sequestration of calcium, production of steroids, synthesis and storage of lipids (Galili et al., 1998).

Structure and morphology of the endoplasmic reticulum

The ER can be subdivided into three domains with different functions: the rough ER (RER), the smooth ER (SER) and the nuclear membrane. The RER is called rough because ribosomes bound to the cytosolic face of the membrane give it a granular structure in electron micrographs. With these ribosomes, the RER plays the major role in protein translation and translocation. The SER is morphologically and functionally different from the RER at it does not carry ribosomes and is involved in lipid biosynthesis, detoxification of xenobiotics and calcium regulation(Vertel et al., 1992).

The nuclear envelope is a specialized extension of the ER. The outer membrane of the nuclear envelope is studded with ribosomes and is thus a part of the rough ER. It contains the specialized nuclear pore complexes which connect the cytoplasm and the nucleoplasm.

Proteins targeting to the endoplasmic reticulum

Soluble and membrane proteins destined to enter the secretory pathway are simultaneously translated and translocated across or inserted into the ER membrane (Gomord and Faye 1996). This corresponds to the first step of protein biogenesis in the secretory pathway (Deshaies and Schekman 1987).

Proteins will enter the lumen of the RER only if they carry a specific signal sequence.

The signal sequence required for the proper translocation of soluble proteins into the ER is located at the N-terminus end of the nascent polypeptide and is generally 10 to 50 amino acids long (Von Heijne 1988). It begins with an N-terminal region carrying 1 to 5 positively charged amino acids, and ends with a polar C-terminal region containing a proteolytic cleavage site. Between these two regions the H-region is constituted of 7 to 16 mostly hydrophobic residues (von Heijne 1986; Gomord and Faye 1996). As soon as it emerges from the ribosome the signal peptide is recognized and bound by a cytosolic ribonucleoprotein complex, the signal recognition particle (SRP), which consists of a 7S RNA and of 6 protein subunits. The nascent protein bound to the SRP forms a complex which stops translation (High and Dobberstein 1991). The SRP-ribosome complex also targets the ribosome–nascent chain complex in a GTP-dependent manner to the SRP receptor. The SRP-preprotein complex is then recognized by a membrane receptor, the docking protein (DP) which is anchored in the ER (Rapiejko and Gilmore 1997; Nagai et al., 2003). Subsequently, the SRP is released and the ribosome–nascent chain complex is delivered to the ER translocation machinery. The translocation complex, the translocon, consists of the Sec61p protein which forms the translocation pore, the signal peptidase (SP), the oligosaccharyltransferase (OST) and the translocating chain associated membrane (TRAM) protein. It is suggested that a conformational change of Sec61p and TRAM opens the translocation channel and that SP cleaves the signal peptide after its translocation (Deshaies and Schekman 1987; Shelness and Blobel 1990; Görlich and Rapoport 1993). However, some proteins containing a signal sequence can be inserted in an SRP independent manner. They are targeted to, and translocated across the ER membrane after being fully synthesized and released from the ribosome post-translationally.

Type I membrane proteins have a cleavable signal peptide similar to the one of soluble proteins and a TM sequence. In eukaryote, type II and III membrane proteins have an internal uncleavable signal sequence which functions as well as a transmembrane domain. Insertion of these proteins in the ER membrane is oriented based on the distribution of charge residues flanking the transmembrane segment, positive charges tending to remain on the cytosolic face of the membrane (Hartmann et al., 1989; Wahlberg and Spiess 1997).

Protein retention and recycling of ER resident soluble and ER integral membrane proteins

Retention and recycling of the ER resident protein.

Newly synthesized proteins need to be correctly folded in the ER before their transport into different organelles. The correct folding and quality control of newly made proteins is made possible by several ER proteins: PDI, BiP (Hsc70 family) chaperones, and calreticulin. Only properly folded proteins are transported from the ER.

Reticuloplasmins are ER resident proteins which remain in the ER and the lumen of the ER contains proteins involved in some modification of secretory proteins describe above: e.g. BiP, PDI (protein disulfide isomerase). These proteins remain in the ER while their substrates are transported away. The retention in the ER is due to the C-terminal signal, which in mammals is usually KDEL and in yeast HDEL (Denecke et al., 1992; Gomord and Faye 1996). In plants, both tetrapeptides have been identified (Denecke et al., 1992; Gomord and Faye 1996; Gomord et al., 1997). For example, the alfalfa PDI harbours 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). 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; Napier et al., 1992; Boevink et al., 1996; Gomord et al., 1997)

Retention and recycling of the ER integral membrane proteins.

A di-lysine motif (i.e. KKXX or KXKXX) end has been identified at the cytosolic C-terminal in many types 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 di-lysine motif mediates retrieval of type I membrane proteins from the GA back to the ER in mammalian (Jackson et al., 1993) and yeast cell (Gaynor et al., 1994) by interacting with receptor which react with the COPI coat.

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

Retention and retrieval mechanisms appear to co-exist for ER resident membrane proteins. Many types I ER membrane proteins have been identified from different plant species (Huang et al., 1993; Hasenfratz et al., 1997) and similar motifs in their CT could also be involved in their ER-retention. When fused with GFP the TMD and the 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).

Protein quality control

In eukaryotic cells, DNA, RNA, proteins are subject to quality control (QC) at each step of their synthesis (Ibba and Söll 1999; Lindahl and Wood 1999; Wickner et al., 1999).

QC is a set of processes by which macromolecules are identified not (yet) properly folded and are processed, or if they fail, are targeted for destruction.

For proteins, the QC occurs at the level of, translation, folding and assembly (Ellgaard and Helenius 2003).

It has been estimated that a third of all polypeptides synthesized by animal cells fails to pass the QC and are degraded. Cells recognize improperly folded proteins by using helper proteins which are able to recognize typical features of non-native structures. These chaperones interact with proteins assisting their refolding or diverting improperly folded proteins for degradation. Several chaperones use ATP for this process (Gottesman et al., 1997; Wickner et al., 1999; Ellgaard and Helenius 2003; Hirsch et al., 2004).

The main compartment of the secretory pathway for this QC process is the ER and the mechanisms include protein retention in the ER, ER-associated degradation (ERAD), retrieval to the ER from other organelles and transport via Golgi to lysosomes or vacuoles for degradation. In animal cells the ER-Golgi intermediate compartments (ERGIC) or vesiculo-tubular clusters are also involved in QC (Ellgaard et al., 1999).

Molecules and mechanisms involved in the quality control

The first step in the process is the retention of malfolded proteins by interactions with ER-resident proteins.

While folded proteins are not recognized, misfolded proteins have usually exposed hydrophobic regions, containing wrongly paired cysteines in disulfide bonds and have a strong tendency to aggregate (Ellgaard and Helenius 2003).

Misfolded or unfolded proteins are commonly found associated to chaperones. These proteins have exposed hydrophobic surfaces which can interact with chaperones or with proteases (Wickner et al., 1999). Chaperones Hsp70 (DnaK) and Hsp40 (DnaJ) are both involved in the binding and release of the hydrophobic regions present in misfolded proteins in an ATP-dependent way (Rüdiger et al., 1997; Bukau and Horwich 1998; Frand et al., 2000). The hydrolysis of ATP helps in the refolding of misfolded proteins (Schmid et al., 1994; Fewell et al., 2001). When bound to ATP, HSP 70-like proteins assume an open form in which an exposed hydrophobic pocket transiently binds to exposed hydrophobic regions of the unfolded target protein. Hydrolysis of the bound ATP causes molecular chaperones to assume a closed form in which a target protein can undergo folding. The exchange of ATP for ADP releases the target protein. (McCarty et al., 1995)

Chaperones prevent also protein aggregation by stimulating the correct folding of proteins. This allows a higher yield of correctly folded proteins (Groenendyk and Michalak 2005).

Misfolded proteins accumulating in the ER, are bound by different factors: BIP, calnexin, calreticulin, glucose-regulated proteins (GRP54), and thiol-disulfide oxidoreductase, protein disulfide isomerase (PDI) and ERP57 (Ellgaard and Helenius 2003).

In the cytosol there are also enzymes that select and covalently modify misfolded proteins for recognition by folding and degradation machinery of the cytosol. Ubiquitin, is a small protein that is attached to lysine side chains as a degradation signal (Glickman and Ciechanover 2002) and several enzymes are involved in the process, to activate ubiquitin, select the substrate and ubiquitinate it (McClellan et al., 2005).

When the folding machinery in the ER is not sufficient to promote a native conformation proteins are generally degraded by the ER-associated degradation (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 et al., 1999). It 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).

Heat shock proteins (HSP): HSP70, HSP40, HSP90, and HSP110

One of the central molecular chaperones identified was BIP. It's has been identified as an immunoglobulins heavy chain binding proteins and as a glucose-regulated protein (Haas and Wabl 1983).

Members of the HSP70 family contains two domains: the N-terminal ATPase domain followed by a conserved C-terminal substrate binding domain (Munro and Pelham 1986; Wang et al., 1993)

It has been shown in yeast that the activity of HSP 70 is enhanced by another chaperone family the HSP 40 (DnaJ). This stimulates the activity of HSP70 and induces a stable substrate binding (McCarty et al., 1995; Fewell et al., 2001). It can deliver protein to HSP70 because it binds to hydrophobic 8 amino-acid motifs (Rüdiger et al., 2001).

The heat-shock protein 90 (Hsp90) is a cytosolic molecular chaperone that interacts with folding polypeptide chains at normal temperature. Hsp90 promotes functional refolding to the native state by suppressing unspecific side reactions (Jakob et al., 1995).

The 110-kDa heat shock protein (hsp110) has long been recognized as one of the primary heat shock proteins in mammalian cells. It belongs to a recently described protein family that is a significantly diverged subgroup of the hsp70 family and has been found in organisms as diverse as yeast and mammals *in vitro* heat denaturation and refolding assays demonstrate that hsp110 is highly efficient in selectively recognizing denatured proteins and maintaining them in a soluble, folding-competent state and is significantly more efficient in performing this function than is hsp70 (Oh et al., 1997; Oh et al., 1999).

Other types of chaperones (unconventional chaperones) and maturation of secretory proteins within the ER

Other type of chaperones are the lectin chaperones: calnexin (CNX) and calreticulin (CRT)(Parodi 2000b, 2000a).

These proteins are homologous and reside in the ER. They share similar structures and functions except that calnexin is a membrane-anchored protein and calreticulin is luminal. The luminal N-terminal portion of calnexin is very similar to calreticulin, although one of the repeated segments of calnexin is absent from calreticulin. (Boyce et al., 1994; Krause and Michalak 1997; Schrag et al., 2001) They have been shown to be implicated in some cytoplasmic and nuclear processes and can be retranslocated from the ER lumen to the cytosol (Zhang et al., 1997; Danilczyk et al., 2000; Afshar et al., 2005). They are essential for the folding of glycoproteins because they associate with glycan structures: They bind to the oligosaccharide core Glu_1-Man_9-$GlcNAc_2$ as an initial step in recognizing unfolded glycoproteins. CNX or CRT bind to the monoglucosylated glycans of proteins when a glucose residue is retained by N-glycan in misfolded glycoproteins. The two first glucose residues of the N-glycan extremity are removed by glucosidase I and II. Then glucosidase II removes the last remaining glucose and disrupts the interaction glycan-CNX or -CRT. The free glycoprotein can leave the ER unless it is recognized as unfolded by chaperones.

Incompletely folded glycoproteins are reglucosylated by an UDP-Glc: glycoprotein glucosyltransferase (GT) (figure**2**). The newly glucosylated molecule can associate again with lectin chaperones and the cycle is repeated. The de- and reglucosylation cycle will take place until the molecule is correctly folded (Hammond et al., 1994; Parodi 2000b; Helenius and Aebi 2004).

The three dimensional structures of proteins are often characterized by the presence of intra- or intermolecular disulfide bonds. Disulfide bond formation is catalysed by a protein disulfide isomerase (PDI) which displays thiol: protein disulfide oxidoreductase and isomerase activity and was shown to assist CNX and CRT in the QC system. This is facilitated in the oxidizing environment of the ER lumen (Parodi 2000b). Identified members of the oxidoreductase family that contain thioredoxin-like activity are ERp57, ERp72, ERp44 (Oliver et al., 1999).

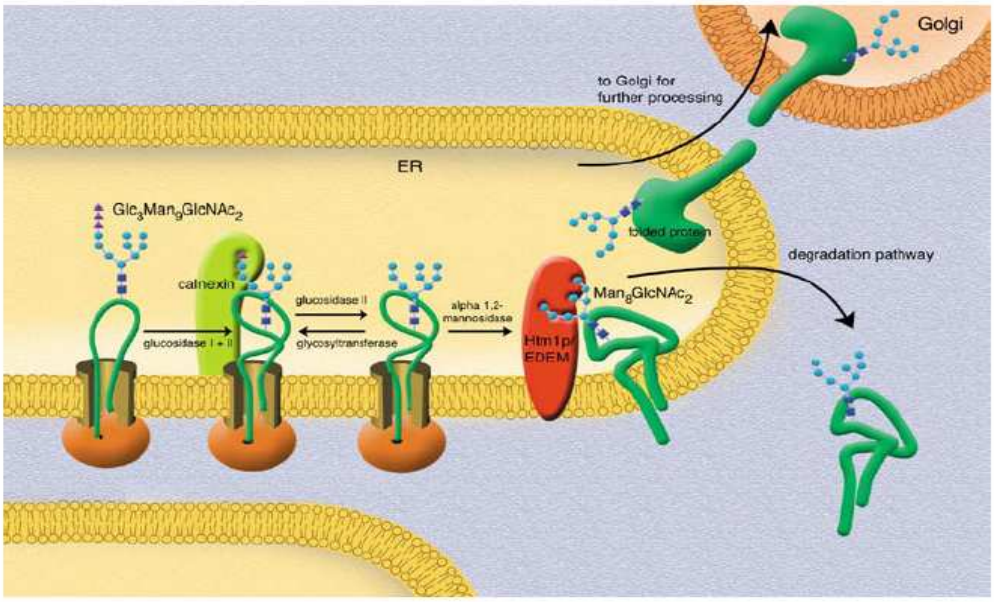


Figure 2: Modifications of the N-linked glycan on proteins**.**
The first, second and third glucose of the protein are removed by glucosidase I and II. The third glucose can be reattached by a glucosyltranferase that recognizes only misfolded and unfolded. Calnexin and calreticulin associate with the monoglucosylated chain to retain immature glycoproteins in the ER, to allow folding to proceed. The ER α 1, 2-mannosidase removes a single mannose, resulting in $Glc_{0-3}Man_8GlcNAc_2$, before transport of the properly folded protein to the Golgi complex where further processing occurs. This figure was taken from Braakman (2001).

Transport between compartments using vesicles.

Secretory and membrane proteins that have successfully passed the quality control are transported within the secretory pathway to specialized regions called ER exit sites.
Protein transport from the ER to the GA are packaged in carrier vesicles that are formed on the ER membrane and selectively fused with the *cis*-Golgi membrane (Bannykh et al., 1996; Watson and Stephens 2005).
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 membranes ("vesicle fusion").

The processes of budding, targeting and fusion are repeated at consecutive transport steps until the cargo reaches its final destination within or outside the cell. To balance the forward movement of vesicles, organelle homeostasis requires the retrieval of transport machinery components and escaped resident proteins from the acceptor compartments back to the corresponding donor compartments (retrograde transport), which is also realized by vesicular transport.

Three types of coat proteins are involved in vesicle budding and in the selective incorporation of cargo in the forming vesicles, COP-I, COP-II and clathrin (Nickel and Wieland 1998; Bonifacino and Lippincott-Schwartz 2003).

COP-I (Coatomer protein) and COP-II

It is generally accepted that COP-II coats mediate the transport of material from the ER to the Golgi (Barlowe et al., 1994; Schekman et al., 1995; Schekman and Orci 1996) and segregate biosynthetic cargo from ER-resident proteins (Barlowe 2002), whereas COP-I plays an important role in the Golgi-to-ER retrieval and in maintenance and function of the Golgi (Cosson and Letourneur 1994; Gaynor et al., 1998b; Mironov et al., 2005). This function was better demonstrated with the cycling of the ER chaperone calreticulin from the ER and its return from Golgi in COP-I vesicles (Pimpl et al., 2000; Contreras et al., 2004; Hawes 2005). Additionally the transport from early to late endosomes also depends on COP-I vesicles (Gu and Gruenberg 1999) Figure**3**.

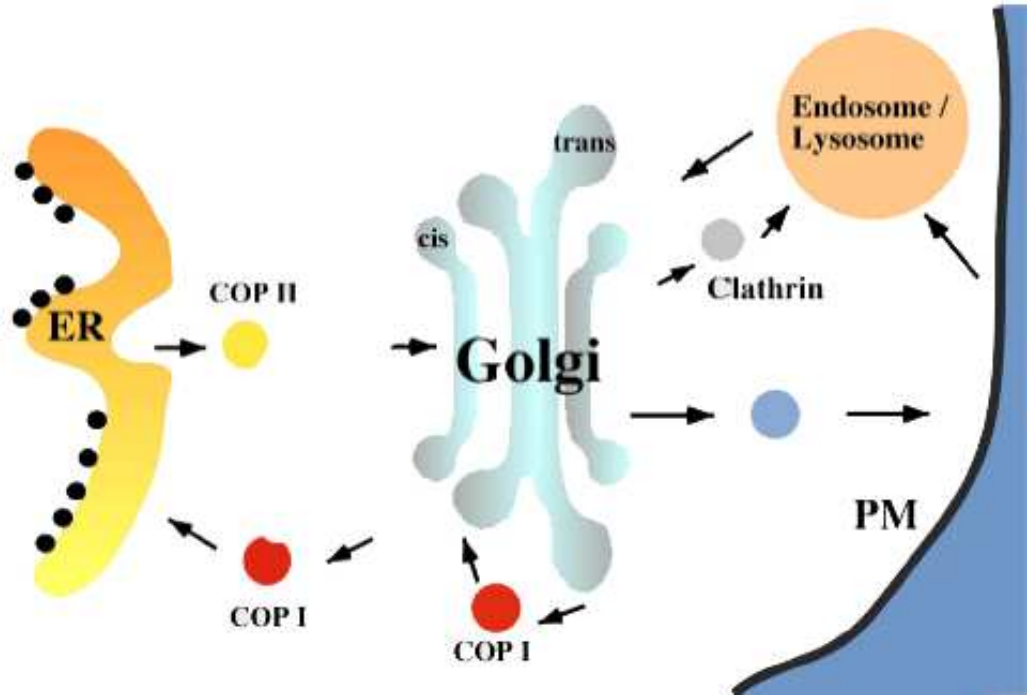


Figure3: **Transport between the endoplasmic reticulum ER and the cis-Golgi is regulated by the COP II and COP I vesicles coat**. COPI mediate a retrograde transport pathway that selectively recycles from the cis-Golgi complex. COPII are required for selective export of newly synthesized proteins from the ER. Vesicular traffic pathways from the ER to and within Golgi complex, to the endosome/lysosome system and to the plasma membrane are shown (PM). Adapted from Duden (2003).

The formation of both COP-II and COP-I vesicles requires the activation of a specific small GTPase, which causes the recruitment of structural components of the vesicle coat to the membrane, resulting in the formation of a vesicle that can then bud from the membrane and travel to its destination (Hanton et al., 2005).

COP-I is a multi molecular complex composed of seven COPs (for *coat* proteins): α, β, β', γ, δ, ε, and ζ. The seven COP polypeptide complex is generally called a coatomer (Waters et al., 1991; Schekman and Orci 1996). The eighth components of the COP-I coat is ADP-Ribosylation Factor (ARF1), a small GTP-binding protein. The coat formation begins by the activation of ARF by an ARF-GEF recruitment to a preassembled coatomer (Orci et al., 1993). In this case, the coatomer directly interacts with membrane-bound ARF•GTP ("activation") via its β- and γ-COP subunits. COP-I dissociation requires GTP hydrolysis by ARF1 which is stimulated by a cytosolic ARF-specific GAP (Goldberg 1998; Spang 2002). In yeast, two subpopulations of COP-I vesicles seem to be involved in the sorting of Golgi resident proteins both containing high amounts of Golgi resident proteins, but only minor amounts of anterograde cargo. This was the case of early Golgi proteins which were shown to

be sorted together into vesicles that are distinct from those containing mannosidase II, a glycosidase of the medial Golgi stack (Lanoix et al., 2001). In plants, homologues to the yeast COP coatomer subunits (Pimpl et al., 2000), and ARF1 have been identified (Regad et al., 2003).

Activation of ARF1 by its ARF-GEF (Sec7p in yeast) is prevented by Brefeldin A, preventing the recruitment of COP I on Golgi. This causes a block of transport through the Golgi apparatus and the fusion of *cis*-Golgi cisternae with the ER (Zeghouf et al., 2005)

The coat complex II (COP-II) was identified and characterized in yeast and in mammalian systems and homologues of several components have been identified in plants (d'Enfert et al., 1992; Bar-Peled and Raikhel 1997). In mammals and in yeast, a small cytosolic GTPase Sar1p was shown to mediate COP-II vesicle formation, and three isoforms of this GTPase have been identified in Arabidopsis. Sarp1 activation is mediated by an ER-localized integral membrane protein called Sec12p, a functional homologue of which has been identified in Arabidopsis (Bar-Peled and Raikhel 1997; Vernoud et al., 2003)

The mechanism by which cargo molecules are packaged into COP-II is that COP-II coat assembles by the gradual deposition of Sar1p●GTP, Sec23p●Sec24p dimers, and Sec13p●Sec31p dimers 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 crosslink the pre-budding complexes. The dissociation of the coat requires GTP hydrolysis by Sar1p which is stimulated by Sec23p (a GTPase protein (GAP)) destabilizing the coat (Kuehn and Schekman 1997; Kirchhausen 2000b; Bonifacino and Glick 2004; Hanton et al., 2005).

Coat complexes

Three types of coat complexes, each mediating different transport steps, are presently known: Clathrin coat COP I and COP II (Scales 2000).

Clathrin

Clathrin coated vesicles (CCVs) are central players in receptor-mediated endocytosis. They also mediate the transport of cargo from the Trans Golgi Network (TGN) to the endosomal /lysosomal compartment reviewed in (Kirchhausen 1999). Clathrin was first purified by Pearse and was shown to be the major component in purified coated vesicles extracts (Pearse 1975). Clathrin is a trimer consisting of 3 heavy chains (~190KDa) (Kirchhausen 2000a) which associate with light chains (~25KDa). Together they form a structure called a triskelion (Robinson et al., 1998b; Fotin et al., 2004). Like COP I and COP II vesicles, the vesicles coated with clathrin must lose their coat to be able to fuse with the target membrane.

Adaptor proteins (APs)

The adaptor proteins, another component of the coat, provide a link between the clathrin triskelion, and transmembrane proteins which will be incorporated into the vesicle (Robinson and Bonifacino 2001). There are four kinds of adaptins: AP-2 is incorporated in the vesicles budding from the plasma membrane, which are composed of an adaptin α, an adaptin β, a polypeptide of approximately 50 kDa (AP50 or μ_2) and a polypeptide from approximately 20 kDa (AP17 or σ_2); another adaptin is named AP-1 and is associated with vesicles budding from the TGN and endosomes. It includes an adaptin γ, an adaptin β ', a polypeptide of approximately 50 kDa (AP47 or μ_1) and a polypeptide of approximately 20 kDa (AP19 or σ_1) (Bonifacino and Dell'Angelica 1999; Traub 2005). Polypeptides μ_1, and μ_2, forming part of adaptors AP-2 and AP-1 respectively, were shown to interact with transmembrane proteins harbouring either a Tyr motif (YXXΦ, where Y represents a tyrosine and Φ an amino acid with a hydrophobic side chain) or di-leucine signals [DE]XXXL[LI]. These motifs are present in several transmembrane proteins (Bonifacino and Traub 2003; Nakayama and Wakatsuki 2003; Traub 2003). In the case of vesicles directed to lysosomes, the adaptor which binds the clathrin also recognizes a motif present in the cytosolic tail of the mannose 6 phosphate receptor 275 kDa (Pearse and Robinson 1990).

Other families of monomeric proteins with adaptin-related domains have been identified. The GGAs (Golgi-localized γ-adaptin ear homology domain ARF binding protein) were described in mammalian cells and in yeast (Dell'Angelica et al., 2000; Hirst et al., 2000; Costaguta et al., 2001) but have apparently no equivalents in plants.

In plants, all members of the Vacuolar Sorting Receptor family (VSR, see below) contain a form of the tyrosine motif (YXXØ) (Paris et al., 1997). VSR_{PS-1} was found to be enriched in CCVs(Kirsch et al., 1994; Robinson et al., 1998b; Hinz et al., 1999; Hillmer et al., 2001), which is also true for some of its *Arabidopsis* homologues, the AtVSRs. It was recently demonstrated that a *Arabidopsis* 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)

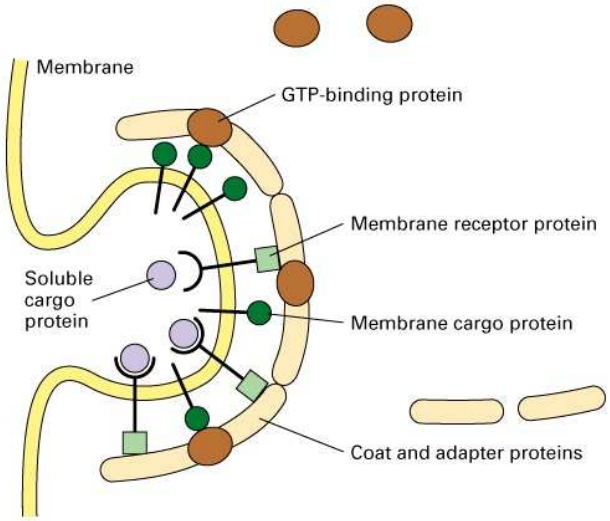


Figure 4: **General Scheme for Vesicle Budding.** Polymerization of coat protein (occurs on cytosolic side of embrane). Adapter proteins select the appropriate cargo proteins. GTP binding protein regulates budding. Membrane fusion and vesicle release

Vesicle fusion with the target membrane

The attachment and fusion of vesicles at their destination is mediated by transmembrane proteins named SNAREs (Soluble NSF (N-ethylmaleimide-sensitive fusion protein) attachment protein receptors , which together with cytosolic factors form complexes that allow fusion of vesicles with specific target organelles (Hanton et al., 2005). A large number of SNAREs (at least 54 different SNAREs) have been found in the Arabidopsis genome, with six SNAREs in the ER membrane and nine in the Golgi membrane (Sanderfoot et al., 2000; Sanderfoot et al., 2001; Uemura 2004). This includes homologues known in yeast as Sec22p, Bet1, Bot1 Sed5 all located to punctate Golgi–like structures (Takeuchi et al., 2000; Chatre et al., 2005; Hawes and Satiat-Jeunemaitre 2005; Weinberger et al., 2005; Flanagan and Barlowe 2006).

There is a type of SNAREs located on the transport vesicle (v-SNAREs) and others on the target membrane (t-SNAREs), which have been reclassified as either R-SNAREs or Q-SNAREs depending on the presence of a conserved Arg or Gln in a central position of the main helix (Jurgens 2004; Chatre et al., 2005; Hong 2005).

The formation of the SNARE complex via conserved coiled-coil domains (Bonifacino and Glick 2004; Hawes 2005) results in membrane fusion. The term "SNARE complex" refers to a machinery including four components: V-SNARE, t-SNARE, SNAP and N-ethylmaleimide-sensitive factor (NSF). Proteins that regulate SNAREs include Rabs which are small GTPases facilitating SNARE complex formation. In plants 52 Rab GTPases have been found, few of them were studied and there is little information on their localization. Rabs also cycle between an inactive GDP-bound and an active GTP-bound conformation with GEFs catalyzing the GDP/GTP exchange reaction and GAPs (Batoko et al., 2000; Ueda 2001; Nebenführ 2002; Preuss et al., 2004) v-SNARE present on the vesicle membrane interacts with t-SNARE present on the acceptor membrane. After fusion, SNAP binds to the SNARE complex then recruiting NSF. ATP hydrolysis by NSF dissociates the SNARE complex, the v-SNARE can then be recycled to the donor compartment by retrograde transport, while the t-SNARE can re-organized for the next round of docking and fusions events (Hong 2005). NSF is required to dissociate *cis*-SNARE complex, present on the membrane after the fusion (Weber et al., 2000)

The Golgi complex

After leaving their production site at the ER, most secretory proteins are transported to the Golgi apparatus (GA), which consists of several flattened cisternae interconnected by tubular elements (Mellman and Simons 1992) except in yeast , where cisternae are dispersed in the cytosol.

In mammalian cells, Golgi stacks are localized in a juxtanuclear position, while in plants they are dispersed in the cytoplasm and they apparently move to locations where their activity is needed (Harris 1986; Andreeva 1998). The GA is divided into four different subcompartments: the *cis*-, the *medial*-, and *trans*-Golgi, as well as TGN can often be distinguished, both microscopically and functionally.

In plants, the GA functions as an important biosynthetic compartment that modifies proteins and synthesizes lipids and polysaccharides (such as hemicelluloses and pectins, but not cellulose). Xylo-glucan and polygalacturonic acid are the two main classes of polysaccharides

synthesized by dicotyledons (Driouich and Staehelin, 1997). The GA is also the site of posttranslational modifications such as remodelling of the N-linked oligosaccharide side chains and biosynthesis of O-linked glycans of glycoproteins. N-glycans are very similar in plants and in mammals but differ in terminal residues: plant N-glycan have alpha-1,3- fucose instead of alpha-1,6-fucose in mammals and beta-1,2-xylose is found in plants while N-acetylneuraminic acid is found in animals (Faye et al.,1992). O-glycosylation is found on 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 stack by numerous Golgi enzymes such as glycosidases and glycosyltransferases.

The other main function of GA is the synthesis of some lipids like ubiquinone and plastoquinone, as described in spinach (Swiezewska et al., 1993; Osowska-Rogers et al., 1994).

The GA is a major sorting point in the secretory pathway. Proteins destined for secretion enter the Golgi at the *cis*-face and subsequently move to the *trans*-face where the majority of proteins exit the stack *en route* to the plasma membrane or to the vacuolar system (Neumann et al., 2003). The GA packages its macromolecular products on the *trans*-face in membrane-bounded vesicles, which are targeted to different destinations within the cell.

Three models have been suggested to explain the vectorial transport of secretory proteins through the GA, which are not mutually exclusive, could be correct to some extend (Pelham and Rothman 2000).

The first model: The vesicle shuttle

In this model, each cisterna constitutes a stable compartment, and anterograde cargo moves via vesicles cisterna to cisterna in a cis-to-trans direction. The transport of Golgi proteins is mediated by COP-I coated vesicles, formed at the Golgi cisterna (Staehelin and Moore 1995). Vesicles are produced at any point and remain associated on the ER/actin network (Boevink et al., 1998; Glick and Malhotra 1998; Batoko et al., 2000). In mammalian cells and in plants, the application of N-ethylmaleimide blocks the fusion process of the vesicle with an acceptor membrane, and causes their accumulation in the vicinity of the Golgi stack (Rothman 1994; Steele et al., 1995). Another experiments to support this was the use of the fungal metabolite brefeldin A, that inhibits the formation of COPI vesicles in mammals yeast, and plants. The transport of sporamin (a sweet potato storage protein) from the ER to GA in transgenic

tobacco cells is blocked with an accumulation in the vicinity of the Golgi stack (Holwerda et al., 1992; Rothman 1994; Satiat-Jeunemaitre et al., 1996). Indeed, in animals cells anterograde cargo proteins have been immunolocalized within COP-I vesicles near the GA (Orci et al., 1997).

The second model: the cisternal progression or maturation

In this model, vesicles are not involved in transport of the secretory cargo, which is instead passively carried forward by cisterna progression, while COP-I vesicles are used only for the retrograde transport to recycle resident Golgi proteins (Pelham 1998). Cisternae mature during their progression through the stacks by exporting "early" Golgi proteins to younger cisternae, while receiving "late Golgi" proteins from the older cisternae (Glick and Malhotra 1998). Genetic studies showed that COP-I vesicles also carry material from Golgi back to the ER (Letourneur et al., 1994; Gaynor et al., 1998a)by moving backward one cisternae at a time. So, in this model, cisternal maturation coexists with anterograde carrier vesicles.

Using light and electron microscopy, the vesicular stomatitis virus membrane glycoprotein (VSVG) was shown to be largely excluded from Golgi-associated vesicles and does not move between cisternae, whereas Golgi enzymes freely enter vesicles as predicted by the cisternal maturation model (Martinez-Menarguez et al., 2001; Pelham 2001). Mironov et al., (2001) showed by using GFP tagged VSVG delivered from ER to Golgi, that only a subset of stacks in the Golgi becomes labelled. Using light and electron microscopy, VSVG was not spreading either into earlier or later cisternae, or into adjacent stacked regions or into vesicles. In contrast, Golgi enzymes were present in the Golgi and appeared to be exchangeable between the mother stacks. This model is the only one compatible with the transport of algal scales or of procollagen, which are too bulky to fit into COPI vesicle. In addition, maturation of the single cisternae of yeast could be directly visualized by the use of different fluorescent Golgi markers (Losev et al., 2006; Matsuura-Tokita et al., 2006).

It is clear that the cisternal maturation does not exclude the possibility of vesicles transport, and vice versa (Pelham and Rothman 2000).

Third model: Transient tubular connections

The third model describes intra-Golgi transport by lateral diffusion through membrane continuities or tubules (Mironov et al., 1997). In fact, tubules are a well-characterized feature

of the Golgi complex, whose stacks are interlinked with tubuloreticular networks (Rambourg and Clermont 1990). Membrane continuities have been implicated in the diffusion of Golgi enzymes within intact Golgi in living cells (Cole et al., 1996; Marra et al., 2001). Golgi tubules are dynamic structures and are affected by local lipid compositions (de Figueiredo et al., 1999; Weigert et al., 1999). However, such connections are absent or rare in the Golgi from other organisms like yeast (Rossanese et al., 1999). In addition, vesicles and tubules appear to co-exist in the pathway (Ladinsky et al., 1999). To what extent tubules contribute to membrane transport between Golgi cisternae still remains to be clarified (Marsh and Howell 2002; Storrie and Nilsson 2002). EM tomography of Golgi stacks demonstrated the formation of tubular connections between stacks when a massive pulse of cargo passed through the Golgi apparatus.

The Trans-Golgi network

The trans-Golgi network (TGN) is the site of cargo sorting and final exit from the Golgi, directing newly synthesized proteins to different subcellular destinations (Gu et al., 2001). It is associated to the trans-side of the Golgi and in electron microscopy it is seen as a sacculotubular network. The structure and the size of TGN vary remarkably from one cell type to another. At the TGN, cargo molecules are sequestered into coated vesicles and directed to their correct destinations. Soluble proteins carrying no specific sorting signals are targeted and transported to the plasma membrane through a so-called constitutive pathway. In the regulated secretory pathway leading to the cell surface vesicles only fuse with the plasma membrane upon stimulation by an extracellular signal store cargo in secretory granules (Traub and Kornfeld 1997).

From the TGN to other specific compartments such as lysosomes or vacuoles, most proteins first transit via an endosome or PVC (Robinson et al., 1998a; Tse et al., 2004).One terminal compartment of the secretory pathway in plant cells is the vacuole (Marty 1999). In animals there may be distinct compartments: lysosomes and melanosomes.

At the TGN, sorting of cargo to different destinations is regulated in many ways. First, biochemically distinct coats are likely to specify protein sorting. Second, cytosol-oriented sorting signals of cargo proteins direct them to the appropriate export site. Third, TGN might be organized into discrete subdomains dedicated to assemble specific coat population (Traub and Kornfeld 1997).

Other selective pathways sort proteins into the intracellular endosomal membrane system (Neuhaus and Rogers 1998; Jiang and Rogers 1999).

Protein sorting to lysosomes in mammalian cells

The correct targeting of newly synthesized lysosomal enzymes to lysosomes is dependent on modification of their N-glycans by the action of enzymes. The Man-6-P residues are generated in two steps. N-acetylglucosamine1-phosphate is attached to the C6-hydroxyl group of mannose residues followed by removal of the N-acetylglucosamine. The first reaction is catalysed by UDP-GlcNAc: lysosomal enzyme N-actylglucosamine1 phosphotransferase (phosphotransferase) and the second by an α–N-acetylglucosaminidase (Pelham et al., 1988; Dittmer et al., 1999).

The second step involves the binding of lysosomal hydrolases to Man-6-P receptors in the Golgi. The Man-6-P receptors recognize and bind the Man-6-P tagged lysosomal proteins, and the complex is transported by CCV to an acidic compartment where it then dissociates, and the soluble ligand continues to the lysosome while the receptor is recycled back to the Golgi apparatus. There are two M6PRs (MPR46 and MPR 300) in mammalian cells, the cytoplasmic tails of which contain 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 to the lysosome (Johnson and Kornfeld 1992). 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 (Ghosh et al., 2003).

Another lysosomal receptor was discovered in animals: sortilin, which does not bind to glycans, is related to the yeast vacuolar receptor Vps10p (Nielsen et al., 2001; Lefrancois 2005).

Protein sorting to vacuole in yeast

Like the mammalian lysosome and the plant lytic vacuole, the yeast vacuole is an acidic organelle that is responsible for the degradation of macromolecules and that also serves in the storage of amino acids, small ions and polyphosphates (Klionsky et al., 1990). In yeast, sorting of soluble and the vacuolar membrane proteins was particularly well studied.

Soluble 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 in the TGN. The best studied cargo, 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 resulting in a 67kDa form (p1CPY) which is then transported to the GA where further oligosaccharide modification produces the 69kDa form (p2CPY)●p2CPY is sorted through a receptor-mediated process that leads to its delivery to the vacuole, where it is cleaved by vacuolar proteases into its active, mature 61kDa form(mCPY). It has been shown that the sequence QRLP (residues 24 to 27) in the propeptide region of the pro-CPY forms the core of the targeting signal required to divert p2CPY away from the default secretion to the extracellular space, into the vacuolar biogenesis pathway. Alteration of this signal results in secretion from the cell (Valls et al., 1987; Valls et al., 1990). Subcellular fractionation studies revealed that CPY transit through the PVC on its journey from the TGN to the vacuole (Vida et al., 1993; Horazdovsky et al., 1995; Stack et al., 1995). Marcusson and co-workers (1994) isolated and characterized the sorting receptor for CPY by complementation screen in a mutant strain that secretes a CPY-invertase fusion. This receptor was named Vps10p. Cells lacking the Vps10p missorted more than 90% of their CPY. Vps10p also cofractionned with Golgi membrane as well as with lighter membrane fractions corresponding to a prevacuolar, endosomal compartment (Marcusson et al., 1994; Cooper and Stevens 1996). 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 The Vsp10p receptor must repeatedly cycle between the TGN and the PVC. 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). First Vps10p binds p2CPY in the TGN, then, the receptor-ligand complex travels to the PVC in Golgi-derived transport vesicles, and finally, 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 (detailed in (Bryant and Stevens 1998).

Integral membrane proteins lacking sorting information are not delivered to the cell surface in yeast. Instead, they are transported to the vacuole (Roberts et al., 1992; Wilcox et al., 1992).

Yeast Golgi membrane proteins such as dipeptidylaminopeptidase A (DPAP A), Kex2p, Kex1p possess within their C-terminus motifs containing tyrosine and phenylalanine residues which prevent their exit form the TGN (Wilsbach and Payne 1993). Removal of these signals results in their delivery to the vacuole where they are subject to vacuolar protease-dependent cleavage (Jones et al., 1982; Cooper and Bussey 1992; Nothwehr et al., 1993). Mutations of the localization signals of resident ER membrane proteins also result in their transport to the vacuole (Gaynor et al., 1994). These results 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).
Mislocalization of vacuolar proteins in yeast allowed the isolation of a large number of yeast mutants especially defective in the delivery of proteins to the vacuole (Bankaitis et al., 1986; Rothman and Stevens 1986; Rothman et al., 1989). *vps* mutants were grouped into six classes (A to F). Mutant from class E accumulate an exaggerated form of the PVC containing CPY (see (Banta et al., 1988; Raymond et al., 1992; Piper et al., 1995; Stack et al., 1995; Babst et al., 1997). *pep* mutants were isolated as yeast strains defective in CPY enzyme activity (Jones 1977). p*ep* mutant is equivalent to *vps* mutants.

Protein sorting to vacuoles in plants

It has been shown that in plant there are different kinds of plant vacuoles in contrary to mammalian cells which possess two types of lysosomes. Plant cell vacuoles are multifunctional organelles that serve physical and metabolic functions for plant life. They share some basic properties with yeast vacuoles and lysosomes of animal cells. They are lytic compartments, function as reservoirs for ions and metabolites, including pigments, and are crucial for detoxification and general homeostasis. They are also involved in cellular responses to environmental and biotic stress factors. In seeds and other specialized storage tissues, they serve as storage sites for proteins and soluble carbohydrates (Taiz 1992; Wink 1993; Okita and Rogers 1996; Marty 1999).

Plant cells can have up to three different vacuoles with different functions in a single cell: The lytic vacuole (LV), the vegetative storage (neutral) and the protein storage vacuole (PSV) (Hoh et al., 1995; Paris et al., 1996; Di Sansebastiano et al., 1998, 2001). Tonoplast intrinsic proteins (TIPs, a family of aquaporins) have been used as maker proteins for different types of vacuoles (Paris et al., 1996; Jiang et al., 2000).

The lytic vacuole (LV)

The LV is an acidic compartment that contains enzymes analogous to the lysosomal enzymes of animal cells. This vacuole is important to maintain turgor pressure, for the storage of metabolites, and the sequestration of xenobiotic compounds (Taiz 1992). The membrane, or tonoplast, of such vacuoles contains the aquaporin γ-TIP (Tonoplast Intrinsic Protein, (Höfte and Chrispeels 1992; Marty-Mazars et al., 1995; Paris et al., 1996; Barrieu et al., 1998; Jiang et al., 2000)

The Protein Storage Vacuole (PSV)

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; Müntz 1998; Herman and Larkins 1999). This vacuole was shown to store also defence proteins in seed (Neuhaus and Rogers 1998). The tonoplast of these vacuoles contains another distinct aquaporin, called α-TIP (Paris et al., 1996; Swanson et al., 1998).

The vegetative storage (neutral) vacuole

Di Sansebastiano et al., 2001 showed that LV and neutral (probably vegetative storage) vacuoles are regenerated by evacuolated protoplasts of tobacco (Nicotiana tabacum) cells.
They are also accumulated in specialized vegetative cells in response to wounding or to the developmental switches, and the membrane of these vegetative storage vacuole contains the aquaporin, δ-TIP (Jauh et al., 1998; Neuhaus and Rogers 1998; Park et al., 2004).

Hybrids vacuoles

However, some vacuoles could be identified with two different TIPs (Jauh et al., 1999), and on the other hand Murphy et al., (2005) showed that when soybean plants were subjected to changed physiological conditions, the γ-TIP marker diminished while the δ-TIP marker became present in the paraveinal mesophyll vacuole indicating the conversion of a lytic vacuole to a vegetative storage vacuole. This makes the classification of vacuoles based on TIPs more complicated. A fourth TIP-isoform called DIP (Dark Induced Protein) was also identified which did not cross react with others TIPs and was found in root tip cells and developing seeds (Culianez-Macia and Martin 1993).

Seeds storage vacuoles with subcompartments

Developing seeds of castor bean accumulate lipids and storage proteins in their endosperm (Jolliffe et al., 2004). The major storage proteins are 7S lectin, 2S albumin and 11S globulins. In seeds the protein storage vacuole is complex and contains globoids and large crystalloids within a soluble matrix. Globoids contain for example aleurain, the crystalloids are composed of crystalline 11S globulin while the matrix compartment is a mixture of 7S lectins and 2S albumins. In seed storage vacuoles DIP, a TIP2 isoform, is associated with the crystalloid membranes and the matrix is surrounded by a tonoplast with both α-and δ- TIP (Jiang et al., 2000).

Tamura et al. (2003) noticed different degradation activity in vacuole. GFP was not stable in acidic vacuoles under the light condition. The fluorescence of a vacuole-targeted GFP was stably observed in the vacuole of plants under dark conditions and this fluorescent rapidly degraded under light condition.

Due to the diversity of plants vacuoles, vacuolar sorting seems more complex than in mammalian systems since correct proteins needs to reach each vacuole type.

Vacuolar targeting in plants

Several vacuolar proteins are synthesized as precursors with a short peptide sequence (named vacuolar sorting determinant VSD) necessary for vacuolar targeting which is proteolytically removed upon deposition of the protein in the vacuole (Neuhaus and Rogers 1998; Matsuoka and Neuhaus 1999).VSDs have been identified in different positions within the vacuolar proproteins. The VSDs are divided into three main groups: (1) “the sequence-specific” VSDs (ss-VSD), (2) C-terminal VSDs (ct-VSD), and (3) the internal or physical structure VSDs (ps-VSD) (Neuhaus and Rogers 1998).

The sequence-specific vacuolar sorting determinants (VSDs)

The best studied examples of ss-VSDs are in the N-terminal propeptides (NTPP) of sporamin, a storage protein of the tuberous roots of sweet potato, and of barley proaleuraine, a protease from barley.

The NTPP of sporamin was shown to be 16 amino acids long, and to be processed after protein sorting (Matsuoka et al., 1990). When expressed in tobacco suspension culture cells,

sporamin is sorted to the vacuole and expression of a mutant lacking the 16 amino acids propeptide results in secretion of sporamin, demonstrating that the propeptide contains the essential VSD (Matsuoka and Nakamura 1991; Nakamura et al., 1993). It was then shown that the essential sequence for vacuolar targeting was SRFNPIRL. The point mutation Asn-26 to Gly caused about 40% secretion, while the mutation of Ile-28 to Gly abolished vacuolar sorting of prosporamine (Nakamura et al., 1993), showing that the sequence of such a propeptide is more important than its position (Matsuoka 2000).

The second protein identified to contain an ss-VSD was barley aleurain which is synthesized as a proenzyme and transported to an acidified, post-Golgi 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 PSV (Holwerda et al., 1990). That is why aleurain was used as a maker to define lytic vacuoles (Paris et al., 1997). The deletion of SNPIR from its N-terminal propeptide lowered the sorting efficiency of aleurain, suggesting that these residues are also critical for proaleuraine sorting.

Although the VSDs 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). Comparison of the prosporamin and proaleuraine VSDs demonstrated the presence of a conserved central motif NPIR suggesting that this sequence might be recognized by a very similar sorting receptor in both plants.

In brazil nut, an ss-VSD was identified in the C-terminal propeptide. Castor bean ricin has the ss-VSD located within an internal propeptide. This toxin was shown to accumulate in the PSV (Hara-Nishimura et al., 1998). It was found to have an internal linker SLLIRPVVPNFN which was required for vacuolar targeting and the mutation of the Ile to Gly led to the secretion of the preproricin (Frigerio et al., 2001b).

The C-terminal vacuolar sorting determinants (ct-VSD).

The C-terminal propeptides of several vacuolar proteins have been shown to be both necessary and sufficient for proper sorting of another protein to vacuoles in plant: those of the barley lectin (Bednarek et al., 1990; Dombrovski et al., 1993) , tobacco chitinase A (Neuhaus et al., 1991b) , β-1,3-glucanase (Sticher et al., 1992) and 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., 1991b), because all partial deletions strongly reduced the percentage of intracellular accumulation. 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% (Neuhaus et al., 1994).

In barley lectin, mutational analysis of the C-terminal VSD confirmed that no common structural determinant is important. Addition of two glycines at the C-terminus or translocation of the glycosylation site to the C-terminus disrupted the sorting (Dombrovski et al., 1993).

Deletion of the last four amino acids AFVY of phaseolin, the 7S storage protein of common bean, caused the complete secretion of the protein in transgenic tobacco cells (Frigerio et al., 1998).

In contrast to the ssVSD signals, no consensus sequence has yet been identified for the ct-VSD, but this domain was shown to be enriched in hydrophobic amino acids (Matsuoka and Neuhaus 1999; Ahmed et al., 2000).

The physical structure vacuolar sorting determinant (ps-VSD)

The third class of proteins 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 involved in vacuolar sorting. The sorting determinant must be carried within the mature polypeptide. This third VSD has been described for the phytohemagglutinin of common bean and for legumin-like proteins. A targeting signal is located in different regions of the polypeptide or in a single large portion respectively, suggesting an important role for higher order structures (Tague et al., 1990; Saalbach et al., 1991; Von Schaaewen and Chrispeels 1993; Vitale and Raikhel 1999). With this type of VSD aggregation was shown to be a possible mechanism of sorting (Vitale and Chrispeels 1992). A form of aggregation is also presented by cereals prolamins which aggregate within the ER to form protein bodies (Okita and Rogers 1996). Determinants for aggregation are often associated with hydrophobic regions on the surface of the molecule formed by folding of their three-dimensional structure. When isolated from ER and Golgi vesicles, pea prolegumin 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).

A recent study showed that some proteins may possess more than one vacuolar sorting determinant. The seed storage proteins of soybean are composed mainly of glycinin, an 11S globulin and of β-conglycinin, a 7S globulin. Glycinin possesses five major subunits: A1AB1b, A1bB2, A2B1a, A3B4 and A5A4B3. The C-terminal stretch of 10 amino acids of A1AB1b was sufficient to direct another protein to the PSV, and functional inactivation of this putative ct-VSD did not block PSV sorting. Three-dimensional structure of this subunit identified a candidate for sequence specific determinant on the same sequence, Ile -297 which seemed critical for sorting. Inhibition of the ct-VSD combined with a mutation of the Ile297 to Gly, still did not abolish the vacuolar sorting of A1AB1b, suggesting that there is a third sorting determinant in addition to ct-VSD and ss-VSD (Maruyama et al., 2006).

Similar to yeast and mammalian cells, the transport of proteins to the plant vacuole is saturable, indicating the involvement of sorting receptors that might interact with these signals at the TGN (Vitale and Raikhel 1999).

Vacuolar Sorting Receptors in plant (VSRs)

From Golgi to lysosome in mammals, to vacuoles in yeast or plants, clathrin-coated vesicles (CCVs) are known to participate in this traffic. An integral membrane glycoprotein of 80 kDa was purified from the membrane of pea CCV and this protein showed ability to recognize barley aleurain and sporamine VSDs (Kirsch et al., 1994) and the C-terminal sorting signal of Brazil nut 2S albumin (Kirsch et al., 1996). This protein was named BP-80, but later renamed VSR_{PS-1}(Vacuolar sorting receptor, for *Pisum sativum*).

Another potential vacuolar sorting receptor of the same family was identified in Arabidopsis and named AtELP. AtELP-related proteins have been identified from several plant species (Paris et al., 1997; Shimada et al., 1997; Miller et al., 1999)

The prototype of the VSR family pea BP80 (VSR_{PS-1}) 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 ending with a cytosolic tail domain (CT) of 37 amino acids. The luminal domain contains a PA (protease-associated) domain, followed by a large VSR-specific domain of 318 amino acids and 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. 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 analyzed by confocal microscopy (Paris et al., 1997). VSR_{PS-1} was found in small punctate structures in pea root tip cells (Paris and Rogers 1996; Paris et al., 1997).

The pumpkin seed-specific receptor PV72 was proposed to sort seed storage protein to 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). PV72 was shown to bind as well to the NPIR motif of the precursor of a cysteine proteinase (Watanabe et al., 2004).

The RMR family

A new family of putative receptors was identified by its homology to the PA domain of the VSR proteins. These proteins are composed of an N-terminal luminal domain restricted to a PA domain, 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 (Cao et al., 2000; Jiang et al., 2000) 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 seeds (Jiang et al., 2000).

The luminal domain of the RMR proteins shares with the VSR proteins the PA domain that is known to participate in ligand binding (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 demonstrated. The genome of Arabidopsis harbours 6 homologues (AtRMR1-6).

A new study on AtRMR1 showed that it was highly expressed in protoplast and that AtRMR1-HA (HA epitope-tagged AtRMR1) labelled a punctate structure colocalized with ST-GFP at the Golgi complex but different from the pattern of phaseolin at the PSV in leaf cells. AtRMR1 interacts with the CTPP of phaseolin at acidic pH. Once the AtRMR1-phaseolin complex arrives at the PSV, the neutral pH of this compartment favours the dissociation of the complex. AtRMR1 function as cargo receptor for PSV-protein by interacting with the CTPP of phaseolin and was shown to colocalize with phaseolin on the way to the PSV (Park et al., 2005) .

Aim of the thesis

The aim of this study was to define the function of the different sorting receptors using GFP as the reporter based on RNA interferences (gene silencing).

RNA interference (RNAi gene silencing) has been successfully used as a tool in many organisms to silence individual genes and multiple members of a gene family (Hannon 2002; Tijsterman et al., 2002). RNAi was shown to be systemic in tobacco plants (Voinnet and Baulcombe 1997). It was possible to create a population of transgenic plants with reduced expression of the gene of interest in plants by inheritance of RNAi transgenes in subsequent generations. In our study, we used both transient and stable assay.

Transient assays were used to silence both putative vacuolar sorting receptor families. The vector used in this assay was the Cabbage Leaf Curl Virus belonging to the geminivirus family. This virus was used first, because it was able to replicate through DNA intermediates in plants instead of RNA like other which replicate though RNA (Laufs et al., 1995; Nagar et al., 1995; Hanley-Bowdoin et al., 1999). Secondly it was used because it was possible to

silence more than one gene at the same time. Finally it was possible to achieve systemic silencing in *A. thaliana* plant without needing to go through meiosis and embryogenesis, avoiding problems of embryo lethality or germination problems.
A stable silencing assay was used to by pass the geminivirus symptoms, using Agrobacterium –mediated transformation.

Outline of the thesis

This thesis is built around 5 chapters.
Chapter 1 is this introduction
Chapter 2 describes how the subfamily AtVSR 3 is involved in proteins sorting to the lytic vacuole in plants.
Chapter 3 gives preliminary results on the functional role of AtRMR after their transient and stable silencing in transgenic Arabidopsis plants.
Chapter 4 describes material and methods important for the experiments reported in the thesis.
Chapter 5 presents the general discussion of the results obtained

Chapter 2

Vacuolar sorting receptors of the VSR subfamily 3 are responsible for vacuolar targeting to lytic vacuoles in plants

Jeannine Okmeni Nguemelieu, and Jean-Marc Neuhaus

Abstract

Plant cells can possess at least two types of vacuoles with different functions. To visualize these vacuole types in plants, we fused the green fluorescent protein (GFP) to different vacuolar sorting determinants (VSD). GFP was targeted to a pH-neutral (vegetative storage) vacuole when fused to the C-terminal VSD of tobacco chitinase A (Chi), whereas the N-terminal sequence-specific propeptide of barley aleurain (Aleu) targeted GFP to an acidic lytic vacuole. Vacuolar sorting receptors (VSRs) have been proposed to mediate sorting of proteins to the lytic vacuoles while RMRs would mediate sorting of proteins to the storage vacuoles. To identify a possible specialisation of VSR subfamilies in the recognition mechanism leading to vacuolar sorting mediated by either VSDs, we caused by gene silencing a deficiency of whole VSR subfamilies in reporter-expressing *Arabidopsis thaliana* plants. Partial VSR sequences were cloned in tandem into a geminivirus silencing vector, which was introduced by biolistics into reporter plants expressing either Aleu-GFP or GFP-Chi to visualize the effect of gene silencing. The inactivation of the subfamily VSR 3 in Aleu-GFP transgenic plants caused the disappearance of the GFP marker from the large lytic central vacuole of leaf epidermal cells and its accumulation in small compartments. Fluorescence could also be seen to disappear from roots. In contrast, the joined silencing of subfamilies VSR 1 and 2 showed little effect. In GFP-Chi plants, silencing of neither VSR subfamily 3 nor subfamilies 1&2 showed any marked visible effect. These results suggest that subfamily 3 VSRs are implicated in the sorting of proteins with sequence-specific VSD to lytic vacuoles, while subfamilies 1 and 2 have other functions.

Keywords: *Arabidopsis thaliana*, GFP, NPIR, vacuolar sorting determinants, vacuoles, vacuolar sorting receptor.

Introduction

Proteins of the secretory pathway are synthesized on the rough endoplasmic reticulum (ER) and then delivered by membrane-bounded transport vehicles to other cell compartments such as Golgi, vacuoles, plasmalemma and the cell wall (Hadlington and Denecke 2000; Jolliffe et al., 2005). Except for the bulk flow of soluble proteins to the apoplast, specific signals carried by the cargo proteins are required for their correct sorting to the different compartments. These signals are found in the polypeptide chain and, especially for vacuoles, in propeptides which can be located at the N- or C-terminal ends of precursors or internally located (Neuhaus and Paris 2005).

Sequence-specific vacuolar sorting determinants (VSD) were found first in N-terminal propeptides (NTPP) of barley aleurain and sweet potato sporamin precursors, both having an NPIR motif (Matsuoka and Nakamura 1991; Holwerda et al., 1992), but they were later also found in internal and C-terminal propeptides (Saalbach et al., 1996; Frigerio et al., 2001a). An essential Ile or Leu was identified in each case. C-terminal VSDs were first identified as C-terminal propeptides (CTPP) in tobacco chitinase and barley lectin (Bednarek and Raikhel 1991; Neuhaus et al., 1991b) and have no conserved motifs, but must be accessible from the C-terminus. These two types of VSD indicate therefore the existence of at least two different mechanisms by which a soluble secretory protein can be directed to a vacuole. Two types of vacuoles were immunologically distinguished by antisera against tonoplast and soluble proteins (Hoh et al., 1995; Guivarch et al., 1996). These lytic and storage vacuoles are known to have different functions because the former contain hydrolytic enzymes and some secondary metabolites (Wink 1993) while the latter contain storage proteins. In many cells, these vacuoles may fuse to a hybrid vacuole (Dombrovski et al., 1993; Jauh et al., 1999). Vacuoles can convert from a lytic to a storage vacuole and back depending on the physiological needs of the plant (Murphy et al., 2005). In seeds a third sorting mechanism involves condensation in either the ER or the Golgi of storage proteins, which are transported to seed storage vacuoles by precursor-accumulating (PAC) vesicles or dense vesicles, respectively (Hoh et al., 1995; Shimada et al., 1997). Seed vacuoles can further become organised in a complex storage vacuole with internal compartments (Jiang et al., 2001).

A vacuolar sorting receptor that binds to a sequence-specific vacuolar sorting determinant was first identified in clathrin-coated vesicles from pea cotyledons (BP-80, Kirsch et al., 1994). Homologues were later identified and cloned in different plants such as pumpkin (PV72, Shimada et al., 1997) and *Arabidopsis* (AtELP, Sanderfoot et al., 1998; Laval et al., 1999). These vacuolar sorting receptors (VSR) are type I integral membrane proteins with a large

luminal domain containing a PA (protease-associated) domain and three EGF repeats, a single transmembrane domain and a short cytosolic tail (Paris et al., 1997) containing an adaptin-binding tyrosine motif (Happel et al., 2004). Sorting to lytic vacuoles was suggested to depend on VSR binding a sequence-specific VSD on protein propeptides. However, a sequence-specific VSD was also identified in *bona fide* storage proteins such as 2S albumins (Shimada et al., 2002). PV72, a pumpkin VSR, was identified in PAC vesicles containing storage proteins (Shimada et al., 1997) and a knock-out mutation of the gene for the Arabidopsis AtELP1 caused a defect in protein storage in seeds (Shimada et al., 2003). Different localisations of VSRs have been reported in different systems, mostly in Golgi and prevacuoles (Jiang and Rogers 1999), but also in small vacuoles (Paris et al., 1997) and at the plasma membrane (Laval et al., 1999). Proteomics of fractionated Arabidopsis suspension cells indicated a different localisation for one VSR isoform than for three other isoforms (Dunkley et al., 2006).

Furthermore, ligand binding was shown to be pH-dependent for pea BP-80, but Ca^{2+}-dependent for PV72 (Hara-Nishimura et al., 1998). It is thus probable that there are different functions and localisations for different VSRs.

The genomes of *Arabidopsis thaliana*, poplar and rice encode seven, seven and six VSR homologues, respectively. A phylogenetic analysis of these VSRs indicates that they can be grouped into three subfamilies (Figure 1), which are also supported by cDNA and EST sequences in legumes and grapevine. What are the functions of these subfamilies in plants? Considering the redundancy of VSR genes and the lack of phenotype of single knock-out mutations in Arabidopsis (with the exception of AtVSR1;1 in seeds, cf. Shimada, 2003), we chose the strategy of silencing whole gene subfamilies at once. To visualize the effects on vacuolar sorting, we used transgenic plants expressing either of two fluorescent reporters, the soluble GFP fused with the C-terminal VSD of tobacco chitinase A (GFP-Chi, Di Sansebastiano et al., 1998) or with the N-terminal sequence-specific VSD of barley aleurain (Aleu-GFP, Di Sansebastiano et al., 2001). In protoplasts, these two reporters were found to label neutral or acidic vacuoles, respectively. In transgenic *A. thaliana* the two constitutively expressed reporters labelled different vacuoles: in epidermal cells, Aleu-GFP labelled the large central vacuole while GFP-Chi labelled small peripheral grains. In contrast, in the mesophyll the central vacuole was labelled by GFP-Chi while Aleu-GFP was often not visible (Flückiger et al., 2003).

A silencing vector derived from a geminivirus allowed to observe the effects of silencing of VSR subfamilies in whole plants without the need to go through meiosis and embryogenesis,

which could be disturbed by the loss of VSR function (Laval et al., 2003). We found that the inactivation of the VSR subfamily 3 in Aleu-GFP transgenic plants caused the loss of vacuolar fluorescence in the epidermal cells of rosette leaves, while GFP was concentrated in small compartments, while silencing of subfamilies 1 and 2 appeared to have no effects. We conclude that subfamily 3 VSRs are necessary for proper sorting of proteins sequence-specific VSD to lytic vacuoles in vegetative tissues.

Results

Phylogeny of VSR subfamilies

A comparison of VSR sequences from various plants revealed the existence of conserved subfamilies with more related sequences. All VSR sequences from the genomes of Arabidopsis, rice and poplar were aligned with the VSR sequences of pea (BP-80) and pumpkin (PV72). The phylogeny of VSRs was determined using the fastDNAML program (Olsen et al., 1994). Based on this phylogenetic tree (Fig.1), VSRs were subdivided into three subfamilies VSR1, VSR2 and VSR3. Using a nomenclature based on this subdivision (Neuhaus and Paris 2005), the Arabidopsis genes are renamed AtVSR1;1 (AT3g52850) and AtVSR1;2 (AT2G30290); AtVSR2;1 (At2g14720) and AtVSR2;2 (At2g14740), and AtVSR3;1 (AT4G20110), AtVSR3;2 (At2g34940) and AtVSR3;3 (At1g30900). The same three subfamilies are also found in rice and poplar and are represented by abundant EST sequences from legumes, solanaceae and grape. EST sequences from pine support the existence of at least two of the three subfamilies in gymnosperms. In contrast, moss (*Physcomitrella patens*) VSR sequences do not particularly correspond to any subfamily, but they allow placing the root of the family tree between subfamily 3 and the other two. The conservation of three subfamilies in angiosperms and probably also in gymnosperms suggests that they reflect an ancient functional divergence. Within subfamilies, there is some redundancy, which may explain why there was no detectable phenotype in single knock-out Arabidopsis plants, except for VSR1 (AtVSR1;1) which only affected seed storage tissues (Shimada et al., 2002). It should be noted that this VSR belongs to the same subfamily 1 as pumpkin PV72, which is also involved in storage protein sorting (Shimada et al., 2002), while the original pea BP-80 belongs to subfamily 2.

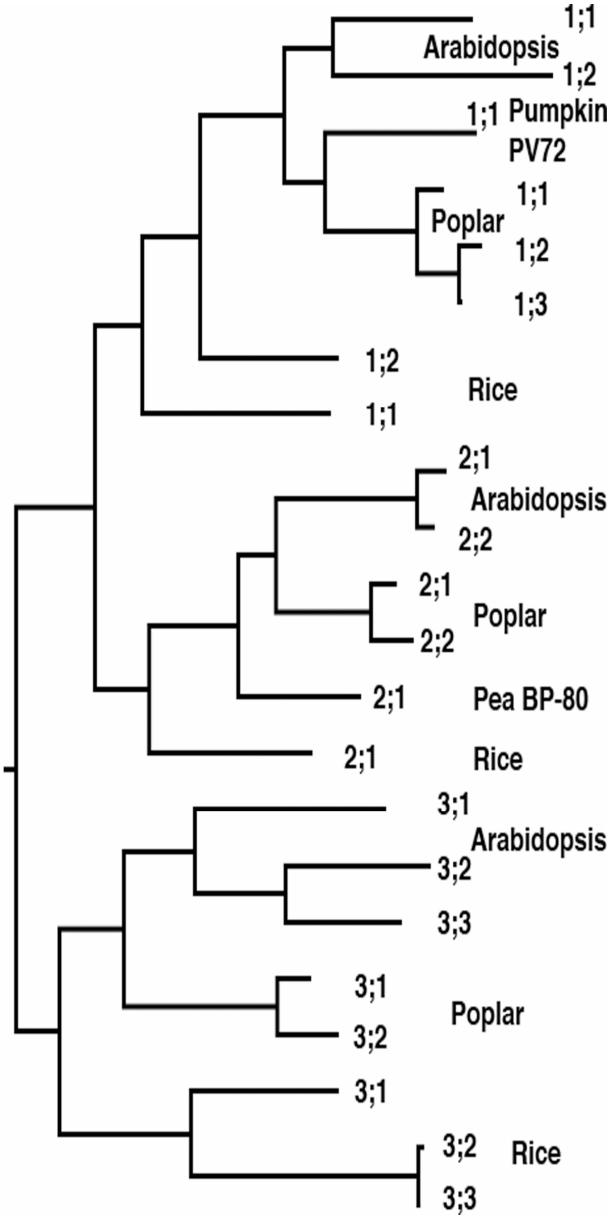


Fig. 1 Phylogenetic tree of VSR sequences from the three fully sequenced plant genomes, *A. thaliana,* rice and poplar. Pea BP-80 and pumpkin PV72 are also included. The most likely trees were determined by the FastDNAML program (Olsen et al., 1994). They only differ by minor rearrangements within the subfamilies. The root was placed by using moss sequences as out-group. This analysis emphasizes the ancient divergence of the three subfamilies, suggesting a functional divergence during the evolution of flowering plants. Arabidopsis VSR1;1 and pumpkin PV72 are involved in trafficking of seed storage proteins.

Expression of VSR in leaves and roots

Before silencing them, we verified by RT-PCR the expression level of the VSR genes in leaves and roots of Arabidopsis. All genes were expressed in leaves and roots except for AtVSR 3; 1 which was not detected (Fig. 2). The strongest expression was observed for AtVSR1;1 and AtVSR3;2 while AtVSR1;2 and AtVSR3;3 had a lower level of expression and the two receptors of subfamily 2, which are highly homologous and were not

distinguished here (as also handled in microarrays), were only detected at a low level. Laval et al.(2003), presented similar results but they found a stronger expression in leaves of AtVSR2;1 than AtVSR1;1. In microarray results, AtVSR3;2 is also the strongest expressed isoform in leaves, while AtVSR1;2 and 3;1 are hardly detected at all, while in roots all three isoforms of subfamily 3 were detected (summarized in Neuhaus and Paris 2005).

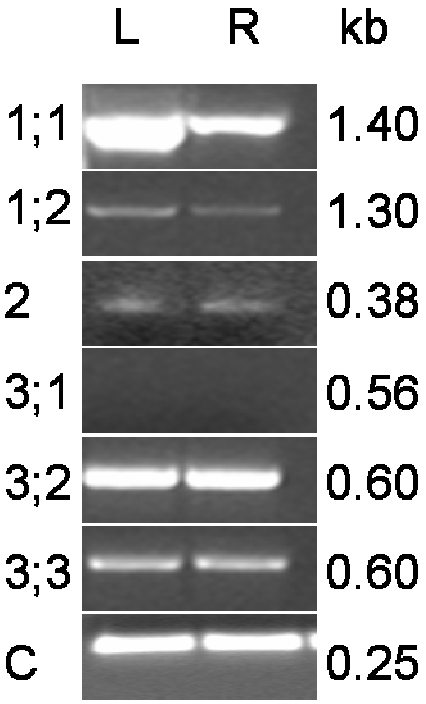


Fig. 2 Expression of VSR genes in leaves (L) and roots (R) of A. thaliana. The expression was analysed by RT-PCR from total RNA of the two tissues. Genes 2;1 and 2;2 were not distinguished. Actin was used as control (C).

Silencing of VSRs with the geminiviral vector

Since the knock-out results for each single VSR gene suggested that redundancy could obscure the effects of a loss of receptors, we chose to silence whole subfamilies at once. On the other hand, an antisense AtVSR1;1 plant had produced non-germinating seeds. In order to avoid the need for plant regeneration and germination, we chose the strategy of systemic silencing in grown plants. For efficient systemic spreading in Arabidopsis we chose the geminiviral vector pCbLCV007, which is derived from the A genome of Cabbage Leaf Curl Virus (CbLCV Turnage et al., 2002). PCR amplified gene fragments for each member of either subfamily 1 and 2 or subfamily 3 were cloned in tandem into the pCbLCV007 vector and shot together with the CbLCV B genome into leaves of reporter Arabidopsis plants expressing either Aleu-GFP or GFP-Chi (Flückiger et al., 2003). General viral symptoms were observed for all the bombarded plants in newly emerging leaves between 3 to 4 weeks post inoculation. They included yellow spots, leaf curling, stunted growth, necrosis and

variegation (Fig.3 A) as had been observed previously in Nicotiana benthamiana (Peele et al., 2001) and Arabidopsis (Turnage et al., 2002). The symptoms spread on young leaves until they became completely yellow. This can be explained by the enlargement and fusion of yellow spots. Symptoms were helpful to follow the spreading of the virus inside the leaf and to the whole plant. The viral spread also affected the localization of the vacuolar marker in trichomes (Fig. 3 B-D).

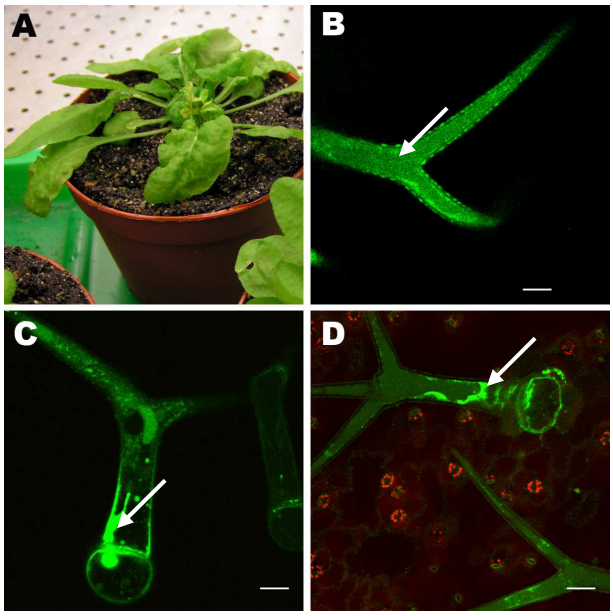


Fig. 3 Viral symptoms and changes in the localisation of vacuolar GFP due to the vector. Visible phenotype after bombardment with the virus (**A**). Homogenous GFP fluorescence in the trichome of a non-infected plant (**B**). Strongly labelled structures within trichomes of plants infected with the empty virus (39 dpi, **C**) and within trichomes of silenced leaves (49 dpi, **D**). Scale bars=100 µm.

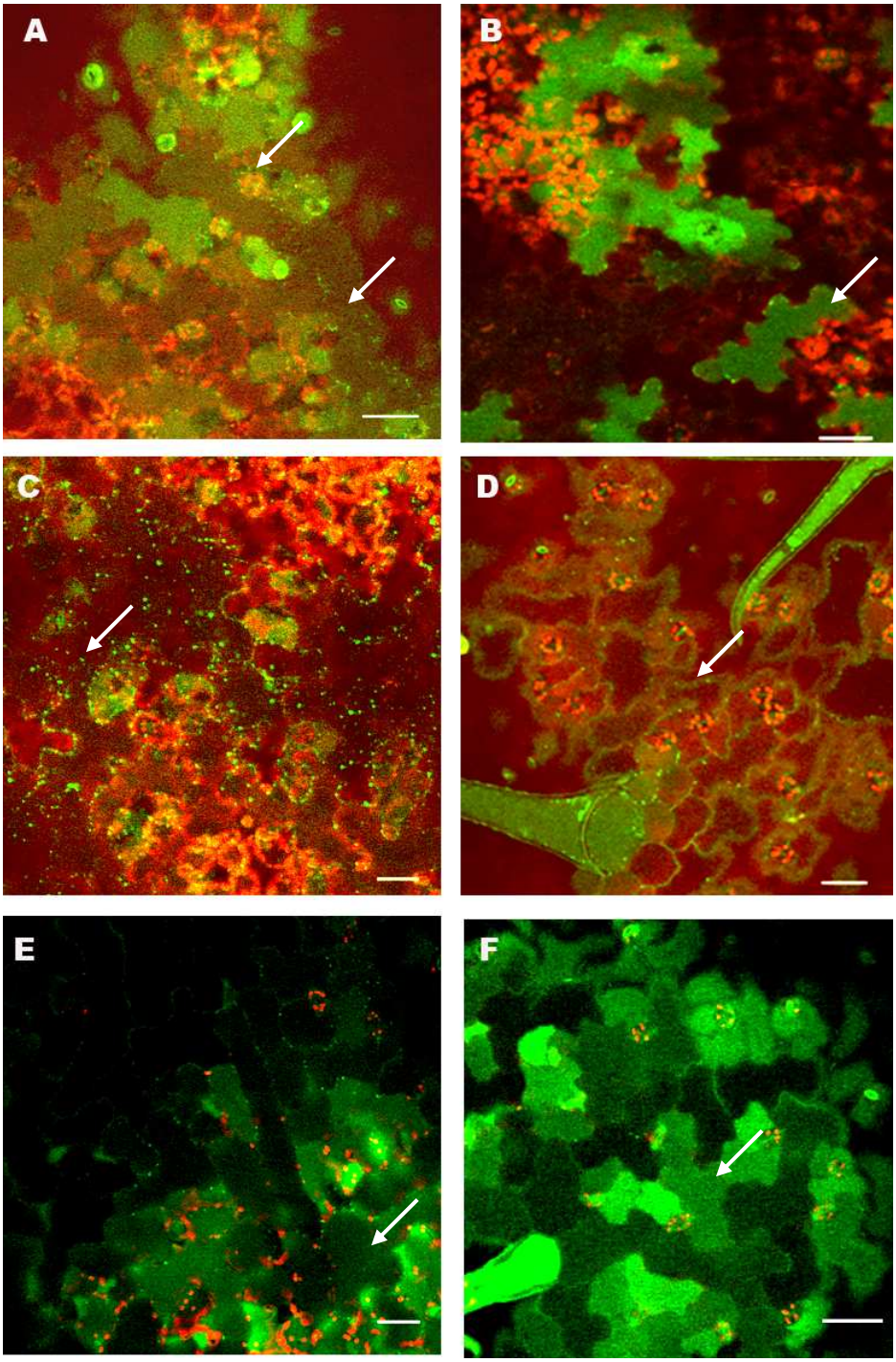
Aleu-GFP redistribution upon silencing of the VSR subfamily 3

Leaves of inoculated Aleu-GFP plants were excised when viral symptoms became visible and were analysed by confocal microscopy. Leaves from plants inoculated with the empty vector showed the previously described distribution of GFP fluorescence (Flückiger et al., 2003). GFP strongly accumulated in the large central vacuoles of epidermal cells (Fig. 4 A) and was also visible as a faint diffuse fluorescence in meristemoids(See arrow). Vacuoles from mesophyll cells in very young le aves showed strong fluorescence, which disappeared in mature leaves (Fig. 4 A and B). This indicates that the spread of the viral vector does not markedly alter the vacuolar compartments of the leaves, except in the trichomes (Fig. 3).

Plants inoculated with the vector silencing subfamilies 1 & 2 showed the same pattern (Fig. 4 G and H).

Silencing of VSR subfamily 3 altered the pattern of fluorescence in Aleu-GFP plants. At 39 d.p.i. (days post inoculation), the large central vacuoles of epidermal cells lost their fluorescence and GFP was concentrated in small compartments near the plasma membrane (Fig. 4C). This pattern resembles the pattern of accumulation of GFP-Chi in epidermal cells or in elongating cells (Flückiger et al., 2003). Later in infection, the GFP fluorescence decreased in the peripheral compartments and a more continuous fluorescence appeared as a line around the vacuole (Fig. 4D). GFP remained absent from mesophyll cells.

Fig. 4 Effects of silencing of VSR subfamily 3 on the localisation of Aleu-GFP, the marker for lytic vacuoles. A and B control plants inoculated with the empty vector. C and D plants inoculated with the virus containing sequences of all subfamily 3 genes. E and F plants inoculated with the virus containing sequences of all subfamily 1 and 2 genes. The plants were examined by confocal microscopy 39 dpi (A, C, E) and 49 dpi (B, D, and F). Arrow indicate the GFP fluorescent in cells Scale bars= 100 µm



The silencing could also spread into the roots of Aleu-GFP plants (Fig. 5 A). While after 39 dpi there still was a strong fluorescence in roots (Fig. 5 B and C), after 49 d.p.i. silencing of the VSR subfamily 3 had a massive effect, causing the almost complete disappearance of GFP from root cells (Fig. 5 D) without affecting the morphology of the roots.Arrow: GFP in cells Scale bar=100µm

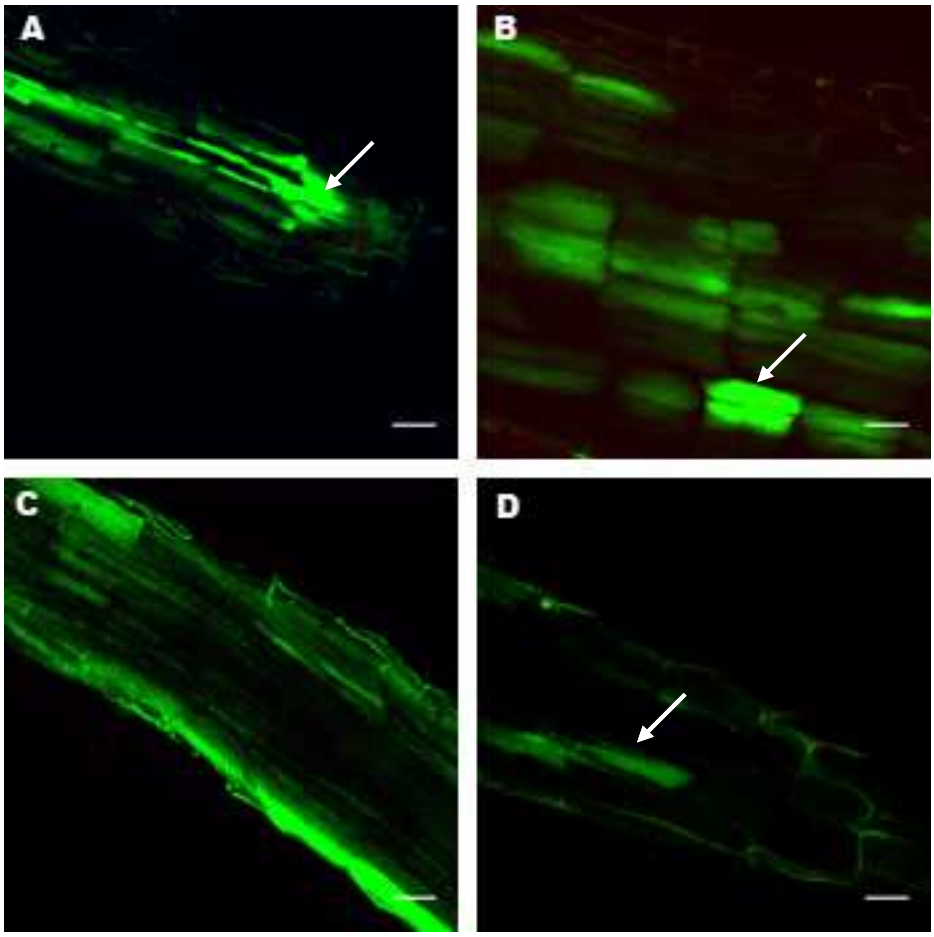


Fig. 5 GFP distribution in roots of control and silenced Aleu-GFP transgenic plants.
Roots showed fluorescent vacuoles 39 d.p.i. in both control (A) and silenced plants (B). GFP disappeared from roots 49 d.p.i. in plants silenced for subfamily 3 (D) and still detected when subfamilies 1and 2 were silenced (C). Scale bars= 100µm

Localization of vacuolar GFP-Chi is unaltered in plants silenced for VSR genes

The GFP-Chi reporter is sorted by a different mechanism to vacuoles of a different type than the Aleu-GFP reporter (Di Sansebastiano et al., 2001; Flückiger et al., 2003) . VSRs are not thought to be involved in sorting of GFP-Chi to a pH-neutral vacuole. GFP-Chi accumulates in leaf epidermal cells of transgenic plants in small peripheral compartments, possibly neutral prevacuoles or small vacuoles (Fig. 6 A and B) but accumulates in the central vacuole of mesophyll cells (Fig. 6 B) and strongly labels guard cells. This distribution was not

affected by the inactivation of the whole subfamily 3 (Fig. 6 C and D) nor of the whole subfamilies 1 & 2 (Fig. 6 E and F).

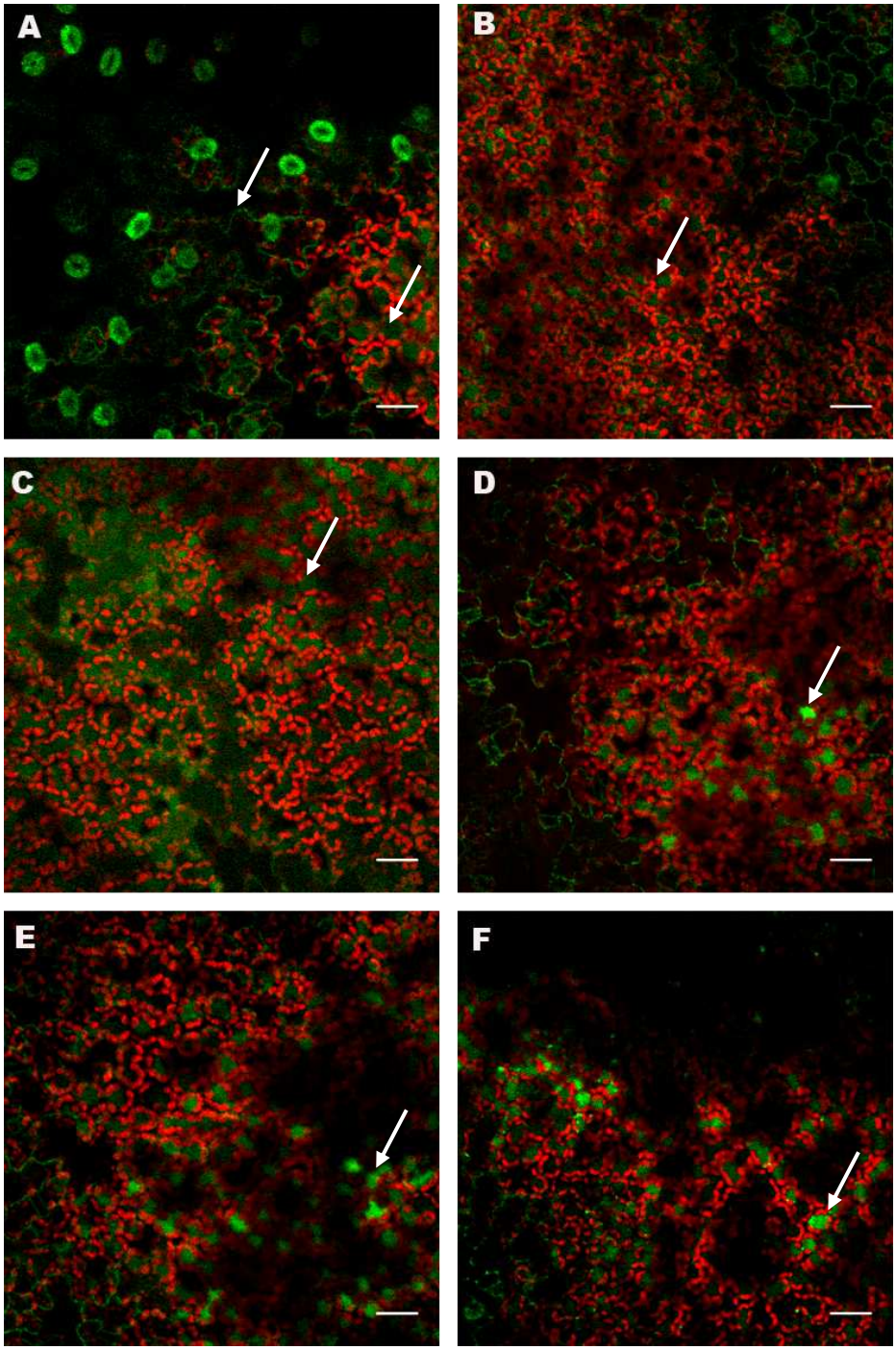


Fig. **6** Effects of silencing of VSR subfamily 3 on the localisation of GFP-Chi, the marker for neutral vacuoles. A and B control plants inoculated with the empty vector. C and D plants inoculated with the virus containing sequences of all subfamily 3 genes. E and F plants inoculated with the virus containing sequences of all subfamily 1 and 2 genes. The plants were examined by confocal microscopy 39 dpi (A, C, E) and 49 dpi (B, D, and F). Arrows indicate the GFP marker localisation in cell. Scale bars= 100 µm

Tissue and cell expression patterns of VSRs

The different effects of silencing subfamilies 1 & 2 or 3 could be due to differential expression in leaf or root tissues. RT-PCR had indicated the abundant presence of mRNA from one member each of subfamilies 1 and 3 in both leaves and roots and a lower abundance of several other homologues (Fig. 2). To identify expression differences between tissues within these organs, their transcripts were localized by *in situ* hybridization in leaves and in roots.

The sections hybridized with antisense probes for *At*VSR2, 3;1 and 3;3 were undistinguishable from control sections (tissues hybridized with corresponding sense probes). The most strongly expressed AtVSR1; 1 and 3; 2 showed strong signals in both leaves and roots (Fig. 7). They were detected in epidermis and mesophyll cells of leaves and were found to be expressed particularly strongly in trichomes (Fig. 7 A and C). On root sections, mRNA signals of these receptors were also strong and were present in epidermis, cortex, xylem and phloem (Fig. 7 B and D). No signal was detected for any sample hybridized with sense RNA. In essence there was no differential tissue expression for these two strongly expressed VSRs, indicating that the different effects of silencing their respective subfamilies were most probably linked to their different functions.

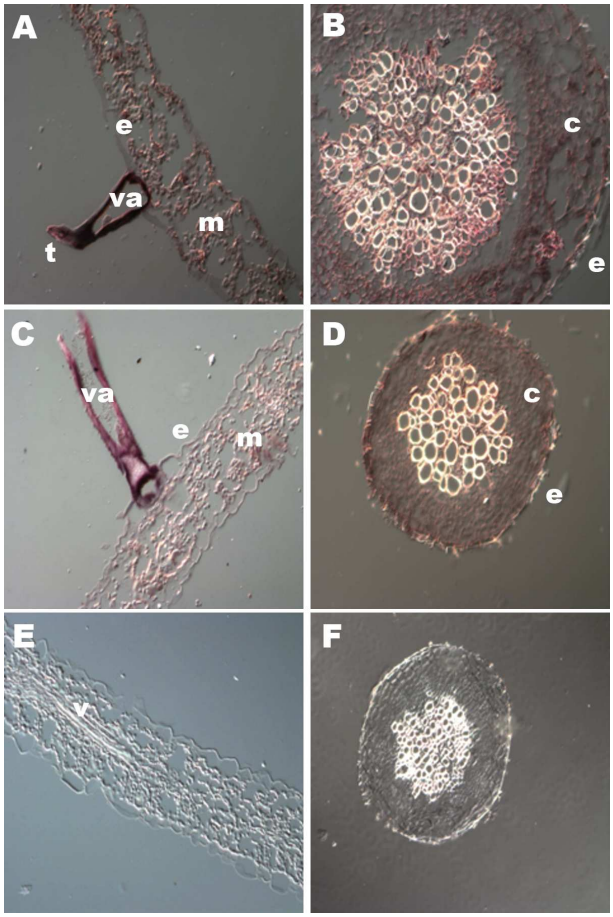


Fig. 7 Tissue-specific location of major VSR transcripts was detected by *in situ* hybridization in sections of leaves (A, C) and roots (B, D) from Arabidopsis control plants. Detection of AtVSR1;1 with an antisense probe (A, B). Detection of AtVSR3;1 with an antisense probe (C, D). Control hybridization with a sense probe (E, F). e, epidermis; c, cortex ; m, mesophyll cells; V, vascular bundle; t, trichome; Va , vacuole

VSR gene expression in leaves after silencing of subfamilies 3 or 1&2

In leaves of control plants all genes except for *At*VSR3;1 were expressed, as previously reported. After transient silencing of each subfamily, the expression of target genes was not completed. It is possible that gene still detected came from the non silenced part of the leaf. Since during RNA extraction, the whole leaf was taken and it was not possible to separate yellow spoted part from green part.

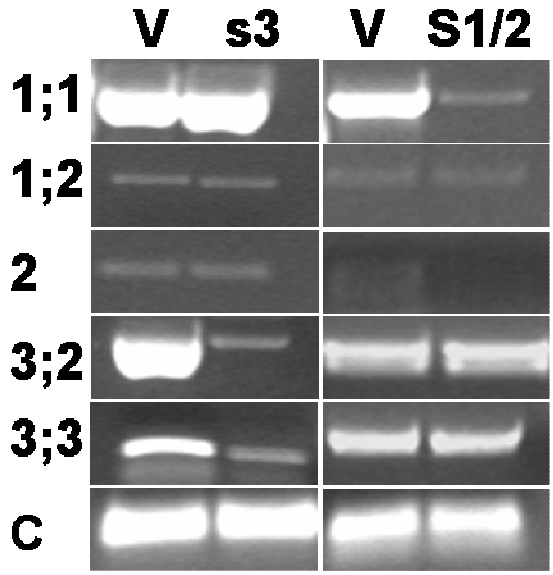


Fig. 8 Expression of VSR genes in leaves after silencing of subfamilies 3 or 1& 2. The expression was analysed by RT-PCR from total RNA as in Figure 2.

Expression was compared between plants shot with the empty vector (lane 1 and 3), with the virus silencing subfamily 3 (lane 2) and with the virus silencing subfamilies 1&2 (lane 4). AtVSR3;1 was not detected (not present in the picture). Actin as control gene was unaffected by the silencing viruses (**C**).

Fate of vacuolar GFP in silenced plants

To determine what happens with the vacuolar Aleu-GFP when its receptors are missing due to the silencing of their genes, we extracted total proteins from leaves of control and silenced plants. Immunoblot analysis indicates that the reporter protein is degraded when its normal targeting has been suppressed (Fig. 9). This was to be expected, as it has been described that GFP rapidly disappears when secreted into the apoplast of Arabidopsis. Plants used in the experiment were the same plants used for the RT-PCR and came from the same clone.

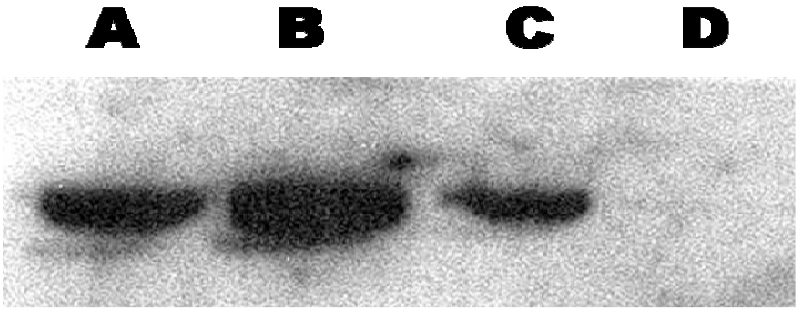


Fig. 9 Fate of vacuolar Aleu-GFP in leaves silenced for the VSR genes

Immunoblot analysis of GFP extracted from E. coli cells(A), control leaves (B), leaves silenced for subfamilies 1 & 2 (**C**), and leaves silenced for subfamily 3 (D).

Discussion

BP-80 was the first VSR protein identified and purified by affinity chromatography from pea cotyledons using as affinity ligand the sequence-specific VSD from the propeptide of barley aleurain. Soon after the cloning of BP-80, a VSR from pumpkin was cloned (Shimada et al., 1997) and a homologue from Arabidopsis was identified by several groups (Sanderfoot et al., 1998; Laval et al., 1999). The antibodies produced against these proteins were used to determine their intracellular localisation. The sequence-specificity of VSRs was tested systematically *in vivo* in tobacco (Matsuoka and Neuhaus 1999) while the affinity for various VSD motifs of VSRs solubilised from several plants was tested *in vitro* by affinity chromatography. The completed genomic sequence of Arabidopsis revealed the existence of a small gene family, which could result in functional redundancy, hiding the effect of gene knock-outs. Indeed the systematic analysis of knock-out mutants for each individual Arabidopsis VSR revealed no phenotype, except for the knock-out of AtVSR 1 (AtELP) which caused surprisingly a partial defect in protein storage in seeds (Shimada et al., 2003). Comparison with other plant genomes and EST databases on the other hand revealed the existence of VSR subfamilies, opening the possibility of more specialised functions. We chose an experimental approach which addressed both redundancy and divergent subfamily functions.

GFP as reporter protein was used to better understand the mechanism of proteins sorting in different organisms. In our lab, GFP was used to focus vacuoles distribution in a single plant cell.

We took advantage of the previously reported different localisation in transgenic Arabidopsis of two different vacuolar GFP reporters addressed either to lytic or neutral (vegetative storage) vacuoles. A geminivirus-derived silencing vector provided the possibility to produce and spread at once dsRNA specific to several VSRs (Turnage et al., 2002) without the need to go through embryogenesis risking embryo lethality or non-germinating seeds.

In this paper, we analysed the GFP redistribution after inactivating the VSR subfamilies 1, 2 and 3 in Aleu-GFP and GFP-chi reporter plants. Silencing of the four genes of subfamilies 1 and 2 had no effects on the vacuolar localisation of either reporter GFP while silencing of the three genes of subfamily 3 caused the disappearance of Aleu-GFP from the central vacuole of epidermal and root cells. The reporter is presumably secreted into the apoplast, where GFP fluorescence has been reported to disappear. Vacuolar localisation of GFP-Chi in mesophyll cells was unaffected. This clearly implicates one or two VSRs of this subfamily (the third is expressed at very low level if at all in our plants) as receptors for vacuolar sorting to lytic

vacuoles in these tissues. On the other hand, receptors of the other two subfamilies are not implicated or not essential for this process, particularly AtVSR1;1 (AtELP) which is strongly expressed in the same tissues as AtVSR3;2. No macroscopic or microscopic phenotype was reported for the knock-out of any of the subfamily 3 receptors, but no specific reporter was used to test their function, except for the confirmed affinity of AtVSR1;1 for the precursor of AtAleu (Shimada et al., 2003). This last result stresses that there is little if any difference in sequence specificity between at least members of the subfamilies 1 and 2.

Laval et al., (2003) demonstrated using an antisense construct for AtVSR1;1 that VSRs are involved in making seeds able to germinate. No knock-out of any single VSR gene had been reported to have this effect. However, the RT-PCR analysis of germinating and non-germinating seeds revealed that the antisense suppression had affected both genes of subfamily 2 in addition to the targeted AtVSR1; 1, while the other four genes were not expressed in either category. Thus there is an involvement of at least two of these three genes in normal seed development. We observed the same defect in germination of seeds from plants silenced for subfamilies 1& 2. This confirms the value of our strategy to silence whole subfamilies at once. Seeds obtained from plants silenced for subfamilies 1 and 2 at once were found by SEM to lack the columella and had thus lost their mucilage (results not shown). Debeaujon et al.(2000) isolated mutants with related testa defects and showed that the mucilage is implicated in the germination process. The receptors of subfamilies 1 and/or 2 are thus implicated in the morphological change transforming epidermal cells into columella and the secretion of mucilage (Western et al., 2000). However, attempts to rescue non-germinating seeds indicated that the embryo itself, while looking normal, also has problems and cannot be rescued by dormancy-breaking treatments (Laval et al., 2003).

How can receptors with a very similar binding specificity and a similar tissue distribution have different functions? Immunolocalisation of VSRs revealed their presence in two compartments, TGN and PVC (Li et al., 2002). This was interpreted as reflecting a steady-state distribution of one family of receptors between the two compartments. It could however also be interpreted as reflecting the superposition of the distribution of two different subfamilies, one being localised in the Golgi and the other in the PVC. Indeed, there are indications of different localisations. Anti-peptide antibodies directed against AtVSR1;1 detected the receptor in the plasmalemma fraction of purified membranes from suspension cells (Laval et al., 1999), while immunolocalisation by polyclonal antibodies had not been reported for this compartment. A proteomics analysis of membranes derived from Arabidopsis suspension cells detected 4 different VSRs (Dunkley et al., 2006). In a Principal

Component Analysis AtVSR1;1 clustered with ER markers while the other 3 were found at the periphery of the Golgi cluster, AtVSR2;1 and 2 being close together while AtVSR3;1 was at some distance. This indicates that AtVSRs have at least two but possibly three different distributions within the secretory pathway. There are also at least two modes of binding regulation: binding of a cognate peptide to pea BP-80 (subfamily 2) was found to be pH-dependent, while it was Ca^{2+} concentration-dependent for pumpkin PV72 (subfamily 1). Neither binding specificity nor release mechanism has been tested for any VSR of subfamily 3.

We thus come to a model where different VSRs recognize similar VSDs but mediate their transport along different routes (Neuhaus and Paris 2005). According to the results presented here, subfamily 3 VSRs mediate transport from the (*trans*-)Golgi to the lytic prevacuolar compartment in leaves and roots while subfamily 1 VSRs could mediate anterograde transport from the ER or retrieval from ER-derived compartments such as PAC vesicles (in pumpkin seeds, Shimada et al., 1997) to the Golgi and/or retrieval of escaped proteins from the apoplast to the Golgi (in Arabidopsis seeds, Shimada et al., 2003). It is not clear yet what the function of subfamily 2 VSRs could be, but BP-80 was the major isoform in clathrin-coated vesicles from pea cotyledons and the two isoforms of Arabidopsis are strongly induced in senescent leaves, where they could contribute to the biogenesis of a senescence-associated vacuole (Otegui et al., 2005). The VSRs share a common membrane-proximal part of their cytosolic tails including a tyrosine motif that could mediate convergent transport to the Golgi but differ in the distal end of the tail which could harbour divergent Golgi- exit signals.

Further clarification of the function of the VSR subfamilies will require the production of stable silenced transformants free of viral symptoms and the separate silencing of subfamilies 1 and 2, although in Arabidopsis the single knock-out of AtVSR1;1 could well suppress the whole subfamily except in flowers and mature pollen. Comparative analysis of the trafficking signals will also clarify the picture.

Materials and methods

Plant material

Seeds from Aleu-GFP and GFP-Chi expressing transgenic *Arabidopsis thaliana* ecovar. Wassilewskaja (Flückiger et al., 2003) were surface-sterilized and plated on Murashige and Skoog (1962) agar medium containing kanamycin and incubated in a growth chamber with 8

hours of light at 20°C. Seedlings were transferred to soil in pots and returned to the same growth chamber.

Silencing constructs

Fragments of approx. 150 bp were amplified for each AtVSR gene and assembled in tandem for simultaneous silencing. Each fragment contained a single restriction site at each end for later recombination. One tandem sequence was assembled for the 4 genes of subfamilies 1 and 2 and another for the 3 genes of subfamily 3. The primers, their restriction sites and their annealing temperatures are indicated in Table 1.

Each fragment was first amplified by PCR (3' denaturation at 94°, then cycles of 45'' at 94°, 45'' at the annealing temperature and 1' at 72° and a final extension at 72° for 10') from a corresponding cDNA clone, using first 5 cycle with the lower annealing temperature Tm1 and then 30 cycles at the higher annealing temperature Tm2.

To assemble the fragments in tandem we amplified in a single PCR reaction 1µl of each PCR product (AtVSR3;1, 3;2 and 3;3) using the AtVSR3;1 5' and AtVSR3;3 3' primers . The same amplification program was used with annealing temperatures Tm1= 56°C and Tm2= 62°C. The resulting DNA was cloned into a pGEM-T easy vector (Promega,Wallisellen, Switzerland) and sequenced. The plasmid was digested with KpnI and BglII and the VSR tandem fragment was cloned into the corresponding sites of the pCbLCV007 genome A vector (Turnage et al., 2002).

Plant transformation

The reporter Arabidopsis plants were transformed using the particle bombardment technique. Vectors CbLCVA containing each subfamily were mixed with the CbLCV B genome and bombarded into three weeks-old plants as described by Kjemtrup et al. (1998) or Turnage et al.(2002), using the Particles Delivery System (PDS) 1000 He Particle gun (Bio-Rad). Gold particles of 1µm in size (Bio Rad) were used as micro carriers and their coating was done as described by the manufacturer: 3 mg of gold were coated with 5µg each of the CbLCVA clone and of the CbLCVB component followed by addition of 50µl of 2.5M $CaCl_2$, 20µl 0.1 M spermidine. The rupture disks, stopping screen, macrocarriers and microcarriers holders were sterilized by soaking in 70% ethanol.10 µl of the coating suspension was used for each transformation. Plants were bombarded three times and were then grown under 8h of light at 20°C until yellow spots were observed on leaves.

Confocal Laser Scanning Microscopy

Leaves from control and silenced Aleu-GFP and GFP-Chi plants were examined in water under glass cover slips using a confocal laser scanning microscope with the TCS 4D operating system (Leica). The Argon laser line at 488nm was used for excitation. GFP was detected with the filter set for FITC and the chloroplasts with the filter set for TRITC.
The stored images were pseudocolored as red or green images using Adobe Photoshop 7.0 in correspondence to the real red and green colours before merging.

RNA isolation and RT-PCR analysis

Leaves from control and silenced plants were frozen in liquid nitrogen and stored at -80°C until extraction. Total RNA was extracted from ground leaf powder using an extraction buffer (2M Tris-HCl pH8.0; 0.5M EDTA pH 8.0; 20%SDS), extracted with an equal volume of phenol/chloroform/isoamylalcool (24/1/1 v/v) and precipitated with one volume of 6M LiCl. First strand cDNA was synthesized from 2µg total RNA with the Improm-II Reverse Transcription System from Promega using oligo $(dT)_{15}$ as primer according to the manufacturer. The PCR reaction was performed using gene specific primers (Table 2). The amplification conditions were: pre-denaturation at 95°C for 3'; 35 cycles of 45'' at 94°C, 45'' at Tm (56°C for all VSR except 50.2°C for the subfamily 2 and 54° for actin), 1' at 72°C and a final extension step of 10' at 72°C.

Protein extraction and western blotting

52 days after bombardment (dpi), two leaves were ground in an Eppendorf tube containing 50 µl 0.5M phosphate buffer (pH7.0) and a small quantity of quartz beads. Leaves were ground with a pestle on ice. The solution was centrifuged at 14,000 rpm at 4°C for 10'. The soluble fraction was mixed with the same volume of 2X loading buffer containing β-mercaptoethanol and incubated at 95°C for 5'. SDS-PAGE and blotting followed standard procedures (Laemmli 1972). Anti-GFP serum was used for immunodetection at the dilution 1:5000.

In situ RNA hybridization

A partial cDNA sequence of each VSR was amplified by PCR and cloned into pGEM-T vector (Promega) and sequenced (Microsynth). Depending of the insertion of the sequence different restriction enzymes were used for the sense and the antisense. Sense and anti-sense

probes were generated with the riboprobe transcription kit according to the manufacturer (Roche, Mannheim, Germany). After transcription, RNA probes were treated with RNAse-free DNAse (Roche) and precipitated with 20µl 10M ammonium acetate and x µml absolute ethanol and dissolved in 50µl DEPC water. Deionised formamide was added and probes were kept at -20°C. Before hybridization, probes were denatured at 80°C for 2 min. The anti-sense probes were used as specific probes while sense probes were used as controls for background hybridization.

Samples were taken from plant roots and newly emerging leaves. The tissues were cut into about 1 cm sections and fixed overnight in freshly prepared 4% formaldehyde, then rinsed with cold PBS and subsequently dehydrated in cold ethanol series (10, 30, 50,70, 85, 90, and 100%) at 4°C. In the final dehydration step 0.1% eosin was added to visualize samples in the paraffin. Samples were washed in a series of Histoclear at different concentration (25, 50, 75 and 100%), then they were heated at 60°C and washed for one hour with a solution of histoclear/paraplast (1/1 v/v). Two more washings in paraplast 100% (Sigma, Germany) were performed and finally the samples were embedded in moulds and kept at 0°C.

The paraffin-embedded tissues were cut into 7µm sections using a microtome (Leica Microsystems, Nusslo Gmbh, Germany) and mounted on DAKO microscope slides (Menzel-Glaser, Germany). Sections were hydrated as described by Jackson(1991). Sections were incubated with a proteinase K solution (1µg/ml in Tris-EDTA buffer) for 30 min at 37°C and treated sequentially with solutions of glycine (0.2% in PBS), 4% Para-formaldehyde and acetic anhydride (0.5%) in triethanolamine (0.1M). Sections were dehydrated in ascending concentrations of ethanol as described by Jackson (1991).Sections were hybridized for more than 12 hours at 50°C with a DIG-UTP RNA probe (either sense or anti-sense) in the hybridization solution (50% deionised formamide; salt solution: 3M NaCl,100mM Tris pH 8, 10mM EDTA; 10X dextran sulphate, 50X Denhardt's solution, 100µg/ml tRNA). After several washings in 0.2X SSC (0.03M NaCl, 3mM NaAcetate), single stranded RNA was removed with a preheated (55°C) solution of TNE containing 20µg/ml RNAse at 37°C for 30 min. For the detection of DIG labelled hybrids, slides were first incubated in a blocking solution, then in another solution containing 1% BSA, 0.3% Triton X100 . A blocking solution containing 1.25 units/ml of alkaline phosphatase-conjugated anti-DIG Fab fragments was added on the slides. The reaction was incubated at room temperature for 1 hour. Slides were washed three times with the same blocking solution containing BSA and Triton X100. Finally the staining buffer containing Levimasole (1mM) and the NBT/BCIP substrates for the phosphatase (5µl of NBT 75mg/ml in dimethylformamide 70% and 3.75 µl of BCIP

50mg/ml in pure dimethylformamide) were added on the slides and left overnight for development. The staining was stopped and slides were air dried and mounted with faramount (DAKO). Pictures were taken with a camera (Leica) connected to a light microscope (Leica).

Table 1. Primers for amplification of VSR fragments and their assembly in tandems

Primer	Sequence	Restriction site	Tm1	Tm2
AtVSR3;1 5’	G**GGTACC**TAGTCAACGGGAGAGCTTC	KpnI	54°	65°
AtVSR3;1 3’	GACCA**CTCGAG**GTGCTTCGACCTCATCTC	XhoI		
AtVSR3;2 5’	AGCAC**CTCGAG**TG GTC AAC GGG TTT TCA TC	XhoI	52°	60°
AtVSR3;2 3’	TTATG**TCTAGA**GACCA AAA GTT TGC TAT GG	XbaI		
AtVSR3;3 5’	TGGTC**TCTAGA**CA TAA AGG AGC CAC CTT GGC	XbaI	56°	62°
AtVSR3;3 3’	A**AGATCT**GGC TGC GTC GTG CTT AGA	BglII		
AtVSR1;1 5’	GGAGT**GTCGAC**GGCTTTTCACTCTCTCGTTTC	SalI	58°	65°
AtVSR1;1 3’	CAAAC**TCTAGA**CTCCGAAATTACCAATGGCAC	XbaI		
AtVSR1;2 5’	G**GGTACC**ACGACGAATGTATGGT	KpnI	50°	61°
AtVSR1;2 3’	AAGCC**GTCGAC**ACTCCTCTGATGGACTC	SalI		
AtVSR2 5’	CGGAG**TCTAGA**GTTTGTCGGTGACGTCG	XbaI	52°	61°
AtVSR2 3’	A**AGATCT**CGGATAAACCACCGTACCA	BglII		

Table 2. Primers for RT-PCR

Primer	Sequence
AtVSR1;1 5’	CCTCGAGGGCTTTTCACTCTCTCGTTTC
AtVSR1;1 3’	GGTACACTTATTTCTGTTTGTGGC
AtVSR1;2 5’	CCCATGGACGACGAATGTATGGT
AtVSR1;2 3’	TTATGCAAATGTCGTGTTCTCTTATG
AtVSR2;1 and 2 5’	GATCAGAGCCATAATGGCAC
AtVSR2;1 and 2 3’	TTCTCCGAAGCTACATCGAAG
AtVSR3;1 5’	GTC TCT GGT TTG TGA TTG AGC
AtVSR3;1 3’	TGTAAAGCCGGTCACCAGA
AtVSR3;2 5’	GCT TTG AAG ATA TGG AAC GG
AtVSR3;2 3’	GTA TAG ACT CAC TCC AAT CCA TC3’
AtVSR3;3 5’	GCA TTA AAG GTA TGG AAC GGT C
AtVSR3;3 3’	GAC TCA CTC CAG TCT ATC TTC AGG
Actin 5’	ATATGGAGAAGAATCATGGCATCATCAC
Actin 3’	GTTTCAGTGAATTACCTAGCT

Chapter 3

Preliminary results on the biological function of *At*RMR receptor in *Arabidopsis thaliana*

Jeannine Okmeni Nguemelieu, Sophie Marc-Martin and Jean-Marc Neuhaus.

Summary

Vegetative plant cells contain both lytic vacuole and vegetative protein storage vacuoles (PSV). Proteins reach vacuoles because they contain vacuolar sorting determinants that are recognized by corresponding sorting receptors proteins. While vacuolar sorting receptors VSRs (such as pea BP-80) sort proteases to lytic vacuole via a lytic prevacuolar compartment (PVC), relatively little is known about receptors for PSV. Receptor homology region-transmembrane domain-RING-H2 (RMR) is a putative receptor for the PSV pathway in tobacco and Arabidopsis. Park et al. (2005) showed that one gene of this family, *At*RMR1, may function as the sorting receptor of phaseolin for its trafficking to the PSV. Here we study the functional roles of all six Arabidopsis RMR (*At*RMR). Toward this goal, we cloned in sense and antisense orientation the six RMRs from Arabidopsis in pCAMBIA vector and generated transgenic Arabidopsis plants missing the receptors. Confocal microscope observations demonstrated that stable silencing of RMRs affects the localization of vacuolar GFP reporters. In GFP-Chi plants, GFP was found to be localized faintly in the central vacuole of epidermal cells but was also detected in the apoplast. Vacuoles of mesophyll cells lost their strong fluorescence compared to the control. In Aleu-GFP plants, GFP was also present in apoplast and the epidermal vacuoles were no more strongly fluorescent. Therefore we conclude that RMRs are involved in the sorting to both PSV and lytic vacuoles.

Introduction

Lytic and protein-storage vacuoles seem to be end points of the plant secretory pathway (Vitale and Hinz, 2005; Jolliffe and Frigerio, 2005). While soluble proteins can reach the apoplast by a default pathway, vacuolar proteins need to be sorted to vacuoles in a receptor mediated manner. To be specifically sorted and directed to their final destination, proteins must possess vacuolar sorting determinants that are recognized by vacuolar receptors. Proteins are first translocated into the ER and then travel through the Golgi complex. From the Golgi complex there are at least two pathways to vacuoles: The first pathway involves protein-sorting to lytic vacuole via PVC/MVB (prevacuolar compartment or multivesicular bodies; Tse et al., 2004). In this route, a vacuolar sorting receptor (initially called BP-80) is thought to mediate the sorting by recruiting clathrin coats through binding of clathrin adaptator complexes (Paris et al., 1997; Paris and Neuhaus 2002). The second pathway is possibly not receptor-mediated and uses dense vesicles. Hara-Nishimura et al., (1998) showed that a third pathway can exist by passing the Golgi. This was shown in developing pumpkin cotyledons where 2S albumin accumulated in ER-derived precursor-accumulating vesicles that directly fused with PSV. In plant cells, lytic and storage vacuoles can coexist and can fuse to form a hybrid vacuole (Di Sansebastiano et al., 2001).

Up to now two types of protein-sorting receptors known as VSRs (Vacuolar Sorting Receptors) and RMRs (Receptor Membrane RING-H2) have been identified. Several publications have provided information on VSR functions in Arabidopsis (Laval et al., 2003; Shimada et al., 2003). In contrast, the RMR receptor-family is not well known and less information is available. The RMRs were first identified as a new putative receptor family by homology to the first domain of the VSRs; this domain constitutes the whole N-terminal luminal domain of RMRs and is followed by a transmembrane domain, and a cytosolic domain with a RING-H2 and a serine-rich region (Jiang et al., 2000). Antibodies against RMR1 detected the same organelles as antibodies against the TIP isoform DIP, i.e. the crystalloid compartment in *Arabidopsis* and tomato (Jiang et al., 2000). The luminal PA domain, also found in VSR proteins is known to participate in ligand binding (Cao et al., 2000). This raised the possibility that RMR proteins function as vacuolar receptors. Coexpression of AtRMR1 mutants with altered localization inhibited the trafficking of phaseolin to the protein storage vacuole and co-immunoprecipitation indicated a direct interaction between the two proteins (Park et al., 2005). The genome of Arabidopsis harbours 6 homologues, *At*RMR1-6, while rice has two. No functional subfamilies can be identified.

To obtain additional information on the biological role of these receptor proteins, an investigation of loss-of-function mutations on whole plants was used. To visualize the effects on vacuolar sorting, we used transgenic plants expressing either of two fluorescent reporters: the soluble GFP (green fluorescent protein) fused either with the C-terminal VSD of tobacco chitinase A (GFP-Chi, \Di Sansebastiano, 1998) or with the N-terminal sequence-specific VSD of barley aleurain (Aleu-GFP, \ Di Sansebastiano, 2001). In protoplasts, these labels were found in neutral or acidic vacuoles, respectively. In transgenic *A. thaliana* the two constitutively expressed reporters labelled different vacuoles: in mesophyll cells, Aleu-GFP labelled the large central vacuole while GFP-Chi labelled small peripheral grains (Flückiger et al., 2003). In contrast, in the mesophyll, the central vacuole was labelled by GFP-Chi while Aleu-GFP was often not visible.

Two strategies were developed: transient silencing using a silencing vector derived from a geminivirus and stable transformation of reporter plants with a silencing construct.

In GFP-Chi plants, silencing of genes of the *At*RMR family most strongly affected mesophyll cells GFP being absent from the large central vacuole while accumulating in the apoplast. We also found that the inactivation of the RMR family in Aleu-GFP transgenic plants caused the loss of vacuolar fluorescence in some epidermal cells of leaves, and a concentration of GFP in the apoplast of mesophyll cells. We conclude that the *At*RMR receptor family is involved in the sorting of proteins both to the lytic and storage vacuoles.

Results

Expression of AtRMRs in different plant organs

Multiple RMR isoforms may be linked to differential gene expression in different tissues. An analysis of 2507 experiments with Affimetrix 22K arrays using Genevestigator (Zimmermann et al., 2004) indicates however that the relative expression levels are rather constant in different tissues, with *At*RMR1 being the strongest expressed isoform (45-60%), followed by *At*RMR2 and *At*RMR4. *At*RMR3 and *At*RMR5+6 (not distinguished) have the lowest expression level. Only in seeds *At*RMR5+6 are second in abundance.

We determined gene expression level of RMR genes by RT-PCR in *A. thaliana* ecovar Wassilewskaja in fresh siliques, flowers, leaves and dried seeds (Figure 1). *At*RMR1 showed the strongest expression in all tissues, followed by *At*RMR4 expressed in siliques, where *At*RMR5 have a higher expression level. In fresh siliques, all RMR genes were strongly expressed while *At*RMR3, 5 and 6 had low levels of expression in leaves. *At*RMR3 and 5

have similar patterns of expression. We can conclude that all RMRs are expressed in most plant tissues. They could however have different functions within the cells.

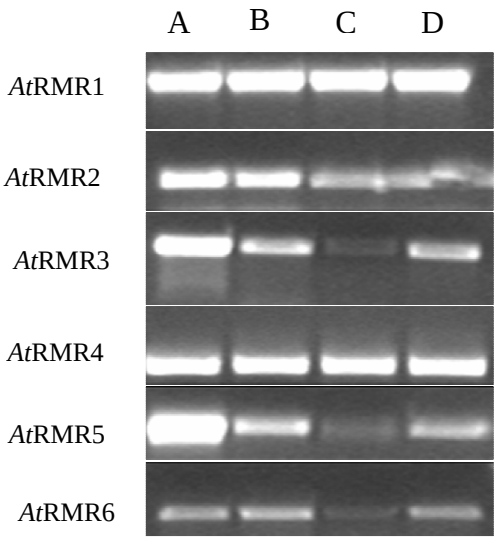


Fig 1. Expression of RMR genes in *A. thaliana*. The expression was analysed by RT-PCR from total RNA of fresh siliques (**A**), flowers (**B**), leaves (**C**) and dried seeds (**D**).

Patterns of fluorescence in leaves of reporter plants

As described previously (Flückiger et al., 2003), the marker for lytic vacuoles, Aleu-GFP, is visible in the central vacuole of most epidermal cells in *A. thaliana* (Fig.2A). In contrast, for GFP-Chi, the marker for neutral storage vacuoles, the fluorescence was restricted in leaf epidermal cells to the ER and small peripheral compartments which were proposed to be neutral prevacuoles or small vacuoles (Fig. 2B). Further, the guard cells also have their ER labelled by GFP.

In mesophyll, the pattern is very different: strong GFP-Chi fluorescence is visible in the central vacuoles of most cells (Fig. 2C), while in Aleu-GFP expressing plants most vacuoles are non-fluorescent (Fig. 2D, Fig. 6B).

The distribution of the two markers in different root cells types has been described previously (Flückiger, 2003). In fully elongated root cells, most central vacuoles are labelled by Aleu-GFP (Fig. 2E), while in most cells GFP-Chi is restricted to small peripheral compartments (Fig. 2F) with occasionally a small vacuole.

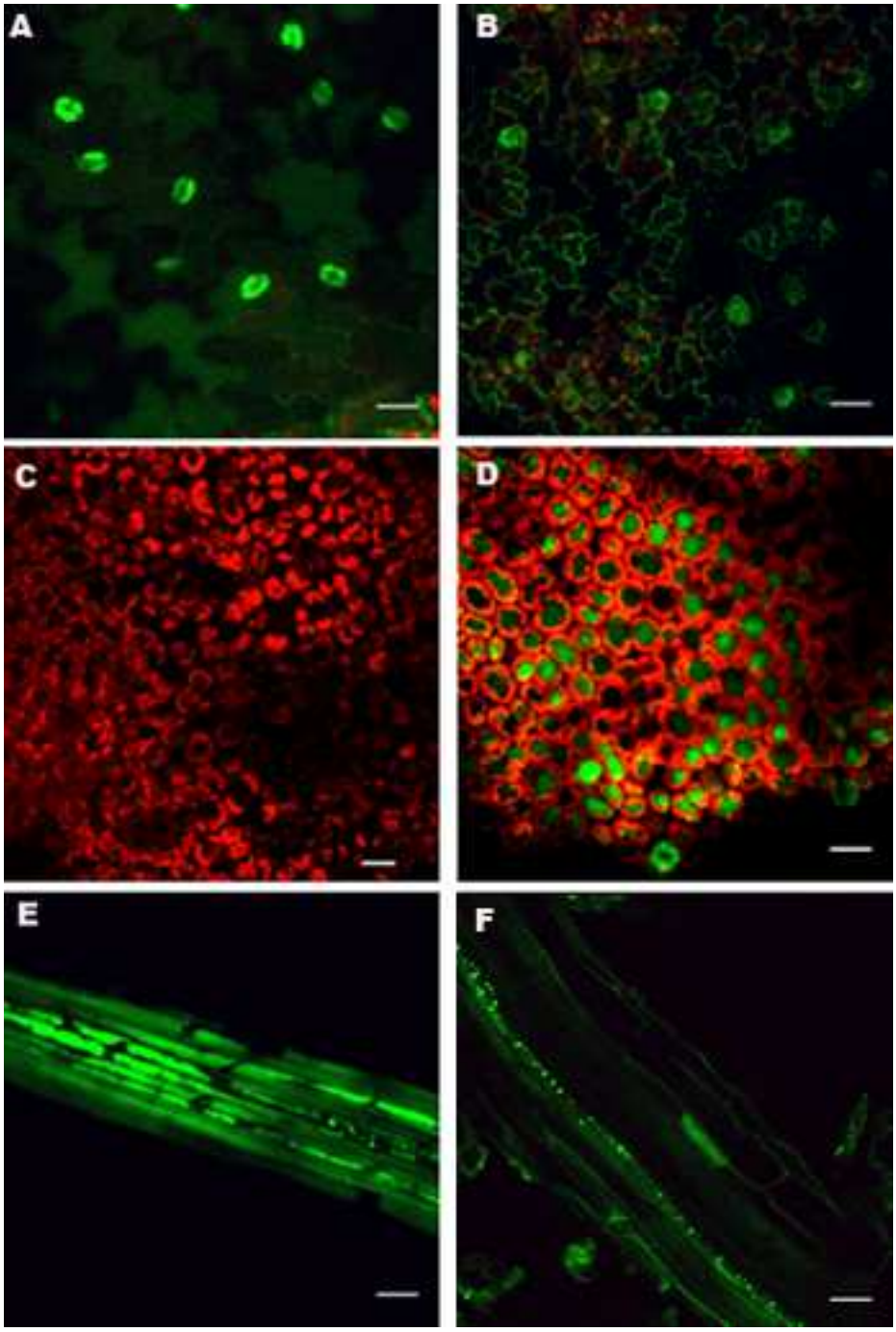


Fig 2. GFP distribution in non inoculated reporter plants. Aleu-GFP distribution in epidermis (**A**), mesophyll (**C**) and roots (**E**). GFP-chi distribution in epidermis (**B**), mesophyll (**D**) and roots (**F**). Scale bar= 100µm

Phenotype of virus-silenced plants

General symptoms were observed in all bombarded plants. Viral symptoms were observed in new emerging leaves between 3 to 4 weeks post-inoculation. These symptoms include yellow

spots, leaf-curling, stunted-growth, necrosis and variegation. They were observed previously in *Nicotiana benthamiana* by Peel et al., (2001)or in *Arabidopsis* by Turnage et al., (2002). These symptoms progressed in young leaves until they became yellow. A general yellow colour can be explained by the fact that yellow spots became bigger until they fused. These symptoms were helpful to visualize the spread of the virus inside the leaf or the whole plant (Fig. **3**). With stable silencing, all transformants germinated, grew and reproduced normally and did not show any visible phenotype (not shown).

Fig3. Viral symptoms caused by the vector for gene silencing. Visible phenotype after bombardment with the vector containing the whole RMR family of genes.

Silencing of AtRMRs in transgenic Arabidopsis plants expressing the marker for neutral vacuoles

Despite the viral symptoms the empty vector did not affect the distribution of GFP-Chi in epidermis or mesophyll (Fig. 4**A** & **B**). Epidermal cells were not strongly affected by transient silencing of the whole RMR family, however, a faint GFP fluorescence was observed in some epidermal cells. Strong labelling at the corners suggest apoplastic accumulation (Figure 4**C** and **E**). In contrast, vacuoles of mesophyll cells were strongly affected by the silencing of RMRs, which reduced the fluorescence in these vacuoles to undetectable levels (Figure 4**D and** 4**F**). When observing the cells 39 days post-inoculation (d.p.i), GFP fluorescence was sometimes detected in the intercellular space between a few mesophyll cells (Figure 4**D**).

Later no GFP fluorescence was observed anymore in the mesophyll, not even in the apoplast (Figure 4**F)**.

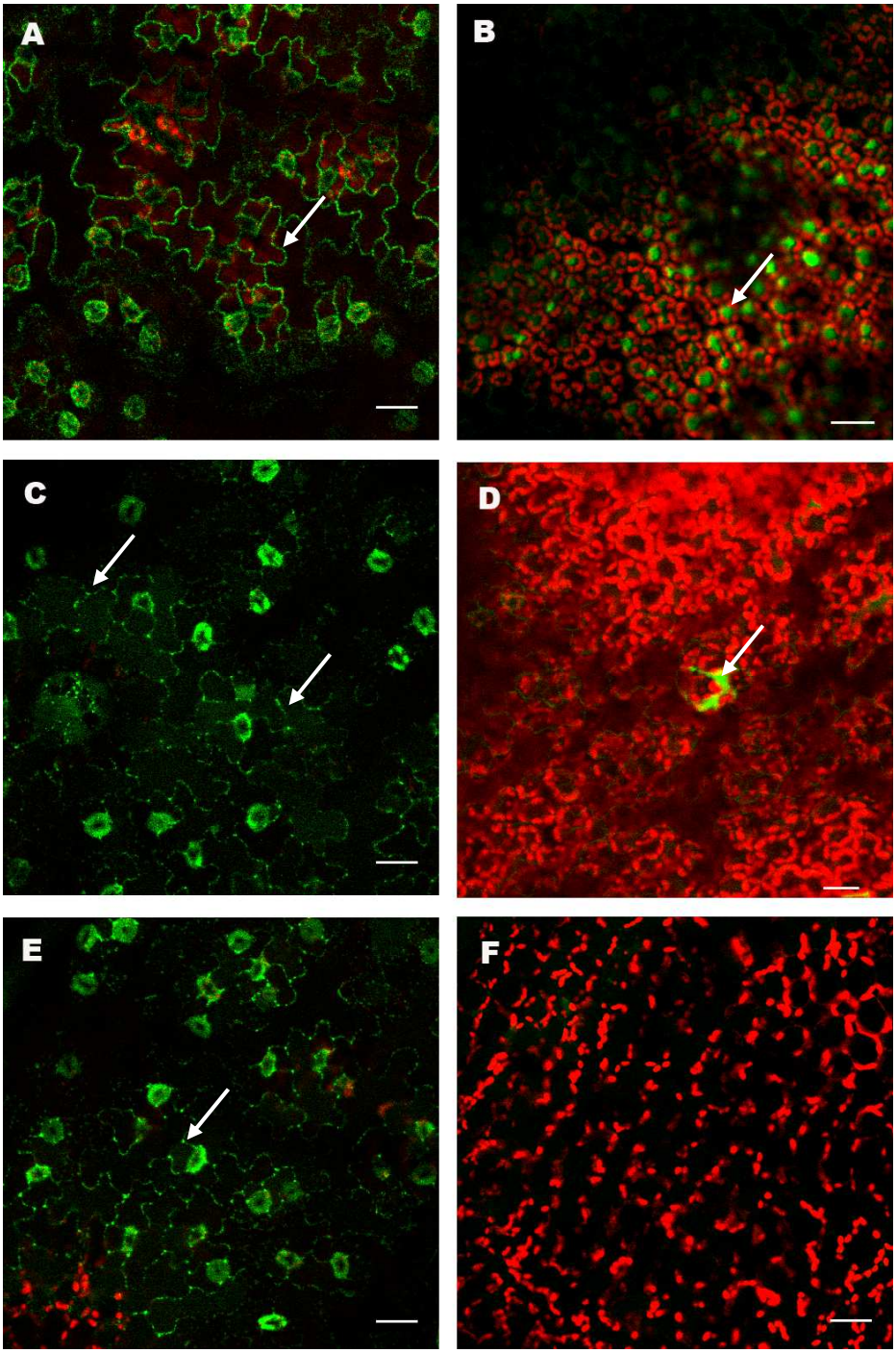
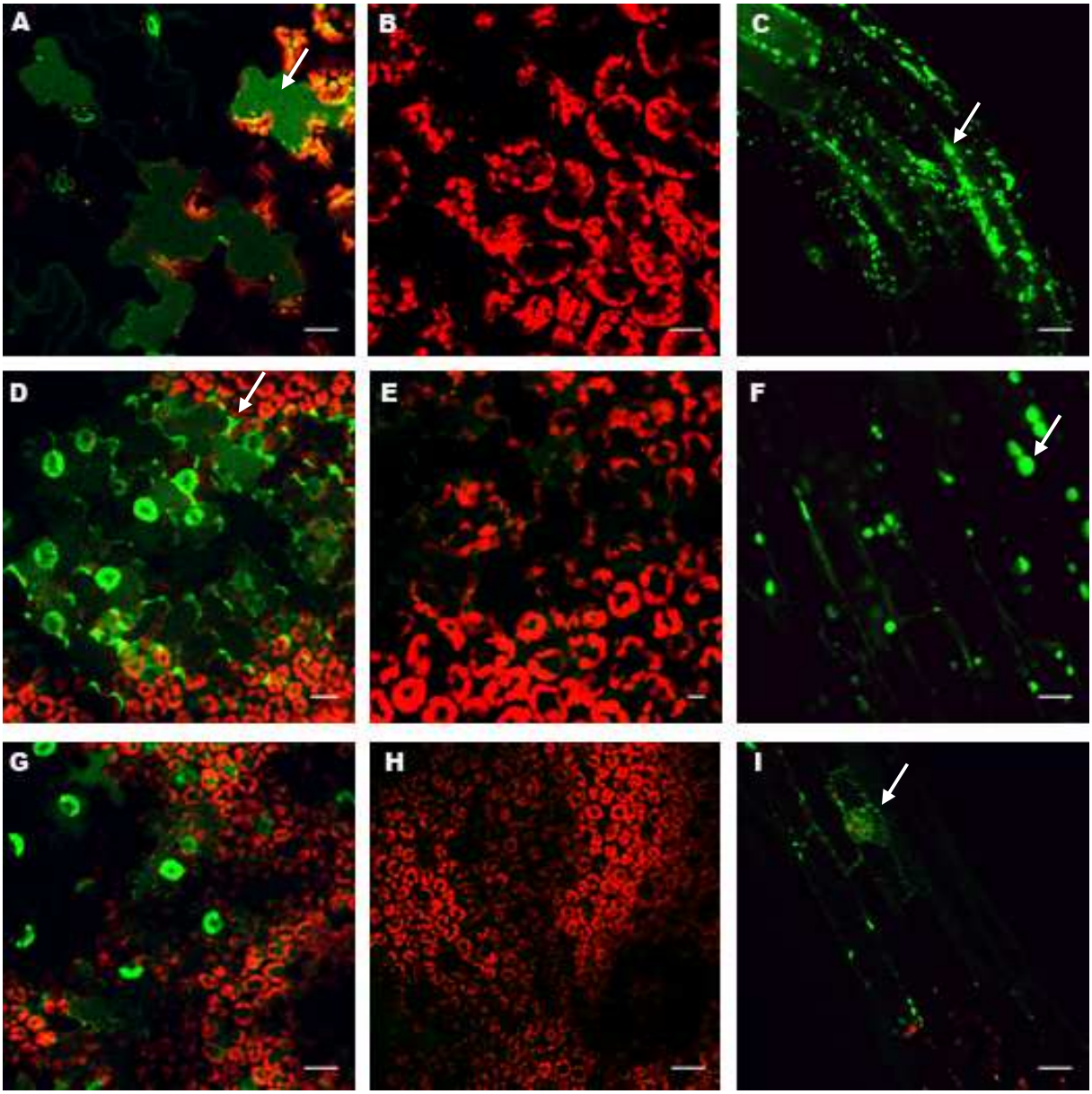


Fig 4. Effects of transient silencing of the AtRMR family on the localisation of GFP-Chi, the marker for storage vacuoles. Control plants inoculated with the empty viral vector **(A** and **B)**, plants inoculated with the silencing construct analyzed at 39 d.p.i (**C** and **D**) and 49 d.p.i (**E** and **F**). Scale bar= 100 µm

Similarly changed patterns were observed when leaves of homozygous stably silenced GFP-Chi plants were excised at different stages (at 8 days, 2 weeks and 4 weeks after germination) and were analysed by confocal microscopy (Fig. 5). Central vacuoles of mesophyll cells were strongly affected as no GFP fluorescence could be detected, nor in the intercellular space (Figure5 B,E and H). In young leaves, GFP-Chi was found to strongly label approximately 20% of central vacuoles of epidermal cells (Figure 5**A**) but 2 weeks after germination, GFP was also detected in peripheral compartments which appear to be the apoplast (Figure 5**D**). This was less visible two weeks later (Figure 3G). In roots of young seedlings GFP-Chi distribution was not affected by the inactivation of *At*RMRs (Figure 5 **C**), but after two or four weeks the green spots were fewer and at two weeks post-germination they were also bigger (Figure 5**D**). Transient and stable silencing thus gave similar results.

Fig. 5. Stable silencing of AtRMRs in GFP-Chi plants. Two-leaf stage of development: epidermis (**A**), mesophyll (**B**) and roots (**C**). 4 weeks old plants epidermis (**D**), mesophyll (**E**) and roots (**F**). 8 weeks old plants: epidermis (**G**), mesophyll (**H**) and roots (**I**). Scale=100µm

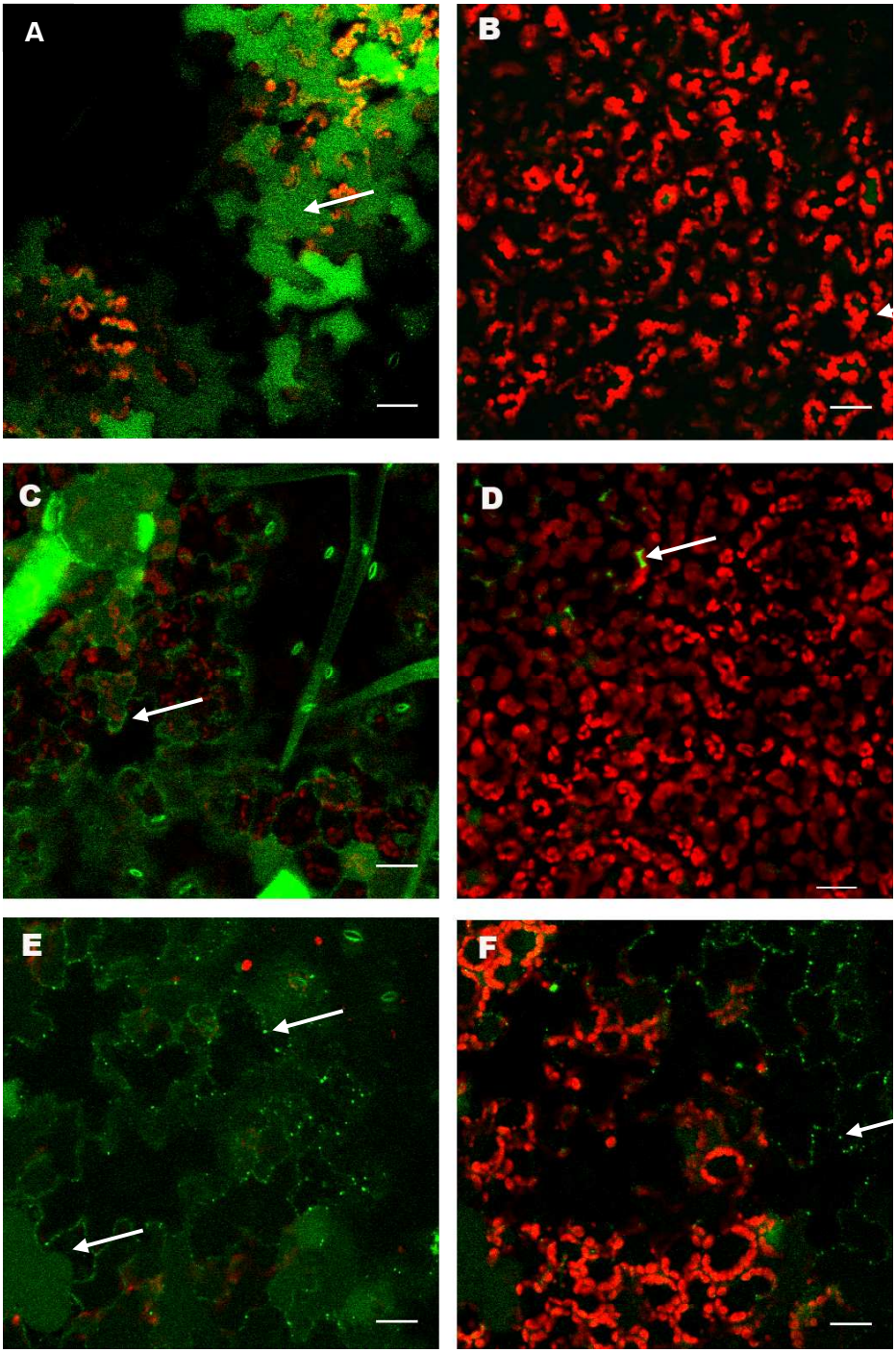


Silencing of AtRMRs in transgenic *Arabidopsis* plants expressing the marker for lytic vacuoles

Leaves from virus innoculated Aleu-GFP plants were excised when viral symptoms became visible and analysed by confocal microscope. Leaves from plants inoculated with the empty vector showed the previously described distribution of GFP fluorescence (Fig. 6A; Flückiger et al., 2003). The large central vacuole of epidermal cells in leaves from these control-plants showed strong GFP accumulation, and the marker was also visible as a faint diffuse fluorescence in guard cells. While GFP fluorescence was present in the central vacuole of mesophyll cells in very young leaves, it was usually not detected in mature leaves that

appeared non-fluorescent (Figure 6 **B**). The Aleu-GFP plants inoculated with the vector for *At*RMR silencing altered the pattern of Aleu-GFP fluorescence in leaf cells at 39 d.p.i. (Figure 6**C**). In the epidermis, many cells did not have a labelled central vacuole but GFP was instead concentrated in small unidentified compartments near the plasma membrane (Figure 6**C**). These small compartments became more visible after longer incubation in a pattern resembling GFP-Chi accumulation in epidermal cells. The remaining epidermal cells only showed a faint fluorescence (Figure 6**E**). In the mesophyll cells vacuoles remained non-fluorescent with some GFP accumulation was observed in the apoplast 39 d.p.i (Figure 6**D**) but not 49 d.p.i. (Figure 6**F**).

Fig. 6. Effects of transient silencing of the AtRMR family on the localisation of Aleu-GFP, the marker for lytic vacuoles. Control plants inoculated with the empty viral vector **(A** and **B)**, plants inoculated with the silencing construct analyzed at 39 d.p.i (**C** and **D**) and 49 d.p.i (**E** and **F**). Scale bar= 100 µm



In stably silenced reporter plants, Aleu-GFP distribution was affected in young leaves with faint fluorescence in central vacuoles of epidermal cells and brighter peripheral small compartments (Figure 7**A).** But the central vacuole later lost any fluorescence and the labelling appeared to concentrate in the apoplast (Fig. 7 D and **G**). In mesophyll cells no significant fluorescence change was observed compared to the control plants (Figure 7B, E, H see arrow).

In roots Aleu-GFP distribution was also strongly affected by AtRMR silencing. GFP fluorescence was detected in vacuoles of young roots (Fig 7**C**) in small compartments in four weeks old plants (Figure 7F) while later GFP was localized as a continuous line around the cells (Figure 7I).

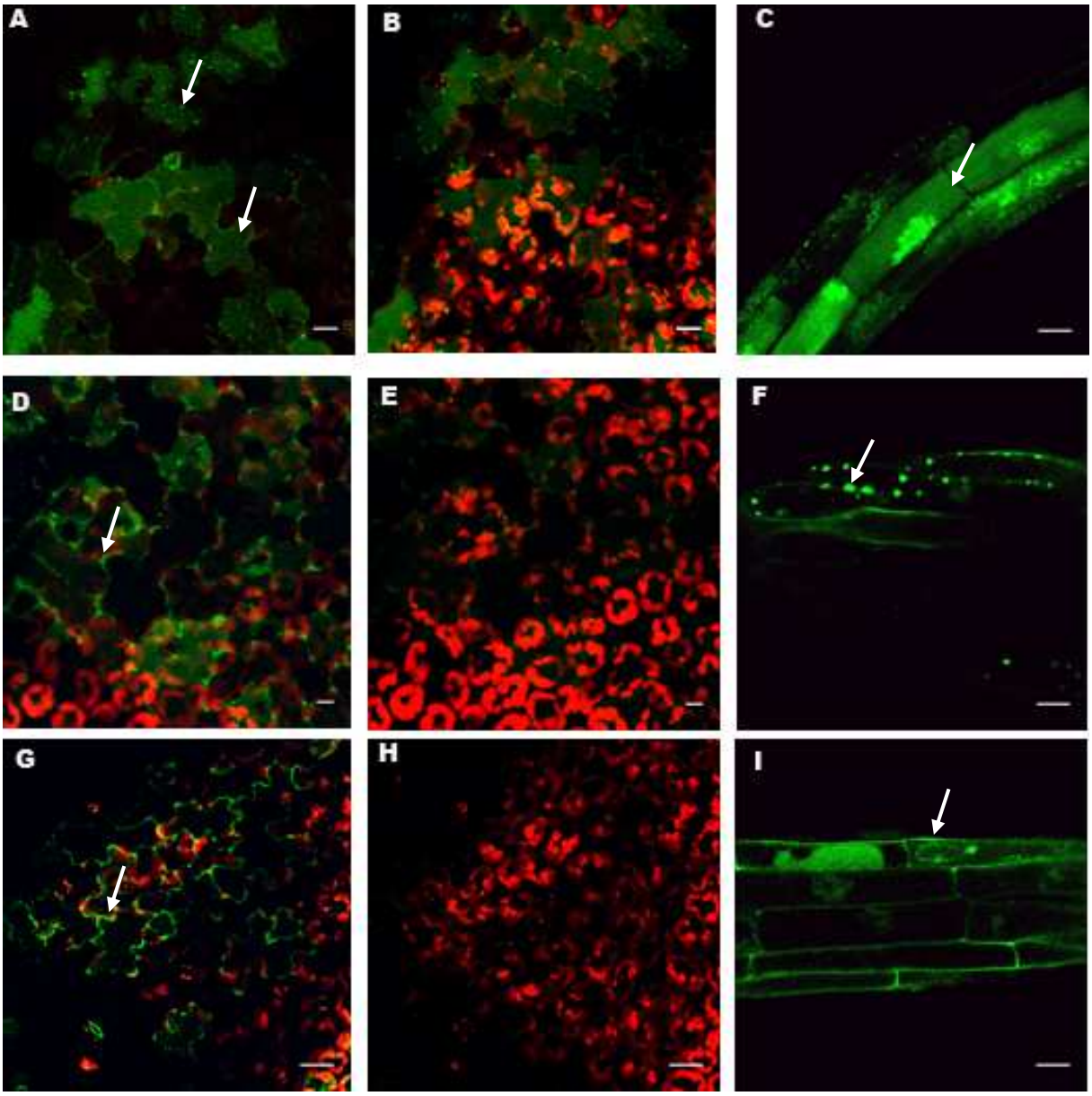


Fig. 7 Stable silencing of AtRMRs in Aleu-GFP plants. Two-leaf stage of development: epidermis (**A**), mesophyll (**B**) and roots (**C**). 4 weeks old plants epidermis (**D**), mesophyll (**E**) and roots (**F**). 8 weeks old plants: epidermis (**G**), mesophyll (**H**) and roots (**I**). Arrow indicated the GFP marker localisation in leaf. Scale bar= 100 µm

RT-PCR analysis of AtRMR genes after their inactivation

In leaves of control plants all genes except for *At*RMR6 were strongly expressed, as previously reported. After transient silencing of the whole AtRMR family, the expression of all target genes was suppressed except for *At*RMR 5, for which a faint band still could be detected by agarose electrophoresis (figure 8). This result confirmed the efficiency of the silencing technique

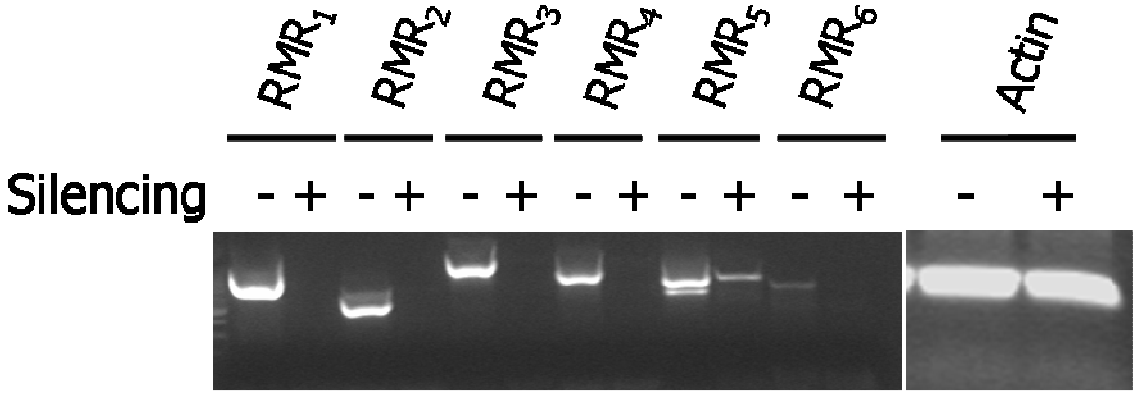


Figure 8: Expression of AtRMR genes in leaves after silencing of the whole family. The expression was analysed by RT-PCR from total RNA. Gene expression in plants inoculated with the empty vector (-) and in inoculated leaves (+).

Immunodetection of GFP in control and in silenced leaves

Proteins were extracted from control Aleu-GFP plants (Figure 9**A**) and from transgenic Aleu-GFP (Figure 9**B**) and GFP-Chi plants (Figure 9**C**) with silenced *At*RMRs. GFP is still detected by western blot in all samples; however, the signal was weak in silenced transgenic plants (Figure 9**C**).

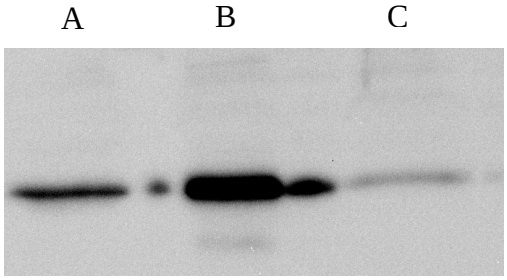


Fig.9: Immunoblot analysis of GFP extracted from control leaves (A), leaves silenced for all *At*RMRs in Aleu-GFP plants (**B**), and in GFP-chi plants (**C**).

Discussion

Protein storage vacuoles were found in cells from storage tissues of seeds, they were marked by the presence of α-TIP (Müntz, 1998; Paris et al., 1996).Vegetative storage vacuoles are found in leaves, petals, tubers and are marked specifically by δ-TIP (Jauh et al., 1998). A putative receptor family, the RMRs, was proposed to be involved in the protein sorting to these two types of vacuoles.

Up to now results obtained in seeds by Jiang et al., (2000) and in leaves by Park et al., (2005) suggested that RMRs are receptors for storage vacuoles. In fact Park et al., 2005 demonstrated that *At*RMR1 interacts with the C-terminal VSD of phaseolin and when HA-tagged *At*RMR1 deletion mutant (a deleted lumenal domain) was coexpressed with this protein, the trafficking of phaseolin to the storage vacuoles was inhibited and thus it was secreted into the medium.

Considering that A. thaliana has 6 RMR genes that they are expressed in most tissues and that phylogenetic analysis does not support the existence of functional subfamilies (in contrast to the VSR subfamilies), we hypothesized these genes are redundant and decided to silence the whole family at once with a single construct. The test plants used for this study were transgenic plants expressing GFP fused to different vacuolar sorting determinants, derived either from tobacco chitinase for storage vacuoles and barley aleurain for lytic vacuoles. The GFP-Chi marker labelled the central vacuole of mesophyll cells and was detected in small grains in the epidermal cells. The Aleu-GFP marker was detected in the large central vacuole of epidermal cells and also appeared in central vacuoles of mesophyll cells of plantlets, but was not detected there when the plants became mature (Flückiger et al., 2003). This change of nature of a vacuole type was not surprising since it was described that paraveinal mesophyll of soybean vacuoles can reversibly convert from a lytic to a vegetative storage vacuole and back depending on the metabolic demand for nitrogen (Murphy et al., 2005).

Our results on silencing of the whole *At*RMR receptor family showed that sorting of GFP-Chi was strongly affected. This corresponded to our expectation that the marker would not be detected in the vegetative storage vacuole anymore. It rather seemed to be transiently accumulated in the apoplast, where it then disappeared.

In contrast, our results obtained with Aleu-GFP plants were more puzzling, because in contrast to our expectation, Aleu-GFP distribution was as strongly affected as GFP-Chi distribution, meaning that *At*RMR receptors are somehow implicated in protein sorting to both

of these different vacuoles. Based on these results, new models have to be proposed and this will be discussed in the next chapter.

Note added in the last moment: We have just realized that the numbering of *At*RMRs by Park et al. (2005) is different from the one used in this work. We will correct our numbering in the revision of this thesis. Their *At*RMR1 is our *At*RMR2 and reverse.

Materials and Methods

Plant material and Growth conditions

Seeds from transgenic *Arabidopsis thaliana* ecovar Wassilewskaja expressing Aleu-GFP or GFP-Chi (Flückiger, 2003) were surface-sterilized and plated on MS (Murashige and Skoog, 1962) agar medium containing kanamycin (50 µg/ml) and incubated in a growth chamber with 8 hours of light at 20°C. Seedlings were transferred to soil in pots and returned to the same growth chamber.

Plants were grown under standards conditions in a growth chamber under the light-dark regime of 16:8h (20ºC) provided by fluorescent tubes, Philips TL-D 36w. For selection of transgenic plants, seeds were sterilized in ethanol and bleach, rinsed with 100% ethanol, and germinated on MS medium containing 30µg/ml Basta (glufosinate ammonium). Basta resistant plants were transferred to soil.

Silencing constructs for transient silencing

Fragments of approximately 250bp from each *At*RMR gene were generated using PCR (3min denaturation at 95°C, then 30 cycles of 45s at 94°C, 45s at the annealing temperature and 1m at 72°C and a final extension at 72°C for 10min) from a corresponding cDNA clone, using first 10 cycles at the lower annealing temperature Tm1 and then 30 cycles at the higher annealing temperature Tm2.

The primers and the restriction sites are indicated in the table I:

All genes were assembled in tandem for simultaneous silencing using a PCR reaction where 1 µl of each amplification reaction and primers AT1fKpn 5’ and AT2rBgl 3’ were used in 50µl of reaction mix. A similar PCR program was used as for the first amplifications, using two different annealing temperatures, Tm1=60°C and Tm2=68°C. The resulting DNA fragment of approximately 1500bp was subcloned into pGEMT-Easy and sequenced (Sanger et al., 1977).

The plasmid was then digested with KpnI and BglII and the fragment was cloned into the corresponding sites of the pCbLCV007 genome A vector (Turnage et al., 2002)to obtain CbLCV007+RMR.

Silencing constructs for stable transformation

The tandem sequences from each AtRMR gene were cloned twice into an intermediary vector pGEM-PIT containing the 35S promoter, adequate restriction site pairs flanquing an intron and the OCS terminator. The inverted repeat interrupted by the intron was then transferred into the binary vector pCAMBIA 3300.

The binary vector pHELLSGATE8 was first modified by removing both copies of the ccdb gene using sequentially XbaI and XhoI digestion followed each by a ligation. The resulting plasmid was digested with NotI and the fragment containing the promoter, the intron, the restriction sites for sense and antisense insertion and the terminator was cloned into NotI-digested pGEMT-Easy vector (Promega). The resulting vector named pGEM-PIT allowed the insertion of the AtRMR gene fragments in sense and antisense orientations. For this purpose, the AtRMR gene fragments were amplified from CbLCVA+RMR by PCR using a forward primer RMRClaI-KpnI (5'CGATCGATGGTACCATGGCAGGT 3', introducing ClaI and KpnI sites) and a reverse primer RMRXbaIEI (5' TCTAGAATTCAGATCTGCATCTAACC3' introducing XbaI and EcoRI sites). The PCR product was digested with EcoRI and KpnI and cloned into pGEM-PIT pre-digested with the same enzymes. The resulting plasmid was digested with ClaI and SpeI and used to clone the ClaI and SpeI digested PCR fragment in the other orientation, producing the plasmid pGEM-PIT-RMR2X. Finally the fragment from pGEM-PIT-RMR2x containing the promoter 35S, the AtRMR fragments in sense and antisense orientation, the intron and the *ocs* terminator, was obtained by digestion with NotI and inserted into a NotI-digested Pcambia3300 vector to obtain pCAMBIA+RMRs.

Biolistic transformation

The Arabidopsis reporter plants were transformed by particle bombardment. Vectors CbLCVA containing sequences from each AtRMR were mixed with the CbLCV B genome and bombarded into three week-old plants, as described by Kjemtrup et al., (1998) or Turnage et al.(2002), using the Particles Delivery System (PDS) 1000 He Particle gun (Bio-Rad). Gold particles (Bio Rad) were used as micro carriers and their coating was done as described by the

manufacturer: 3mg of gold was coated with 5µg of CbLCVA+RMRs and CbLCVB component followed by addition of 50µl of 2.5M $CaCl_2$, 20µl 0.1 M of spermidine. The rupture disks, stopping screen, holders for macro carriers and micro carriers were sterilized by soaking in 70% ethanol. Ten µl of the coating suspension were used for each transformation. Plants were bombarded three times and were grown under 8h of light at 20°C until yellow spots were observed on leaves. Leaves were then examined using a confocal microscope.

Agrobacterium-mediated transformation

Transgenic Arabidopsis plants expressing either Aleu-GFP or GFP-Chi at the six leaves stage were transferred to soil and grown in a green house at 20ºC under continuous light for one week, transferred to growth chamber with 16hours of light and irrigated with water every four days until inflorescences appeared. The main floral stem was cut to induce more stems which were used for the inoculation with *Agrobacterium*.

The pCAMBIA+RMRs plasmid was mobilized into the *A. tumefaciens* strain GV3101 by the freeze-thaw method. Agrobacteria were grown overnight at 28°C in 5 ml of YEB medium with antibiotics rifampicin, gentamycin, and kanamycin at concentrations of 50, 25, and 50 µg/ml, respectively. 300 µL of the overnight culture were added to 300 ml of fresh medium with the same antibiotics and grown at 28° to the stationary phase (OD600 ~2.0). Bacteria were harvested by centrifugation at 4000 rpm for 20 min at room temperature. The pellet was resuspended in infiltration medium (5% sucrose, 0.05% Silwett L-77) to obtain the desired density (OD_{600} of 0.8 -0.1). Plants were inoculated by submersing inflorescences in the agrobacterial suspension for 10s (Clough and Bent, 1998).

Seeds were spread on soil and kept for 2 days at 4ºC. They were then incubated at 20°C with 16 h light and 8 h darkness for approximately 10 days, until plants reached the 4-leaf stage, then Basta (Glufosinate ammonium, 50 µg/ml) was sprayed twice a week on plants to ensure Basta resistance. Transformed plants remained green while non-resistant plants turned white and died.

Confocal Laser Scanning Microscopy and Data collection

Images were taken with a confocal laser microscope (DMR, Leica Microsystems, Heidelberg, Germany) using the TCD 4D operating system. GFP was detected with the filter set for fluorescein isothiocyanate (FITC), whereas chlorophyll fluorescence was detected with the filter set for trimethylrhodamine isothiocyanate (TRITC).

The stored digital images were pseudocolored as red or green images using Photoshop CS2 (Adobe system) in correspondence to the real red or green colour.

RNA-isolation and RT-PCR analysis

Leaves from control and silenced plants were frozen in liquid nitrogen and stored at -80°C until extraction. Total RNA was extracted from ground leaf powder using an extraction buffer (2M Tris-HCl pH8.0; 0.5M EDTA pH 8.0; 20%SDS), re-extracted with an equal volume of phenol/chloroform/isoamylalcohol (24/1/1 v/v) and precipitated with one volume of 6M LiCl. First strand cDNA was synthesized from 2µg total RNA with the Improm-II Reverse Transcription System from Promega using oligo $(dT)_{15}$ as primer according to the manufacturer. The PCR reaction was performed using gene specific primers (Table II). The amplification conditions were: pre-denaturation at 95°C for 3 min; 30 cycles of 45s at 94°C, 45 s at Tm (60°C for all *At*RMR 1 and 2; and 62°C for *At*RMR 3,4,5 and 6), 1min at 72°C and a final extension step of 10 min at 72°C.

Protein Extraction and western blotting

Leaves from control and silenced plants were ground to powder in liquid nitrogen and resuspended in extraction buffer (1x TBS containing protease inhibitor). The mixture was then centrifuged at 14000 rpm for 10min at 4°C. The soluble fraction was diluted with 2X loading buffer, then incubated at 95°C for five minutes and loaded on a SDS-PAGE gel and electrophoresed under standard conditions. After electro-transfer of the proteins to a nitrocellulose filter, anti-GFP serum was used at the dilution of 1:10000 for immunolabelling.

Table 1. Primers for amplification of RMRs fragments and their assembly in tandems

Primer	Sequence	Restriction site	Tm 1	Tm 2
At1fKpn	GACGGTACCATGGCAGGTAACTCTGGAGGTATAAGG	KpnI	54°	65°
At1rXho	CCCCCCATCTCGAGAGGACCGAGATGTCCGCCTTCTT	XhoI		
At3fXho	TCGGTCCTCTCGAGATGGGGGGAGACTCGGACGGTATAAAG	XhoI	52°	60°
At3rBam	CGCTCCATGGATCCGGCACATCCCATTGAATTGAGATGTAGAGT	BamHI		
At5pfSal	TATGCCAAGTCGACATGGCAGGAAATTCATCTGGTGTCTATATAC	SalI	56°	62°
At5prNhe	ACCTTCATGCTAGCTTGGCATACGAGAATGTCCTTGGCC	NheI		
At2fNhe	TATGCCAAGCTAGCATGAAGGTGAACCCTCAGGACATTAC	NheI	58°	65°
At2rBgl	GTCAGATCTGCATCTAACCTGATGGTCCTGGTG	BglII		
At4fBam	GATGTGCCGGATCCATGGAGCGAAACCCCTCTGGTG	BamHI	50°	61°
At4rXba	CCTCCATTCTAGATGGGCATACGATGAAAGTCATTGCC	XbaI		
At5fXba	TATGCCCATCTAGAATGGCAGGAAATTCATCTGGTGTGG	XbaI	52°	61°
At5rSal	CCTGCCATGTCGACTTGGCATACAAGAAAGTCCTTGGCC	SalI		

Chapter 4

Material and methods

Bacterial strains.

The bacterial strains used were:

- *Escherichia coli* XL-1 Blue: *rec*A1, *end*A1, *gyr*A96, *thi-1 hsd*R17 (rk-,mk+), *sup*E44, *rel*A1, λ-, *lac*- . This strain was used to multiply plasmid. As the strain is resistant to tetracycline, it could not be used for plasmids containing a tetracycline marker.
- *Agrobacterium tumefaciens* strain GV3101 was described to be the most efficient to infect *Arabidopsis thaliana* ecotype *Wassilewskaja*.

Plasmids and vectors

Plasmids and vectors used for this study are listed in the table according to their relevant characteristics and references.

Table 2.1 Plasmids and vectors

Plasmids/vectors	**Relevant characteristics**	**references**
pGEM-T-Easy	Amp^r , Sp6 and T7 promoter	Promega
CbLCVA007d	Amp^r, 35S promoter and terminator	Turnage et *al., 2002*
CbLCVB	Amp^r, 35S promoter and terminator	Turnage et *al.,2002*
CbLCV008	Amp^r, 35S promoter and terminator. Contain gene for the magnesium chelatase	Turnage et *al.*,2002
pCAMBIA	Kan^r, $Basta^r$	

r: resistant

Primers

Primers used in this work were from Microsynth GmBH (Balgach, Switzerland) as indicated in the table below.

Table 3.1: Primers for VSR amplification

Names	Sequence(5'→ 3')	Length
VSR AMPLIFICATION		
SAT_1b	GGAGTGTCGACGGCTTTTCACTCTCTCGTTT C	32
RAT_1b	CAAACTCTAGACTCCGAAATTACCAATGGCAC	32
SAT_2b	CGGAGTCTAGAGTTTGTCGGTGACGTCG	28
AAT_2	AAGATCTCGGATAAACCACCGTACCA	26
SAT_3	GGGTACCTAGTCAACGGGAGAGCTTC	26
RAT_3b	GACCACTCGAGGTGCTTCGACCTCATCTC	29
SAT_4b	CCCATGGACGACGAATGTATGGT	23
RAT_4b	AAGCCGTCGACACTCCTCTGATGGACTC	28
SAT_5b	AGCACCTCGAGTGGTCAACGGGTTTTCATC	30
RAT_5b	TTATGTCTAGAGACCAAAAGTTTGCTATGG	30
SAT_6b	TGGTCTCTAGACATAAAGGAGCCACCTTGGC	31
AAT_6	AAGATCTGGCTGCGTCGTGCTTAGA	25

Table3.2: RMR amplification

At1fKpn	GACGGTACCATGGCAGGTAACTCTGGAGGTATAAGG	36
At1rXho	CCCCCCATCTCGAGAGGACCGAGATGTCCGCCTTCTT	37
At3fXho	TCGGTCCTCTCGAGATGGGGGGAGACTCGGACGGTATAAAG	41
At3rBam	CGCTCCATGGATCCGGCACATCCCATTGAATTGAGATGTAGAGT	43
At5pfSal	TATGCCAAGTCGACATGGCAGGAAATTCATCTGGTGTCTATATAC	45
At5prNhe	ACCTTCATGCTAGCTTGGCATACGAGAATGTCCTTGGCC	39
At2fNhe	TATGCCAAGCTAGCATGAAGGTGAACCCTCAGGACATTAC	40
At2rBgl	GTCAGATCTGCATCTAACCTGATGGTCCTGGTG	33
At4fBam	GATGTGCCGGATCCATGGAGCGAAACCCCTCTGGTG	36
At4rXba	CCTGCCATTCTAGATGGGCATACGATGAAAGTCATTGCC	39
At5fXba	TATGCCCATCTAGAATGGCAGGAAATTCATCTGGTGTGG	39
At5rSal	CCTGCCATGTCGACTTGGCATACAAGAAAGTCCTTGGCC	38
SCb007-7	CCAATCACATGCCGCCTGACAAGGT	25
ACboo7-7	CTTCGATGAGAGCTTCTGGGCGGAC	25
SCb007-8	CCAATCACATGCCGCCTGACAAGGT	25
ACboo7-8	CTTCGATGAGAGCTTCTGGGCGGAC	25

Table3.4: RT-PCR

SAT1N	TGGATGCGGAAATTAGAGGG	20
AAT1N	GGTACACTTATTTCTGTTTGTGGC	28
SAT2Nb	GATCAGAGCCATAATGGCAC	20

AAT2N	TTCTCCGAAGCTACATCGAAG	21
SAT3N	GTCTCTGGTTTGTGATTGAGC	21
AAT3Nb	TGCATTTGTATCCACCCCATG	21
SAT4N	AGATGTGAATGAGTGTGAGGAGAA	24
AAT4N	TTATGCAAATGTCGTGTTCTCTTATG	26
SAT5N	GCTTTGAAGATATGGAACGG	20
AAT5N	GTATAGACTCACTCCAATCCATC	23
SAT6N	GCATTAAAGGTATGGAACGGTC	22
AAT6N	GACTCACTCCAGTCTATCTTCAGG	24

Antibiotics used in selective media

Antibiotics used in this study are listed in the table below
Table 4.1: Antibiotics used

Antibiotics	**Working concentration (µg/ml)**	**Stock solution(mg/ml)**
Ampicillin	50	50
Kanamycin	50	50
Tetracyclin	50	50 in 70%Ethanol
Rifampicin	50	50 in DMSO
Gentamycin	25	25
Glufosinate ammonium(Basta-herbicide)	30	30

Enzymes, chemicals and Kits

Enzymes

Enzymes	**Buffer used**	**Manufacturer**
XbaI	D/2	Promega/Biolabs
KpnI	J	promega
XhoI	D	promega
EcoRI	E	promega
BglII	D	promega
SalI	D	promega
NheI	B	promega
Acc65 I	D	promega
T4 DNA Ligase	10X ligase buffer	Promega
Taq polymerase	10X Taq buffer	Promega

Chemicals and other materials

Chemicals/Materials	Manufacturers
Agarose	Gibco BRL
Coomassie Brillant blue	Serva
dNTPs	Promega
Ethidium Bromide	Fluka
Chloroform	Acros Organics
Phenol/chloroform/isoamylalcohol	Sigma
Ethanol (HPLC)	Romil
Isopropanol	Reactolab

Kits

- Plant RNA purification reagent (Invitrogen, Lucerne, Switzerland) for RNA-extraction:
- Improm-II Reverse Transcription System (Promega, Wallisellen, switzerland) for RT-PCR
- pGEM-T and pGEM-T easy vector system(Promega,Wallisellen, switzerland) for subcloning

Media, solutions and Buffers

Most of media and solutions were prepared with deionised milli Q water and were autoclaved 20 min at 120°C for sterilization. Solutions and techniques were used according to the Molecular cloning book (Sambrook et al.,2001).Antibiotics were added after autoclaving.

Table 6.1 Media for bacterial culture

LB-medium	0.5% NaCl; 0.5%(w/v) yeast extract, 1% (w/v) bacto tryptone in H2O
LB-Amp-medium	LB-medium with 50µg/ml Ampicillin
LB-Kan-medium	LB-medium with 50µg/ml Kanamycin
LB-medium-plates	1.6% bacto agar was added to the liquid medium
YEB-medium	0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5%sucrose, 2mM MgSO4
YEB-medium plates	1,6% Agar was added to the liquid medium
YEB-Kan-Gent-Rif-medium	YEB-medium with 50µg/ml kanamycin,

	25µg/ml gentamycin, 50µg/ml rifampicin
YEB-Rif-Gent-medium	YEB-medium with 25µg/ml gentamycin, 50µg/ml rifampicin

Table 6.2 Other media for seeds selection

MS- medium	0.22% MS salt, 0.05% MES,1%sucrose,the pH was adjusted to 5,8 using a solution of 1 M KOH
MS-medium plates	0.8% agar was added to the liquid medium
MS-Kan-medium	MS-medium with 50µg/ml Kanamycin added after autoclaving
MS-Basta-medium	MS-medium with 30µg/ml Basta(Glufosinate ammonium) added after autoclaving

Table 6.3 Buffers and solutions

Buffer I	25.0 mM Tris/HCl pH8.0; 50mM glucose; 10mM EDTA pH8.0
Buffer II	0.2N NaOH, 1%SDS
Buffer III	3M potassium acetate, 5M acetate solution
TE-buffer	10mM Tris/HCl, 1mM EDTA,pH8.0
Bromophenol blue-mix	0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll
Ethidium Bromide	0.5µg/ml
0,5X TBE buffer	45 mM Tris base, 45 mM boric acid, 1 mM EDTA pH 8.0
KOAc	3.0 M K Acetate , pH 5.5
RF-1 Buffer	100mM KCl;30mM $MnCL_2$; 30mM KOAC pH 7,5; 10mM $CaCl_2$; 15% glycerol; adjust the pH to 5.8 with 200mM acetic acid and filter sterilised
RF-2 Buffer	10mM MOPS pH 6.8; 10mM KCl; 50mM CaCl2; 15% glycerol, was adjusted to pH 6.8 with 1M NaOH and filter sterilised
RNA 2X Loading buffer	3% Ficoll; 0.05% Xylene cyanol; 0.05% bromophenol blue
RNA extraction Buffer	1Vol of 2M Tris pH 8.0; 2 Vol of EDTA pH 8.0; 1 Vol 20% SDS
6M Lithium Chloride	
70% EtOH	70ml of 100% ethanol diluted in 30ml of DEPC water
DEPC water	500µl of DEPC in 1l of water stir very well and autoclaved.
Protein Electrophoresis buffer	196mM glycine / 0.1% SDS / 50mM Tris-HCl pH 8.3
0,5M Tris-HCl pH6,5	60,57 g was dissolved in water then the pH was bring to 6.5 using a solution 2N HCl. Autoclaved for sterilisation
1.5M Tris-HCl pH 8,8	181.71 g was dissolved in water then the pH was bring to 8,8 using a solution 2N HCl. Autoclaved.
10% SDS	10g of SDS in 100ml of water
10% APS	10g of APS in 100ml filter sterilized
Protein Transfer buffer	3.03 g Tris –base; 14.4 g Glycine; 200ml Methanol
2X protein loading buffer	125mM Tris-HCl pH 6.8, 10% 2-mercaptoethanol, 10% SDS, 10% glycerol
PBS buffer	NaCl : 8g, KCl: 0.2g, Na_2HPO_4: 1.15g, KH_2PO_4: 0.21g, pH was adjusted to 7.4
Blocking buffer	8% skimmed milk in 1 x PBS
Buffer for antibodies dilution	8% skimmed milk, 0, 2% Tween ,1 x PBS
Washing buffer	1x PBS containing 0.2 % Tween
Stripping buffer	2%SDS, 50mM Tris pH 6.8, 100mM, β -mercaptoethanol
Protein extraction buffer	50mM Tris-HCL buffer pH7.6 supplemented with 150mM NaCl, 5mM EDTA, 0.1% SDS, and 0.1% β-mercaptoethanol

Bacterial culture

Freezing and storage of *E.coli* strains.

200µl of sterile 87% glycerol was added to 800µl of an *E.Coli* culture mixed and was frozen at -80°C.

Standard growth conditions for *E.coli*

E.coli bacteria were grown in LB-medium at 37°C. Liquid cultures were shaken at 280 rpm. Depending on the resistance of plasmids antibiotics were added to the media.

Heat-shock competent *E.coli* XL1-Blue cells

5 ml LB medium containing Tetracycline (50mg/ml) as antibiotic was inoculated with a single bacterial colony and incubated for 16 hours at 37°C and 250 rpm. This preculture was diluted 1:100 into 100 ml LB-medium and incubated for 2 to 3 hours at 37°C and 250 rpm until the OD_{600} reached a value of 0.5. Soon after, the culture was then chilled on ice for 15 min, and the bacteria were pelleted for 15min at 4°C and 5000 rpm in the Sorvall GSA rotor. The pellet was resuspended in 32ml of RF1 buffer and was left for 20min on ice. Following a centrifugation step of 15min at 4°C and 5.000 r.p.m in the Sorvall GSA rotor, the pellet was taken up in 8ml RF2 and incubated for 20 min on ice. The Eppendorf tubes for freezing aliquots of competent bacteria were precooled in cold room. The bacteria suspension was portioned in aliquot of 100µl and was frozen in liquid nitrogen. Tubes were stored in a -80°C freezer.

Transformation of competent *E.coli* cells by heat-shock

This protocol was used for E.coli strains and plasmids, which showed good transformation efficiency by means of heat-shock.
First, competent cells were thawed on ice and 1µl of purified DNA sample or 5µl of a ligation mixture were added. After mixing, an incubation of 45 min on ice followed. The heat-shock was performed by placing the cells for 2 min into a heating block at 42°C. Then the mixture was placed on ice for 10min. At the end, all of the bacterial suspension was plated on LB-plates with the corresponding antibiotics and was grown overnight at 37ºC.

Isolation and purification of DNA

Isolation of plasmid-DNA from *E.coli*

Small scale or mini-preparation (Birnboim and Doly 1979)

For mini-DNA preparations, a single bacterial colony was transferred into 5mL of LB medium containing appropriate antibiotic in a loosely capped 12ml tube. The culture was grown overnight with shaking at 37°C and 250 rpm. Then, 1.5mL of this culture was centrifuged for 5min at 4°C and 16000 rpm in a table top centrifuge and the bacterial pellet was resuspended in 100µL of ice-cold solution I. The mixture was kept in ice for 10min. After this step, 200µL of a freshly prepared solution II was added into the tube and the content were mixed well by inverting tube rapidly five times until the lysate became clear and then kept on ice for 10 min. After incubation on ice, to the viscous cell lysate was added 150µL of ice-cold solution III, the tube was mixed by votexing and was incubated on ice for 15 min.

After centrifugation at 4°C, 12 000 rpm for 10 min, the supernatant was added with 2 volumes of phenol: chloroform and mixed by vortexing. The mixture was centrifuged for 10min at RT and at 12000 rpm. 400µl of the upper phase was transferred to a fresh tube and 400µl chloroform was added to it and was mixed by vortexing. After centrifugation for 10min at RT and at 12000 rpm, the supernatant was transferred into a new tube.

To the supernatant, 2 volumes of 96% ice-cold ethanol were added and the mixture was kept at RT for 10 min to precipitate the double-stranded DNA, which was followed by a centrifugation for 20 min at 4°C and at 14000 rpm. The pellet was washed with 1 volume of 70%ethanol ice-cold and was dried for 5 min at 65°C. Finally the pellet was dissolved in 30 µl of distilled water and was digested with 0.3µl of RNase A for 5min at 65°C or 30min at 37°C. The DNA solution can be stored at -20°C for further investigations

Small scale concentrated DNA extraction: modified minipreparation

The bacterial pellet of 3ml culture, grown overnight at 37ºC and 250 rpm, was resuspended in 100µl of ice cold solution I by vigorous vortexing until the pellet was dissolved completely. 200µl of the freshly prepared solution II was added to the tube and the contents were mixed well by inverting the tube rapidly five times and incubated on ice for 15 min. After incubation on ice, 150µl of ice-cold solution III was added to the mixture and mixed by vortexing and kept on ice for 10min. The mixture was centrifuged at 4ºC for 10min and the supernatant was

transferred in a new tube mixed with 1 Vol of phenol/chloroform /isoamyl alcohol. After a short centrifugation for 5 min, at 8000 rpm the upper phase was added with 1 vol of chloroform. After another centrifugation for 5 min at 8000 rpm, the supernatant was added with 1 vol of isopropanol and KOAc (final concentration of 0.2M), vortexed and incubated at RT for 20 min. After centrifugation (RT, 12000 rpm, 20min) the pellet was washed with 1 vol of 70% ethanol and dried at 65ºC for 5 min. DNA was dissolved in 30µl water and stored at -20ºC.

Maxi-preparation of DNA from *E.Coli*

400mL litres of LB medium containing the appropriate antibiotic was inoculated with a single colony of cells with the desired plasmid, and grown for 24 hours at 37°C and 250 rpm. The cells were sedimented from the above two 400mL culture at 15000 rpm for 15 min at 4°C. The pellet was resuspended in 8ml of solution I. Then, 16 ml of Solution II was added, and mixed by inverting tubes several times until the suspension became translucent and viscous; and tawn on ice for 10 minutes. 8 ml of Solution III was added and the mixture was vortexed chilled on ice for 10min then centrifuged. After centrifugation at RT, 15000 rpm for 10min (Beckman centrifugator). The supernatant was transferred into a new tube and was treated with 2V of chloroform. DNA in the aqueous phase was precipitated by adding 16 ml of isopropanol and the mixture was incubated at RT for 10minutes.The pellet was rescued by centrifugation at 15000 rpm for 15 minutes at RT and was washed with 70% Ethanol, was resuspended in 2mL of TE, and 2mL of 5M LiCl. The mixture was centrifuged at 3700 rpm for 10 minutes and 4 ml of isopropanol was added in the supernatant. After a centrifugation at 3700 rpm for 10 minutes, the pellet was washed with 3mL of 70% Ethanol, was dried, was dissolved in 500 µl of TE containing the RNAse A (20µg/ml) then was kept at 37°C for 2 hours. DNA was precipitated by adding 1mL of 99% Ethanol. 5 minutes later, DNA was collected by centrifugation at 12000 rpm for 5 minutes and was rinsed with 70% EtOH .Then was dried 15 minutes at RT and resuspended in 400µl of TE at 65°C for 5 minutes.

Precipitation of DNA for concentration enrichment

The DNA-solution obtained after the inactivation of restriction enzymes should be concentrated for further cloning. 0.1 volume of 3M sodium acetate pH 6.8 was added to increase the salt concentration. Then 2.5 volume of cooled (-20°C) ethanol was added and after mixing well the tube was placed at -20°C for at least 30 min for precipitation. The precipitated DNA was centrifuged at 4°C and 16000 rpm for 20min. The ethanol was aspired,

70% ethanol was added and after the following centrifugation step of 5 min the supernatant was removed completely and the DNA was dried at 65°C for 5min. The dry DNA was taken up in sterile water (10 to 20 µl in case a digestion 100µl-digestion had been performed)

Characterisation of DNA molecules

Restriction of plasmid DNA

Analytical digestion of plasmid DNA

In order to verify DNA preparations, digestions with restriction endonucleases were performed. Usually 0.1 to 1 µg DNA were incubated for 2 hours with 2U of each restriction enzyme in a total volume of 30µl. The buffer and the temperature were chosen according to the enzymes and the manufacturer's recommendations. In order to perform a digestion in a total volume of 30µl, 1µl of concentrated mini-preparation or 8µl of mini-preparation DNA was used.

Preparative digestion of plasmid DNA

This type of digestion was used to prepare either linearized plasmids or fragments with "sticky end" for further cloning. The reaction was performed in 30µl volume and 3 to 6µg of DNA were used.

Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyse and isolate DNA fragments of digested plasmid and PCR-products in the presence of ethidium bromide. The agarose concentration varied from 0.7 to 2.5% depending on the size of the expected DNA fragments. The agarose was weighted in a flask and was suspended in 0.5 x TBE and dissolved by heating in a microwave oven for 3min. 2 µl of ethidium bromide was added to 50 ml of agarose, the gel was poured in a tank and a comb was inserted. After gel polymerisation, the comb was removed and the gel was placed into an electrophoresis chamber. The fragments were separated at 90 to 95 V for 15 to 35 min (120 V for a big gel). DNA bands were visualized on a GEL DOC system from Bio-Rad. Under UV light, the desired band was eluted directly with a pipette or was cut out with a blade.

Estimation of DNA concentration with a spectrophotometer

1 or 2 µl of DNA were diluted in 500 µl of water and the absorption at 260nm was measured in a quartz glass cuvette. An OD_{260} of 1 corresponds approximately to 50µg/mL DNA and to 33 µg/ml for a ssDNA (oligonucleotides).

Sequencing

The sequencing reaction was performed according to the dideoxynucleotide method described by Sanger [1977].This experiment needs primers labelled at their 5'end by a special dye. Primers were labelled with the IRDye800 or IRDye700. These primers were synthesised by MWG Biotech (Germany).35 cycles of PCR were carried out in a Tgradient thermocycler (Biometra, Göttingen). In a Thermo Sequenase kit, the enzyme (Taq Polymerase), reaction buffer and nucleotides are pre-mixed and found in four separate tubes called A reagent, T reagent, C reagent and G reagent, each with the appropriate terminators.

Cycle sequencing-Reaction

First step: Reaction beginning

The DNA template and the primers were combined in 0.5mL tube

- 1µl of 500-700 ng template(ds-DNA)
- 1µl of IRD700 forward primer (1.0 pmol/ul)
- 1µl of IRD800 reverse primer (1.0 pmol/ul)
- 3µl of Distilled water
- 1 drop of mineral oil

The components were mixed well by pipetting the reaction up and down several times with the same tip.

The next step consists of labelling a micro plate of 96 wells and labelled a set of four 0.2ml constituting the plate by A, T, G, C for each template/primer combination.

In each well was added: 1ul of the A reagent to the A tube(s), T reagent to the T tubes(s), G reagent to the G tube(s) and C reagent to the C tube(s).

Then was added in each tube 1ul of the appropriate template/primer combination to the A, T, G, and C and mixed well

The micro plate was put in the thermal cycler for the PCR reaction.

Second step: PCR-program:

Table1: PCR program for cycle sequencing

Process	**Reaction**	**Temperature**	**Time**	**Cycles**
Denaturation		95°C	2min	1
	Denaturation	95°C	40sec	
Synthesis	Annealing	55°C	45 sec	30
	Polymerization	72°C	4 min	
Complete synthesis		72°C	10 min	1

When the cycling program was completed, 1 µl of the sample loading buffer was added to the mixture and it was denatured at 95°C for 5 min and was placed on ice.

Depending on the comb used, 1.0 to 1.5 µl of samples were loaded onto a 33cm 7% polyacrylamide gel (25-cm length and 0.25-mm in thickness). The gel was run using 1X TBE buffer. The apparatus used to achieve this process was the LI-COR 4000L sequencer (LI-COR Biosciences).

The data images were automatically collected and simultaneously recorded during electrophoresis.

Following electrophoresis the image file was analysed using LI-COR Gene image IR software.

Cloning

Isolation of DNA from agarose gel

For DNA fragment isolation, two techniques were used

Direct DNA extraction from the gel.

This technique did not need special material. The gel was put under a UV lamp and a 1000µl pipette was used to isolate the DNA. The tip was put inside the gel where the desired DNA

was found and the DNA was sucked out. To maximize the extraction, 10µl of 0.5xTBE was added in the slot before suction.

DNA isolation using Wizard SV gel and PCR clean-Up system (Promega)

For the purification of DNA fragment which could not be extracted with pipette under UV light, the corresponding band was cut out and the DNA was extracted using Wizard® and Gel and PCR clean-Up System Kit (protocol:" Wizard® and Gel and PCR clean-Up System protocol". Handbook, 1/05, p.6-7).

The gel slice was weighted and put in an Eppendorf tube. 10µl of Membrane binding solution per 100mg of agarose gel slice was added. Then the mixture was vortexed and incubated at 65°C for 10 min to melt the gel. The melted gel was transferred to a SV Minicolumn assembly and incubated at RT for 1 min. A centrifugation followed at 16000rpm for 1 min, and the column was washed twice by adding 700µl of Membrane Wash Solution. Between each washing step, the column was centrifuged for 5 min at 16000rpm. The resulting DNA was collected in a new tube by adding 20µl of nuclease free water followed by a centrifugation for 1 min at 16000 rpm. 5µl of the DNA was analyzed on a gel and DNA was pure enough for sequencing and further cloning techniques.

Dephosphorylation of DNA fragments

All enzymes manipulations were carried out according to the recommendations of enzymes providers. To prevent self-ligation of a linearized vector or a vector fragment, the DNA was dephosphorylated. In the case of double digestion as in point **9.1.3** or after precipitation of the DNA, 1µl of CIP (Calf Intestinal Mucosa Phosphatase) was added to a volume of 20µl and the mixture was incubated 1 hour at 37°C. Following enzymatic treatment, CIP or restriction endonucleases was inactivated by incubating the reaction mixture at 75°C for 5 min. The dephosphorylated DNA fragment can be used immediately for ligation (**10.3**)

Ligation of DNA fragments

The ligation was usually performed with T4 DNA Ligase in a total volume of 15µl.

Ligation mixture:

- 4,5 µl of DNA fragment

- 1µl of linearized vector
- 7,5µl of 2x rapid ligation buffer / 1,5 µl of 10x T4 Ligase buffer
- 1µl of T4 ligase(1U/µl)
- Water in the case 10x T4 Ligase buffer was used

Vector and DNA fragment were used in the ratio 1:4. With 2x rapid ligation buffer, the reaction was performed at RT for 1 hour or ON at 4°C while, with 10x ligation buffer the reaction was achieved at 16°C (ON).The ligation product was transformed into XL1-Blue competent cells (as in **7.4**).

Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is used to amplify a segment of DNA that lies between two regions of known sequence. A thermostable DNA polymerase purified from the thermophilic bacterium *Thermus aquaticus* was used to extend the annealed oligonucleotide primers [saiki et al., 1988]. The primers typically have different sequences and are complementary to sequences that first lie on opposite strands of the template DNA and secondly flank the segment of DNA that is to be amplified. First, the template DNA in the presence of a large molar excess of each of the two primers and the four dNTPs was denatured by heating. Then the mixture was cooled to a temperature for annealing of the primers to their target sequences, after which the annealed primers were extended with polymerase. The cycle of denaturation, annealing and polymerization was repeated for many times. The major product of this exponential reaction was a segment of double stranded DNA whose termini are defined by the 5'-termini of the oligonucleotide primers and whose length is defined by the distance between the primers.

To amplify any DNA template, the PCR program should be modified by altering temperature, time, and number of cycle dependent on the DNA sequences. PCR was performed using Biometra Tgradient (Biometra, Goettingen) with standard programs.

Most PCR reactions were made in a final volume of 50µl, or 25µl for PCR on colonies.

Table 2: Typical PCR reaction mixture

Components	volume
10x reaction buffer (100mM KCl,	5µl
dNTPs mixture	5µl (stock 2mM)
DNA	X µl (approximately 0.1 µg)
1st PCR primer	5 µl (stock 10µM)
2nd PCR primer	5 µl(stock 10µM)
Taq-polymerase	1 U
H_2O	complete to 50µl
Total volume	50 µl

PCR-Program

Depending on the required type of DNA-amplification, different PCR protocols were used.

General program

This program was used each time restriction sites were added to the PCR fragment.

Table2: General program used to link receptor sequences in tandem

Process	**Reaction**	**Temperature**	**Time**	**Cycles**
Denaturation		95°C	2 min	1
	Denaturation	95°C	40 sec	
Synthesis	Annealing	Tm1	45 sec	10
	elongation	72°C	1 min	
Complete synthesis		72°C	5 min	1
	Denaturation	95	2 min	
Synthesis	Annealing	Tm2	45 sec	30
	elongation	72	1 min	
Complete synthesis		72	10min	

Table 3: Annealing temperature for each VSR primers

*At*VSR	Tm1(°C)	Tm2(°C)	Restriction sites added
*At*VSR1;1	58	65	SalI/XbaI
*At*VSR1;2	50	61	KpnI/SalI
*At*VSR2	52	61	XbaI/BglII
*At*VSR3;1	54	65	KpnI/XhoI
*At*VSR3;2	52	60	XhoI/XbaI
*At*VSR3;3	56	62	XbaI/BglII

PCR program used to group genes from the same family

Program for the *At*VSR 3;1, 3;2, and 3;3

1µl of PCR product of *At*VSR3;1 amplification

1µl of PCR product of *At*VSR3;2 amplification

1µl of PCR product of *At*VSR3;3 amplification

5 µl of DNTPs (stock 2 mM)

5µl sense primer SAT_3 (stock 10µM)

5µl antisense primer RAT_6 (stock 10µM)

5µl 10x Taq polymerase buffer with MgCL2

1 µl Taq polymerase

26µl water

The PCR reaction were performed as previously but with annealing temperature of 54ºC and 61ºC

Program for the *At*VSR 1;2, 1;1 and *At*VSR 2.

1µl of PCR product of *At*VSR1;2 amplification

1µl of PCR product of *At*VSR1;1 amplification

1µl of PCR product of *At*VSR2 amplification

5 µl of DNTPs (stock 2 mM)

5µl sense primer SAT_4 (stock 10µM)

5µl antisense primer RAT_2 (stock 10µM)

5µl 10x Taq polymerase buffer with MgCL2

1 µl Taq polymerase

26µl water

Annealing temperatures were 52ºC and 59ºC.

The same strategy was performed for RMR receptors.

Table 4: **A**nnealing temperature for each RMR primers

*At*RMR	Tm1(°C)	Tm2(°C)	Restriction sites added
*At*RMR $_1$	60	70	KpnI/XhoI
*At*RMR$_2$	60	68	NheI/BglII
*At*RMR$_3$	63	70	XhoI/BamHI
*At*RMR$_4$	60	69	BamHI/XbaI
*At*RMR$_5$	60	70	XbaI/SalI
*At*RMR$_{5'}$	62	68	SalI/NheI

Tm1 (°C) corresponds to the annealing temperature of the first part of the primer while Tm2 (°C) is for the whole primer

Program for *At*RMRs.

1µl of PCR product of *At*RMR1 amplification

1µl of PCR product of *At*RMR2 amplification

1µl of PCR product of *At*RMR3 amplification

1µl of PCR product of *At*RMR4mplification

1µl of PCR product of *At*RMR5 amplification

1µl of PCR product of *At*RMR5' amplification

5 µl of DNTPs (stock 2 mM)

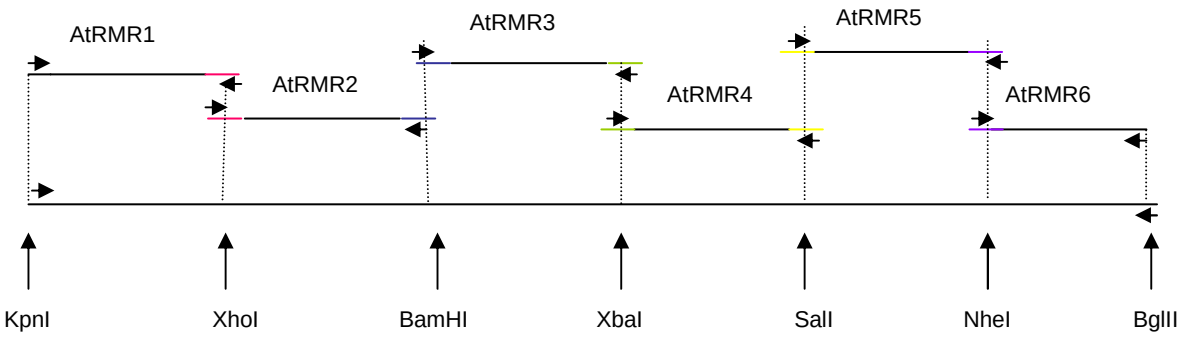
5µl sense primer At1fKpn (stock 10µM)

5µl antisense primer At2rBgl (stock 10µM)

5µl 10x Taq polymerase buffer with $MgCL_2$

1µl Taq polymerase

13µl water.



PCR on colony.

This protocol was used to rapidly detect successful transformations by using standard primers for the determination of correct ligation products by size screening.

This experiment was realized using bacterial colonies as template and available primers (M13 forward and M13 reverse when the pGEM-T vector was used for the subcloning).

A PCR master mix which contained dNTPs, Taq polymerase buffer with MgCl2, forward and reverse primers, Taq polymerase enzyme and water was prepared. This master mix was aliquoted into 25µl into PCR tubes. The following volumes are for five PCR reactions:

12, 5 µl M13 forward primer (stock 10µM)

12,5 µl M13 Reverse primer (stock 10µM)

12, 5 µl dNTPs (stock 10µM)

12, 5 µl 10x Taq polymerase buffer

14, 6 µl H2O

12,5 µl Taq buffer containing MgCl2

Tubes were heated at 95°C and 0.4µl of Taq (1 U) was added. The reaction program as follows:

Table 5: Colony PCR program

	Reaction	**Temperature**	**Time**	**Cycle**
Denaturation		95°C	3min	1
	Denaturation	94°C	30sec	
Synthesis	Annealing	56°C	45 sec	35
	Polymerization	72°C	1 min	
Complete synthesis		72°C	10 min	1
Cool down		10°C	∞	

PCR products were run in an agarose gel as in **9.2**.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).

Small scale RNA extraction.

All material used in this experiment was treated with 0.5M NaOH in order to be RNase-free and to avoid RNA degradation. Also, all solutions were prepared with DEPC water and were autoclaved 20 min at 160°C. During each step of purification hand gloves were used.

• Mortar, Eppendorf tubes, spatula and pestle were cooled in liquid nitrogen.

• Plant material was frozen and was ground thoroughly in the precooled mortar. The powder was then transferred into the precooled Eppendorf tube with a precooled spatula.

• 500µl of an extraction solution 1:2:1(1 Vol of 2M Tris pH 8.0, 2 Vol of 0.5M EDTA pH 8.0 and 1Vol 20% SDS) was added to the ground plant material and was vortexed for 30 sec at RT

• Then, the mixture was centrifuged for 3 to 5 min at 16000 rpm at RT

•The supernatant was transferred to a new Eppendorf tube and 500µl of phenol/chloroform/isoamylalcohol was added. The tube was thoroughly shaken.

•The preparation was centrifuged down for 10 min at RT and 16000 rpm and the supernatant was transferred to a new Eppendorf tube. 500µl of chloroform was added and the tube was centrifuged again.

•The water phase was transferred to a fresh tube, 500µl of 6M lithium chloride was added and the solution was kept ON at 4°C.

• The following day, the sample was centrifuged for 20 min at 4°C and 16000 rpm.

• The pellet was resuspended in 200µl of DEPC water at 4°C for 15 min.

• The RNA was precipitated by adding 20µl of 3M NaAcetate and 600µl of precooled 99%Ethanol

• The tube was centrifuged and the pellet was washed with 500µl of precooled 70% Ethanol. RNA was air dried at 4°C for 5min, and finally was resuspended in 30µl of Nuclease-free water.

RNA isolation with Tri Reagent

TRI Reagent® is a patented reagent for the simultaneous isolation of RNA, DNA and proteins. The reagent is an improved version of the popular single-step method for total RNA isolation [Chomczynski, 1987]. It is a solution containing phenol, guanidine thiocyanate and chloroform. RNA isolation is complete in less than one hour.

This protocol was used when we were dealing with small quantity of material. Leaves were frozen in liquid nitrogen and were ground in a precooled mortar. They were transferred into an Eppendorf tube and 500µl of Trizol was added. The mixture was vortexed for few seconds and was allowed to stand for 5 min at room temperature. After spinning down at 4°C, 14000 rpm for 10min, the resulting supernatant was transferred into a new Eppendorf tube and 200µl of chloroform was added. The sample was vortexed 10 sec and was kept at room temperature for 3 min then was centrifuged at 4°C and 14000 rpm, for 20minutes. The aqueous phase was transferred into a new tube and 500µl of isopropanol was added. The tube was kept at room temperature for 25 min and then centrifuged at 4°C, 14000 rpm for 15 min. Finally the pellet was washed with 1ml of 75% Ethanol (made with DEPC water) and was resuspended in 15µl of nuclease-free water.

DNase treatment of RNA

For the RT-PCR reaction it was necessary that the RNA should be free from DNA, to avoid amplification from residual DNA alone. 1µl of DNAse was added to the RNA extract and the reaction mixture was completed to a final volume of 20µl and incubated 1 hour at 37°C. Following enzymatic treatment, the DNAse was inactivated by incubating the reaction mixture at 75°C for 10 min.

Quality test of RNA in agarose gel

The quality of the RNA isolation was examined in a normal 1.5% agarose gel. Each slot was loaded with 1 µl of RNA diluted into 1µl of Bromophenol blue. For running conditions 1x TAE buffer was used. With good RNA preparations the 16S rRNA and 23 rRNA must be visible.

Photometric determination of the RNA concentration

The RNA concentration was determined by measuring the extinction at 260nm against H_2O in a photometer (Gene Quantum, Pharmacia). An extinction of 1 corresponds to a concentration of 40µg/ml RNA.

Reverse transcriptase Reaction

The Reverse transcriptase reaction copies mRNA into cDNA. The ImProm-II Reverse Transcription System from Promega was used for efficient synthesis of first–strand cDNA in preparation for PCR amplification. The system enables full-length cDNA synthesis for a reproducible analysis starting with either total RNA or poly (A)+mRNA.

The reaction was primed by the oligo (dT) $_{15}$ primer contained in the kit. This primer initiates first-strand synthesis by annealing with the 3' end of any polyadenylated RNA molecule. The synthesized cDNA was used directly for PCR amplification or was kept at -20°C for further experiments.

The experiment was divided in two parts:

Combination of oligo $(dT)_{15}$ primer on target RNA and Denaturation

All following steps were done at 4°C (or on ice). RNA was thawed on ice.

Experimental reaction:

1µl RNA (2µg/µl)

1µl oligo$(dT)_{15}$ (0,5µg/µl)

3µl Nuclease-free water

Final volume: 5µl

• The tube containing the mixture was tightly closed and was placed into a preheated 70°C heating block for 5min.

• Then the tube was centrifuged at 4°C, 800 rpm for 5 sec.

• The reaction was immediately chilled on ice for 15 min.

During this time the reverse transcription reaction mix was prepared

Reverse transcription

This step was prepared in a sterile PCR tube on ice.

Experimental reaction:

4µl Nuclease free water

4 µl ImProm-II 5x Reaction buffer

4µl $MgCl_2$ (25mM)

1 µl dNTP mix (10mM)

1µl RNase inhibitor (20U)

1µl ImProm-II Reverse transcriptase

Final volume: 15µl

Then 5µl of the RNA-oligo$(dT)_{15}$ mixture was added to the 15µl RT cocktail and mixed by pipetting up and down, and was centrifuged briefly.

For the reaction a thermocycler for PCR was used. The following program was used:

Reaction	Temperature	Time
Anneal	25°C	5 min
Extend	42°C	2 hours
Reverse transcriptase inactivation	70°C	15 min

1 µl of the first strand cDNA was directly used in PCR reaction in a final volume of 50µl. The reaction condition was the same as in typical PCR reaction mixture (see **11 PCR**).

Protein techniques

Protein extraction

We extracted proteins from small quantity material in the simplest way. 0.1g of leaves was cut and put into an Eppendorf tube and 30µl of 50mM pH 7. 0 phosphate buffer was added. A small spoon of glass beads and a pestle were used to ground leaves. The mixture was centrifuged at 4°C and 12000 rpm for 10min. The supernatant was transferred into a new Eppendorf tube, 1 vol of 2x protein loading buffer was added and the sample was heated at 95°C for 5 min. The protein extract was used for SDS-PAGE. Proteins obtained were sufficient for one western blot

SDS-PAGE

SDS-PAGE was performed according to Laemmli (1970). Two gels could be cast at once in the gel casting chamber “Mini protean III”(Bio-Rad). Gels with 15% acrylamide/bisacrylamide were used. After pouring the separating gel, it was overlaid with water. After polymerisation, the water was removed and the stacking gel was poured. 20µl sample in protein loading buffer were loaded per lane. Gel electrophoresis was performed at 25 mA until the dye front reached the bottom of the gel. The gel was then blotted.

Component	Separating gel (15%)	Stacking gel (4%)
H2O	1, 88 ml	3, 02 ml
1,5M Tris pH 8, 8	2, 00 ml	
Protogel	4, 00 ml	0,650ml
0,5M Tris pH 6, 5		1,25ml
10% SDS	0, 08 ml	0,05ml
APS 10%	0, 04 ml	0,025ml
TEMED	0,005ml	0,015ml
Final volume	**8ml**	**5ml**

Protogel stock: 30% Acrylamide/Bisacrylamide. See table 3 for media preparation.

Electrophoretic transfer of proteins

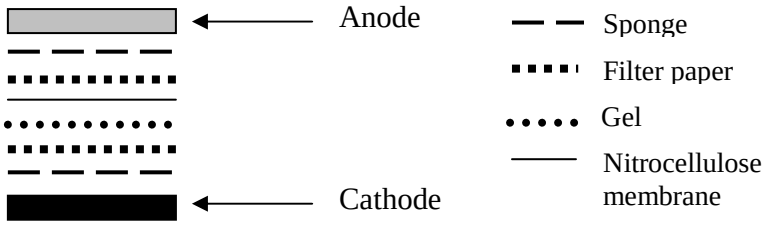
After SDS-PAGE, the proteins were transferred from the gel onto a Millipore membrane (Immobilon™-PSQ 0.2µm Membrane).

The Mini Protean III system (Bio-Rad) is a fast way to transfer proteins and the material supplied with the system makes the transfer easy.

The membrane was prepared by being soaking in 96% Ethanol for few seconds and then submerging in transfer buffer in order to equilibrate the membrane.

The next step was to assemble the sandwich. The cassette used in the transfer has two sides, black and white.

One sponge was placed on the white side followed by a blotting paper (mini trans-blot paper), the membrane, the gel, another blotting paper and finally another sponge on the black side.



The transfer direction is from black side (gel) to white side (membrane). The sandwich was rapidly submerged in the tank which was filled with transfer buffer, the black side facing the anode.

The transfer was done at 30V and 122mA for 1 to 2 hours. After this time the blot could be used for probing.

Western Blot

The chemiluminescence substrate from Bio-Rad was used to detect immunolabeled protein bands which had been electrophoretically transferred to a membrane.

In order to block unspecific antibody-binding sites, the transfer membrane was incubated in blocking buffer (8% skimmed milk, 1 x PBS) for 1 to 2 hours at RT. The blot was incubated with buffer containing the primary antibody (in 8% skimmed milk, 0. 2% Tween, 1 x PBS) (1:10,000) for 16 hours at 4°C on a shaking platform. After this step, the blot was washed 4 times for 10 min (in 8% skimmed milk, 0.2 and 2% Tween, 1 x PBS). Then, the secondary antibody coupled to peroxydase (in 8% skimmed milk, 0. 2% Tween, 1 x PBS at a the dilution of 1:10,000) was added followed by gentle shaking for 1 to 2 hours at RT and the blot was washed again 4 times in 1x PBS containing 0. 2 % of Tween.

After the last wash, the blot was placed between two plastics sheets, 800µl of substrate was added and the air bubbles were gently smoothed out. The blot was kept darkness for 10min and then moved to an X-ray film cassette. The blot was exposed to a chemiluminescence-sensitive film in this cassette for 5 s to 15 min. The film was developed by an automatic machine.

Western blot stripping

This method was used to remove antibodies from the blot by denaturing proteins.

The stripping buffer was heated at 50°C in a water bath .The blot was incubated in the stripping buffer in a water bath at 50°C for 15 to 30min with shaking every 5 min. Then, the blot was rinsed 10 times for 10min each with 1x PBS. After the washes, it was blocked and probed as described in **13.4.**

Plant handling and plants transformation techniques

Plants used

During my thesis, three lines of *Arabidopsis thaliana* plants ecotype Wassilewskaja were used: (1) the wild type, (2) the transgenic line expressing the Aleu-GFP in the lytic vacuoles and (3) the transgenic line expressing the GFP-Chi in the storage vacuoles (Flückiger et al., 2003).

Seed sterilisation

Seeds were poured in Eppendorf tubes, and the tube was filled with 70%EtOH. The mixture was well hand-shaken for 1 min, and then was incubated at room temperature for 30 seconds to 3 min. Under a hood, EtOH was pipetted away and seeds were washed in sterile water and were drained immediately. 1 ml of a solution containing 2% bleach water, 0.005% triton X-100 was added and seeds were shaken vigorously for 15 min using a bench top shaker. The solution was removed with a Pasteur pipette, then seeds were washed four times with ethanol 100% (Schlesier et al., 2003). After the final wash seeds were left under the hood to dry. A tooth pick was used to distribute seeds one by one on solid Murashige & Skoog medium (Murashige and Skoog, 1962) including Gamborg B5 vitamins. Plates contained kanamycin or Basta as selective agent when adequate. Petri dishes were sealed with parafilm and incubated for 48 h at 4°C(stratification) in complete darkness, then transferred to a growth chamber with a 16h/ 8h dark photoperiod at 22°C.

Germination conditions

Plant germination was carried out in growth cabinet at a temperature of 22°C. Lighting was provided by fluorescent lamps (PHILIPS, Holland) with 16h/ 8h dark photoperiod per day with a light intensity of 120 $\mu E\ s^{-1}\ m^{-2}$.

After 3 to 4 weeks in Petri dishes plants were transferred individually in to the soil and were put in a growth chamber with 8h of light. They were used 3 weeks later for transformation by biolistics and were observed 36 days or more post inoculation.

Plant transformation

The biolistic technique was as described by Bio-Rad using the PDS 1000/He gene gun delivery system.

Plant inoculation by biolistic

Conditions and settings.

Parameters used to optimize the results were chosen as:

- Microparticles Size : 1,0µm gold
- Target Distance : 9cm
- Helium Pressure : 1,100 PSI
- Vacuum (Inch Hg): 25

Particle preparations

Gold particles as microcarriers were prepared as described by Bio-Rad and used a final concentration of 60mg/ml:

Gold micro particles (60 mg) with a diameter size of 1µm were weighted into 1.5ml microfuge tube and were mixed with 1 ml of EtOH 70%. The suspension was vortexed for 5 min and the particles were allowed to soak in 70%EtOH for 15 min. The micro particles were pelleted by briefly spinning at 16000 rpm for 1 min. The supernatant was removed and discarded. The gold particles were washed with sterile water (MilliQ and autoclaved). This step was repeated three times and after each washing the suspension was centrifuged at RT and 16,000rpm for 1 min. Then the gold particles were resuspended in 1 ml sterile 50% glycerol and were kept at room temperature until they were used.

Preparation of DNA-coated gold particles

The following procedure was used for 12 shootings. The microcarriers prepared in 50% glycerol were vortexed for at least 10 min on a bench top vortexer. In each 1.5ml centrifuge tube 116.7 µl of microparticles were added and tubes were held on the bench vortexer under continuous vortexing. While vortexing, 12µl of different plasmid DNA (1µg/µl) containing the sequence to be silenced were added and the same quantity of the CbLCVB (1µg/µl) was added too (this plasmid was used for virus spreading inside the plants). Under continuous vortexing, 116µl 2.5 M CaCl2 (3.86 g CaCl2.2H2O in 10ml distilled water, filter sterilized) and 46,7 µl of 0.1 M spermidine (145mg spermidine in 10ml autoclaved milliQ water) were added and tubes were vortexed at maximum speed for 3 min. Then tubes were kept at RT for 30 minutes, then vortexed again at RT and 800 rpm for 1min.The supernatant was discarded and the pellet was resuspended in 150 µl of 70% ethanol(HPLC grade), then recentrifuged

again at RT and 800 rpm for 1 min. The supernatant was removed again and the gold particles were resuspended in 150µl of 100% ethanol (HPLC grade). A final centrifugation was realized, the supernatant was removed and the pellet was resuspended in 140µl 100% ethanol (HPLC grade).Finally tubes were sealed with parafilm and kept on ice.

Shooting

Stopping screens and macrocarriers were sterilized in 70% ethanol and left to dry before use. 10ul of the suspension was pipetted as evenly as possible onto the centre of a macrocarrier sheet. Then, the macrocarrier with the mixture was kept under a sterile bench to let the ethanol evaporate. The gold particles were shot at 1,100 psi with a vacuum at 22 inches Hg onto plants. Plants had been grown in small pots for 30 to 40 days in short day light before bombardment.

Plant transformation of *Arabidopsis thaliana* by floral dip

Transformation of Arabidopsis thaliana was performed based on the protocol of Clough et al., (1998). Plants were grown for three weeks under short day conditions (8h light, 16 hrs dark) and transferred to long day (16 hours light, 8 hours dark). After three weeks, the emerging bolts were cut to induce growth of multiple secondary bolts. Floral dip of plants with *A. tumefaciens* culture was done one week after clipping. Bacteria were grown until DO_{600}> 2.0, harvested by centrifugation and were resuspended in one volume of infiltration medium (5% Sucrose with 0.005% of silvett), at a DO of approximately 0.8. Entire shoots of the plants were submerged into the *A tumefaciens* suspension in a baker for 5 seconds. After flowering and siliques formation, seeds were sterilized by Na-hypochlorite as described in **14.2.** before plating on MS selection medium containing 30mg/l Basta. After two weeks, Basta-resistant plants were transferred to soil, grown and their seeds were collected.

Preparation of competent *Agrobacterium tumefaciens* cells.

An overnight culture of A*grobacterium tumefaciens* in YEB medium was diluted with fresh medium 1:100 (final volume 400ml) and was grown at 28°C until OD_{600} reached the range 0.5 to 0.8. Cells were collected by centrifugation at 4ºC and 3000 rpm for 20 min and were washed twice in 10ml of cold sterile TE. After centrifugation at 3000 rpm for 5 min, the cells were resuspended in 20 ml of cold sterile YEB. 100µl aliquots of the cell suspension were stored in -80ºC freezer.

Cold Shock transformation of competent *Agrobacterium tumefaciens* cells.

One to two mg of plasmid DNA was mixed with an aliquot of competent *A.tumefaciens* cells which were then frozen in liquid nitrogen for 5min. Then cells were transferred to the 37ºC water bath and were incubated for 5 min. One ml of YEB medium was added to the cells. The cells were mixed and incubated for 2 to 3 h at 28°C with shaking. Then they were centrifuged for 5s and resuspended in 200µl of YEB, and cells suspension were spread onto selective YEB plates and were incubated at 28°C for two days in order to receive visible colonies for further propagation.

Characterization of transgenic plants

Characterisation of transgenic plants by RT-PCR

RT-PCR on plant leaves was performed as described in **12.**

Characterisation of transgenic plants by western Blot analysis

Leaves or seeds of plants were extracted with an extraction buffer. Protein extracts were centrifuged at 12000 rpm for 10 min. The protein concentration of the supernatant was measured according to Bradford (1976). Western blot analyses were carried out as described in **13.2**

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 chloroplasts respectively. Digital images were pseudocolored using Adobe Photoshop version 7.0, and green was attributed to GFP and red to chloroplasts. Images with transmitted light were also collected using the same confocal microscope.

Scanning Electron Microscopy

Seeds were mounted on a stub with adhesive Leit C glue and air dried. They were coated with 23nm gold layer in a Baltec SCD 005 sputter apparatus and observed with a Philips FEG ESEM XL40 at the acceleration of 10-20kV .

RNA-RNA *in situ* hybridization using Dig-Labelled probes to sectioned plant tissues

This technique was used to determine VSR gene expression patterns in leaves and in roots. Plant materials were fixed, embedded in parafilm, sectioned, placed on slides and hybridized with DIG probes. Hybrids were detected by applying anti-digoxigenin antibodies to the tissues. The antibody (Roche) used was conjugated to alkaline phosphatase which produced a coloured precipitate.

Tissue fixation, dehydration and embedding of tissues

Tissue fixation

Leaves or roots were cut in small pieces with a blade and were fixed under vacuum in freshly prepared 4% formaldehyde in PBS during 30 min. After prefixation, tissues were left overnight in fresh fixative at 4°C.

Dehydration and embedding in paraplast

Fixed tissues were washed with cold PBS for 1 hour and were dehydrated in an ethanol series (10%, 30%,50% 70%, 85% and 100% containing 0,1 % eosin) at 4°C. Eosin was used in the experiment to stain the sample in pink colour so that the sample will be visible in the paraffin. Tissues were kept overnight at 4°C in ethanol 100% containing 0, 1% eosin.

Dehydrated tissues were embedded in a histoclear series (25% histoclear: 75% ethanol, 50% histoclear: 50% ethanol; 75% histoclear: 25% ethanol and 100% histoclear) at room temperature for 1 hour. Tissues were then moved to a 60°C oven and a solution containing 1vol histoclear/1 vol paraplast was added and kept at 60°C for 1h. A solution of paraplast 100% (60°C) was then added to start embedding at 60°C overnight. The next day, the embedding was continued by replacing twice the paraplast solution. Between the replacements, samples were kept at 60°C for 3 h. Finally tissues were moulded in small aluminium moulds and kept at 4°C.

Sectioning

For this step DAKO slides were used needing no pre-treatment. Slides were placed on a slide warmer at 40°C and DEPC water was put on slides.

Samples were cut to 7µm thick sections using a microtome. In order to get tissues flat, Paint brushes were used to float tissues on a water surface. Water was removed carefully to avoid

tissue bubble. Slides were kept on the slide warmer overnight so that tissues adhered tightly to the slides and were stored at 4°C.

Preparation of RNA probes for *in situ* hybridization

Purification and linearization of plasmid DNA

150 bp of DNA sequence was amplified by PCR, was cloned into the pGEM-T vector, which was purified and sequenced (Microsynth).Ten micrograms of DNA was linearized using the appropriate restriction enzyme (in a total volume of 100µl at 37ºC). The restriction enzyme site was chosen according to the type of probe required (sense or antisense). To verify the correct linearization 2 µl of the mixture were loaded on an agarose gel 1% in 0.5X TBE (80V). Then the linearized DNA was precipitated using phenol/ chloroform as described in **8.2** and was solubilized in DEPC treated water (final concentration 0.5µg/µl).

Preparation of digoxygenin (DIG) labelled RNA-probes.

RNAs were labelled during the transcription reaction using DIG-UTP 1h at 37ºC as described by the manufacturer (Roche, Mannheim, Germany). RNA-DIG was detected afterward using specific antibodies. RNA polymerase used in this experiment was supplied with the kit and whether sense or antisense probes were needed, T7 or SP6 polymerase was used. Then the mixture was treated with DNAse in the presence of tRNA for 15 min at 37ºC. After DNAse treatment, RNA probes were precipitated by adding an equal volume of 4M ammonium acetate and 2 volumes of 100% ethanol and incubating at -20ºC for 30 min. They were centrifuged and the resulting pellet was rinsed with 70% ethanol, air dried in a speed vac, and resuspended in 50µl of DEPC-treated water.

In situ hybridization and detection.

The paraffin-embedded tissues were cut into 7µm sections using a microtome (Leica Microsystems, Nusslo Gmbh, Germany) and mounted on DAKO microscope slides (Menzel-Glaser, Germany). Sections were hydrated as described by Jackson(1991). Sections were incubated with a proteinase K solution (1µg/ml in Tris-EDTA buffer) for 30 min at 37°C and treated sequentially with solutions of glycine (0.2% in PBS), 4% Para-formaldehyde and acetic anhydride (0.5%) in triethanolamine (0.1M). Sections were dehydrated in ascending concentrations of ethanol as described by Jackson (1991).Sections were hybridized for more than 12 hours at 50°C with a DIG-UTP RNA probe (either sense or anti-sense) in the

hybridization solution (50% deionised formamide; salt solution: 3M NaCl,100mM Tris pH 8, 10mM EDTA; 10X dextran sulphate, 50X Denhardt's solution, 100µg/ml tRNA). After several washings in 0.2X SSC (0.03M NaCl, 3mM NaAcetate), single stranded RNA was removed with a preheated (55°C) solution of TNE containing 20µg/ml RNAse at 37°C for 30 min. For the detection of DIG labelled hybrids, slides were first incubated in a blocking solution, then in another solution containing 1% BSA, 0.3% Triton X100 . A blocking solution containing 1.25 units/ml of alkaline phosphatase-conjugated anti-DIG Fab fragments was added on the slides. The reaction was incubated at room temperature for 1 hour. Slides were washed three times with the same blocking solution containing BSA and Triton X100. Finally the staining buffer containing Levimasole (1mM) and the NBT/BCIP substrates for the phosphatase (5µl of NBT 75mg/ml in dimethylformamide 70% and 3.75 µl of BCIP 50mg/ml in pure dimethylformamide) were added on the slides and left overnight for development. The staining was stopped and slides were air dried and mounted with faramount (DAKO). Pictures were taken with a camera (Leica) connected to a light microscope (Leica).

Chapter 5

General discussion

It has been proposed that a plant cell can possess up to three types of vacuoles with different functions linked to their different contents: the lytic vacuole, the vegetative storage (neutral) vacuole and the protein storage vacuole (Hoh et al., 1995; Paris et al., 1996; Di Sansebastiano et al., 1998, 2001). They could be distinguished using tonoplast intrinsic proteins (TIPs, a family of aquaporins) as markers (Paris et al., 1996; Jiang et al., 2000).

The lytic vacuole (LV) is an acidic compartment that contains lytic enzymes analogous to the lysosome of animal cells. The membrane, or tonoplast, of such vacuoles contains the aquaporin γ-TIP (Höfte and Chrispeels 1992; Marty-Mazars et al., 1995; Paris et al., 1996; Barrieu et al., 1998; Jiang et al., 2000).

The second type of vacuoles was identified in leaves, petals, roots or tubers as a vegetative storage vacuole (VSV). It may contain specific vegetative storage proteins (VSPs) and its tonoplast typically contains δ-TIP (Jauh et al., 1998). They were shown in specialized vegetative cells to form or disappear in response to wounding or to developmental switches, such as the transitory storage of nitrogen in leaves until tubers or seeds start to accumulate proteins (Jauh et al., 1998; Jiang et al., 2000; Park et al., 2004; Murphy et al., 2005). Di Sansebastiano et al. (2001) showed that LV and neutral (probably vegetative storage) vacuoles are regenerated by evacuolated protoplasts of tobacco (*Nicotiana tabacum*) cells.

The protein storage vacuole (PSV) is found in cells from storage tissues of seeds, where its major function is the storage of proteins (Okita and Rogers 1996; Müntz 1998; Herman and Larkins 1999). The tonoplast of these vacuoles contains another typical aquaporin, called α-TIP (Swanson et al., 1998). In seeds, the protein storage vacuole is often complex and contains internal compartments, globoids and large crystalloids within a soluble matrix. Globoids contain lytic enzymes, e.g. aleurain; the crystalloids are composed of insoluble 11S globulins while the matrix compartment contains a mixture of 7S lectins and 2S albumins. In seed storage vacuoles DIP, very similar to δ-TIP, is associated with the crystalloid membranes while the matrix is surrounded by a tonoplast with both α-and δ- TIP (Jiang et al., 2000).

Three different types of targeting sequences have been identified in proteins that are targeted to vacuoles through the Golgi complex. Sequence-specific vacuolar sorting determinants (VSD) were found first in N-terminal propeptides (NTPP) of barley aleurain and sweet potato

sporamin precursors, both having an NPIR motif (Matsuoka and Nakamura 1991; Holwerda et al., 1992), but they were later also found in internal and C-terminal propeptides (Saalbach et al., 1996; Frigerio et al., 2001b). An essential Ile or Leu was identified in each case. C-terminal VSDs were first identified as C-terminal propeptides (CTPP) in tobacco chitinase and barley lectin (Bednarek and Raikhel 1991; Neuhaus et al., 1991a) and have no conserved motifs, but must be accessible from the C-terminus. The psVSD type 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 . These three types of VSD indicate therefore the existence of at least three different mechanisms by which a soluble secretory protein can be directed to a vacuole

Evidence for at least two different mechanisms for the transport of soluble proteins with different VSDs to the vacuole in plants was presented. 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 ss-VSDs, which are sorted into CCVs by VSRs at the TGN (Kirsch et al., 1994; Paris et al., 1997; Hinz et al., 1999). The ctVSD and the psVSD are required for the transport to the vegetative (neutral) and seed storage vacuole, respectively (Tague et al., 1990; Neuhaus et al., 1991b). The differential inhibition of protein sorting of proteins with ssVSD or ctVSD by wortmannin also supports the existence of distinct sorting mechanisms (Matsuoka et al., 1995; Pimpl et al., 2006)

In order to explain such signal-based sorting, corresponding receptors were proposed to select the vacuolar protein precursors.

Pea BP-80, the first identified plant VSR, was originally isolated from pea CCVs and was showed to bind the ss-VSD from barley proaleurain *in vitro* (Paris et al., 1997; Kirsch et al., 1994). *A.* thaliana harbours seven homologous genes for which different nomenclatures have been used (Laval et al., 1999; Hadlington and Denecke 2000). Neuhaus and Paris (2005) proposed to rename them based on the different subfamilies defined by phylogenetic analysis which groups VSRs from different plant species into three subfamilies (AtVSR 1, 2 and 3). The existence of the same subfamilies in several plant species suggests that they might be involved in different vacuolar pathways and/or function at different stages of the plant development (Paris and Neuhaus, 2005).

Another family of putative receptors was identified by its homology to the PA (protease associated) domain of the VSRs. This luminal PA domain is known to participate in ligand

binding in VSR proteins (Cao et al., 2000). Antibodies against RMR1 detected the same organelles as antibodies against the TIP isoform DIP, i.e. the crystalloid compartment in *Arabidopsis* and tomato seeds (Jiang et al., 2000). The genome of Arabidopsis harbours 6 homologues, *At*RM*R*1-6 and in contrast to VSRs no functional subfamilies can be identified by phylogenetic analysis.

The main goal of this thesis was to study the functional role of each receptor family in protein sorting to vacuoles. In our study, we were not interested in the special situation of seeds, but concentrated on vegetative tissues, where two types of sorting systems must coexist.

To investigate the role of both receptors families (VSRs and RMRs), we used reporter transgenic Arabidopsis plants expressing either Aleu-GFP or GFP-Chi (Flückiger et al., 2003). We silenced these receptors in whole plants and observed the GFP redistribution. AtVSRs were silenced according to subfamilies, while the whole AtRMR family was silenced at once. For the silencing two strategies were used. First we used a geminiviral vector, which can spread easily in *A. thaliana* and was useful to silence more than one gene at the same time. This vector is introduced into plants by the particle delivery system with gold micro carriers. This system allowed us to observe the effects of silencing in whole plants avoiding meiosis and embryogenesis, which could be disturbed by the loss of AtVSR function (Laval et al., 2003). As a control of the effect of the virus on the secretory pathway, we silenced one component of the magnesium chelatase (necessary for the biosynthesis of chlorophyll) in Aleu-GFP and in GFP-Chi plants (Turnage et al., 2002). We found that the vacuolar targeting was not modified and that the vacuoles remained fluorescent. This indicated that the viral vector was adequate for transient tests of vacuolar sorting receptors. The second strategy was to obtain stably silenced receptors using *Agrobacterium*-mediated transformation by the floral dip method. This strategy was used to avoid the viral symptoms developed in transient silencing (Kjemtrup et al., 1998; Turnage et al., 2002) and to better study receptor function in the whole plant.

Assuming that AtVSRs are subdivided into three subfamilies and that each subfamily has a different role, our main goal was to transiently inactivate them.

Several plant biologists have attempted to demonstrate the function of the VSRs by reverse genetics. Laval et al. (2003) used an antisense approach to inactivate the AtVSR1;1 gene. Transformed plants appeared normal and produced apparently normal seeds which were however not able to germinate. Embryos could not be rescued by adding hormones into the medium or by removing the seed coat. Shimada et al. (2003) tested knock-out mutants of each

of the seven VSRs, where the gene had been disrupted by insertion of transfer DNA. None of the mutants had any visible phenotype, nor was there any germination problem. In the knockout mutant of AtVSR1;1, they observed that while most storage proteins were in the PSV in storage tissues in dry seeds, there was an abnormal accumulation of protein in the intercellular space. Shimada et al. (2002) also showed in pumpkin seeds that PV72 was involved in the sorting of storage proteins and was localized in the precursor accumulating (PAC) vesicles which mediate direct protein transport from the ER to the PSV. It should be stressed that PV72 also belongs to the subfamily VSR1. In our experiment we silenced subfamilies AtVSR1 and 2 in one construct and we obtained normal plants which produced seeds unable to germinate and with abnormal seed coats. The results from Laval et al., 2003 suggested that these receptors are important for germination while our results suggest that they are involved also in seed formation, particularly in maternal tissues. In fact, their anti-sense approach caused silencing of all three expressed AtVSRs in seeds, belonging to subfamilies 1 and 2. Since none of the knock-out mutants of Shimada et al. (2003) had germination problems, and the AtVSR1;1 knock-out plant only had partial sorting problems for storage proteins in seeds, this suggests that these three genes could be have a functional redundancy in seeds. Based on the different results obtained in A. thaliana and pumpkin, we can suggest that the subfamily 1 is implicated in protein sorting to seed protein storage vacuoles, while subfamily 2 is necessary to form some compartment necessary for the germination ability. To better define the function of subfamilies 1 and 2 it will however be necessary to silence them separately. It will also be necessary to test protein sorting in every knockout plant using both Aleu-GFP and GFP-Chi markers and to check the acidity and morphology of vacuoles using Neutral Red.

Some studies support functional redundancy in the AtVSR family. daSilva et al. (2005) used β-amylase as marker to quantify protein secretion in the medium using enzymatic reactions. They fused it with the ssVSD of sweet potato sporamin (amy-spo) to study VSR functions in tobacco leaf protoplasts. They expressed this marker with truncated VSR receptors where the luminal part had been replaced by GFP and found that amy-spo was strongly secreted into the medium. They tested four AtVSRs representing the three subfamilies and obtained the same results. In this experiment truncated receptors compete with the endogenous tobacco receptors. Overexpression of a wild type AtVSR restored vacuolar targeting of amy-spo, indicating that the retrograde, not the anterograde transport of the receptor is limiting. They concluded that all VSRs play the same role and are redundant due to the common tyrosine motif present in all VSRs (daSilva et al., 2006). We think that these results are not sufficient

to support the full redundancy in the AtVSR family. While the cytosolic tails of the AtVSRs share a tyrosine motif known to be involved in clathrin-mediated transport they also differ at the C-terminus. It is possible that the truncated GFP-VSR proteins compete for factors necessary for their retrograde transport to the Golgi, but we think that others motifs could be involved in the anterograde transport from the Golgi to different compartments.

Is there redundancy between subfamilies 1+2 and 3? Our results in leaves do not support this hypothesis, as silencing of subfamilies 1+2 did not affect GFP distribution, while silencing of subfamily 3 strongly affected the GFP distribution in mesophyll. Receptors AtVSR1;1 and AtVSR3;2 are both strongly expressed and are present in the same tissues in leaves. Therefore it appears that AtVSR3;2 is necessary but AtVSR1;1 is not. Our results do not allow determining the redundancy between subfamily 2 and the other two, as its two members are more weakly expressed in leaves.

The analysis of 2507 experiments with Affimetrix 22K arrays using Genevestigator (Zimmermann et al., 2004), indicated that all AtVSR genes are expressed in most tissues, even the AtVSR3;1 gene that we detected neither by RT-PCR nor by *in situ* hybridization. This discrepancy could be due to the different ecotypes, as we used Wassilewskaja while most array experiments were done with the Columbia ecotype. However, Laval et al. (2003) did not detect expression of this gene either, even though they used Columbia.

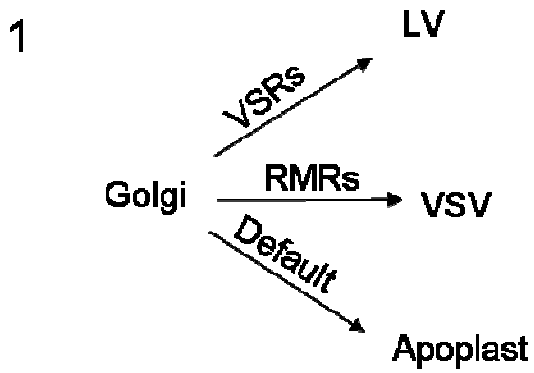
That subfamilies 1 and 3 could have different functions is also suggested by the observation of Dunkley et al. (2006) who detected 4 different VSRs in a global proteomics study of fractionated membranes derived from Arabidopsis suspension cells. In their Principal Component Analysis AtVSR1;1 clustered with ER markers while the other 3 VSRs were found at the periphery of the Golgi cluster. AtVSR2;1 and 2 clustered close together while AtVSR3;1 was at some distance. This indicates that AtVSRs have at least two but possibly three different distributions within the secretory pathway of these suspension cells.

The route to the PSV apparently involves different systems depending on the cell type. In cotyledons of legumes, the storage proteins are accumulated in dense vesicles (DV), which bud from the TGN. The sorting of these proteins and their concentration in lateral buds by an unknown condensation mechanism already begins in the *cis*-Golgi (Hillmer et al., 2001). In pumpkin and castor bean seeds storage proteins are transported in PAC vesicles budding from the ER and bypassing the Golgi. Condensation seems to be an important part of the sorting mechanism in this transport type. In vegetative tissues such a condensation has not been described except when seed storage proteins were ectopically expressed. Evidence for a role of RMRs as receptors for this type of sorting comes from studies on the biogenesis of

complex storage vacuoles in seed tissues of non-leguminous plants, where an RMR was identified in the DIP organelle within the complex protein storage vacuole (Jiang et al., 2000; Jiang et al., 2001). The DIP organelles are believed to be equivalent to the PAC or to the DV vesicles. The RMRs have been proposed to act as the sorting receptors for PSV-destined proteins (Jiang et al., 2000). It may also participate in protein sorting to vegetative storage vacuoles in vegetative tissues: Park et al. (2005) showed first that AtRMR1 was expressed in all tissue. They then showed that coexpression of an AtRMR1 deletion mutant (deleted luminal domain) inhibited the trafficking of phaseolin to the VSV in A. thaliana leaf protoplasts. AtRMR1 was immunolocalized mainly to the DIP-positive organelles but was also found at a lower level in the Golgi complex.

Our experiments on transient and stable silencing of AtRMRs in transgenic plants showed that sorting of GFP-Chi (a marker for vegetative storage vacuole) was strongly affected and was not detected anymore in the vegetative storage vacuoles in mesophyll cells. This result corresponds to those of Park et al. (2005). The silencing unexpectedly also affected the marker of lytic vacuoles (Aleu-GFP) in leaves and the marker was not present anymore in the central vacuole of epidermal cells but was detected in the apoplast, meaning that the protein was secreted. This effect on lytic vacuoles was not expected as our starting model was based on the idea that AtVSRs are the sorting receptors for lytic vacuoles while AtRMRs are the sorting receptors for storage vacuoles. AtRMRs now seem to be involved in both pathways.

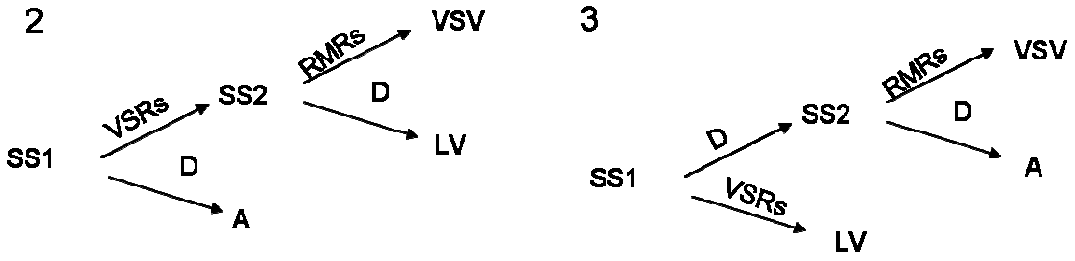
How can we explain the results obtained by the silencing of AtVSRs and AtRMRs? Do AtRMRs interact with AtVSRs? Or do these receptors work in a sequential manner? To answer question on sequential manner, we compared five possible models with our results.



Model 1 is our initial model, where sorting to different vacuoles implicates different receptors working at the same location. It is a trifurcation model. According to this model, if

RMRs are silenced, sorting of Aleu-GFP to LV should remain unaffected, which was not the case.

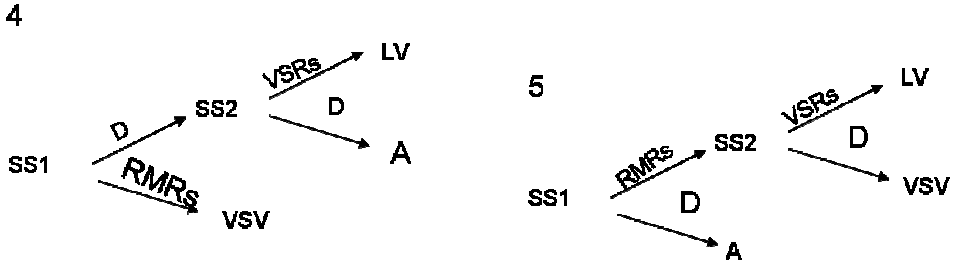
The other four models are based on a sequential sorting process. The sorting sites (SS1 and 2) remain to be defined (cis- or trans-Golgi or prevacuoles). Models 2 and 3 propose that VSRs perform a first selection followed by RMRs.



In Model 2, RMRs select among the proteins preselected by VSRs. Silencing of RMRs would cause both types of fluorescent proteins to accumulate in LV, which thus should remain fluorescent. This model is incompatible with our results.

In Model 3, RMRs select among the proteins that were not first selected by VSRs. In this case GFP-Chi would be secreted when RMRs are silenced but Aleu-GFP would still go the LV, which should remain fluorescent. This model is incompatible with our results.

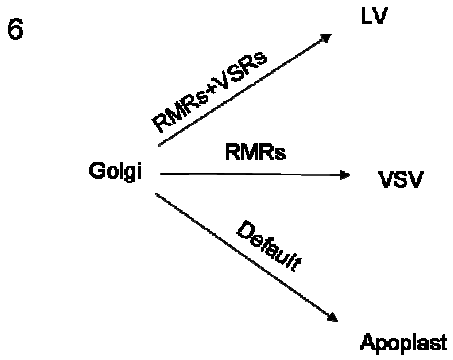
In Models 4 and 5 the RMRs perform the first selection, followed by VSRs. In Model 4, VSRs select among proteins that were not first selected by RMRs. In this case, the GFP-Chi would be secreted when RMRs are silenced but Aleu-GFP would still go the LV, which should remain fluorescent. This model is incompatible with our results.



In Model 5, VSRs select among proteins already selected by RMRs. In this model, when VSRs are silenced Aleu-GFP and GFP-Chi are both sorted to VSV, which remains fluorescent, but when RMRs are silenced fluorescence can be detected neither in the lytic vacuole nor in the vegetative storage vacuole. The latter consequence fits with our results, but in VSR-silenced plants, the Aleu-GFP did not end in the VSV.

This discussion of models means that receptors cannot just function sequentially with RMRs working at the first stage of protein selection followed by protein recognition by VSRs or the reverse.

Another explanation is that some proteins need both receptors for sorting while others are sorted by AtRMRs alone. It this case another model can be added to include a possible interaction to form heterodimers between both receptors There are examples in animal cells of receptors forming heterodimers, e.g the Wnt proteins interact with their receptor Frizzled and a coreceptor Lrp (Lodish et al., 2004).



Our results on silencing of different VSR subfamilies showed that the VSR3 isoforms were the main receptors for protein sorting to lytic vacuoles in leaves. Therefore we can propose that they interact with RMRs for protein sorting to VSV in leaves. At the same time, we cannot decide if the same RMRs are needed for both pathways, as we have silenced them all at once.

In this model, when RMRs are silenced both types of vacuolar proteins are secreted, but when VSRs are silenced only Aleu-GFP is secreted while vegetative storage vacuoles remain fluorescent. This is compatible with our results. If direct interaction between VSRs and RMRs is necessary to sort proteins to lytic vacuoles, co-immunoprecipitation could reveal it. However, it is not necessary for RMRs to interact directly with VSRs but only that they are needed for the formation of the VSR-containing vesicles.

Such a model could mean that VSRs appeared later in evolution than RMRs, to allow a differentiation of vacuoles from ancestral single type. VSR sequences have been found only in land plants (including mosses), while RMRs must be more ancient. Indeed, a protein related to RMRs was found in animals (Jiang et al., 2000), supporting this hypothesis. Differentiation of VSRs into subfamilies occurred later, after the separation of higher plants form mosses. This opens the possibility for further differenciation of VSR-dependent sorting pathways, as for which we present some evidence.

It is difficult to define the exact sorting sites (SS) in these models. We know from immunolocalisation that VSRs are mainly found in the PVC, but also in the TGN (Li et al., 2002), but there may be different localisations for the different subfamilies. RMRs are abundant in seeds and were detected both in DIP organelles and in the Golgi (Jiang et al., 2000; Park et al., 2005). DIP compartments are formed after the sorting event and the first selection by RMRs has occured during the transport through the Golgi. In seeds, it is known that storage proteins start to be sorted laterally within the Golgi and become concentrated at the rim in the cis-Golgi. If RMRs were involved in the sorting of storage proteins in seeds, their action would already start there, preceding the action of VSRs, which were not localised before the trans-Golgi. In animal cells, secretory granules also form in the Golgi involving protein condensation, but the homologues of RMR were never implicated in this mechanism.

During this thesis, different strategies were used to try to define the function of AtVSR subfamilies and AtRMR family in *Arabidopsis thaliana*. The results presented here do not allow proposing a very clear model of the two different sorting mechanisms. Further research will be needed to clarify several points.

When we started this project, we used transient silencing to avoid disturbing the meiosis and embryogenesis by the loss of VSR function (Laval et al., 2003). While we confirmed this problem for subfamilies 1+ 2, there was no such problem when the AtRMR family was silenced. We discovered that VSR subfamilies seem to have different functions in protein sorting to vacuoles. For future experiments it will be interesting to stably silenced each AtVSR subfamily.

Silencing of receptors affected the GFP distribution and the marker was observed in the intercellular space. It would be informative to perform intercellular fluid extraction from silenced and control plants to quantify the secretion. A more readily quantifiable marker (β-amylase, β-glucuronidase) would allow more precise quantification. Other additional analysis

will be to use specific antibodies against well known lytic or storage vacuole proteins to perform western blotting and see if endogenous proteins are also affected. It will also be interesting to develop HA-tagged proteins, to transform stably silenced plants and to determine the protein distribution by immunolocalisation.

Another experiment that can be performed is to cross homozygous knock-out plants and then to transform the resulting double or triple knock-out plants with Aleu-GFP and/or GFP-Chi.

Stably silencing of receptors allows to study the function of each gene by reconstitution assays.

In conclusion, we are still far away from understanding the mechanism of vacuolar targeting in plants.

Bibliography

Afshar, N., B. E. Black and B. M. Paschal (2005). "Retrotranslocation of the chaperone calreticulin from the endoplasmic reticulum lumen to the cytosol." Mol Cell Biol **25**(20): 8844-53.

Ahmed, S. U., E. Rojo, V. Kovaleva, S. Venkataraman, J. E. Dombrowski, K. Matsuoka and N. V. Raikhel (2000). "The Plant Vacuolar Sorting Receptor AtELP Is Involved in Transport of NH2-terminal Propeptide-containing Vacuolar Proteins in Arabidopsis thaliana." J. Cell Biol. **149**(7): 1335-1344.

Andreeva, A. V., Kutuzov, M.A. Evans, D.E.Hawes, C.R. (1998). " The structure and function of the Golgi apparatus: a hundred years of questions." J. Exp. Bot. **49 (325)**(Aug 1998): 1281-1291.

Aridor, M., S. I. Bannykh, T. Rowe and W. E. Balch (1999). "Cargo can modulate COPII vesicle formation from the endoplasmic reticulum." J. Biol. Chem. **274**(7): 4389-99.

Babst, M., T. K. Sato, L. M. Banta and S. D. Emr (1997). "Endosomal transport function in yeast requires a novel AAA-type ATPase, Vps4p." EMBO J. **16**(8): 1820-31.

Bankaitis, V. A., L. M. Johnson and S. D. Emr (1986). "Isolation of Yeast Mutants Defective in Protein Targeting to the Vacuole." PNAS **83**(23): 9075-9079.

Bannykh, S. I., T. Rowe and W. E. Balch (1996). "The organization of endoplasmic reticulum export complexes." J. Cell Biol. **135**(1): 19-35.

Banta, L. M., J. S. Robinson, D. J. Klionsky and S. D. Emr (1988). "Organelle assembly in yeast: characterization of yeast mutants defective in vacuolar biogenesis and protein sorting." J. Cell Biol. **107**(4): 1369-1383.

Bar-Peled, M. and N. V. Raikhel (1997). "Characterization of AtSEC12 and AtSAR1 (Proteins Likely Involved in Endoplasmic Reticulum and Golgi Transport)." Plant Physiol. **114**(1): 315-324.

Barlowe, C. (2002). "COPII-dependent transport from the endoplasmic reticulum." Curr. Opin. Cell Biol. **14**(4): 417-422.

Barlowe, C., L. Orci, T. Yeung, M. Hosobuchi, S. Hamamoto, N. Salama, M. F. Rexach, M. Ravazzola, M. Amherdt and R. Schekman (1994). "COPII: A membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum." Cell **77**(6): 895-907.

Barrieu, F., D. Thomas, D. Marty-Mazars, M. Charbonnier and F. Marty (1998). "Tonoplast intrinsic proteins from cauliflower (Brassica oleracea L. var. botrytis): immunological analysis, cDNA cloning and evidence for expression in meristematic tissues." Planta **204**(3): 335-44.

Batoko, H., H.-Q. Zheng, C. Hawes and I. Moore (2000). "A Rab1 GTPase Is Required for Transport between the Endoplasmic Reticulum and Golgi Apparatus and for Normal Golgi Movement in Plants." Plant Cell **12**(11): 2201-2218.

Bednarek, S. Y. and N. V. Raikhel (1991). "The Barley Lectin Carboxyl-Terminal Propeptide Is a Vacuolar Protein Sorting Determinant in Plants." Plant Cell **3**(11): 1195-1206.

Bednarek, S. Y., T. A. Wilkins, J. E. Dombrowski and N. V. Raikhel (1990). "A carboxyl-terminal propeptide is necessary for proper sorting of barley lectin to vacuoles of tobacco." Plant Cell **2**(12): 1145-55.

Birnboim, H. C. and J. Doly (1979). "A rapid alkaline extraction procedure for screening recombinant plasmid DNA." Nucleic Acids Res **7**(6): 1513-23.

Boevink, P., K. Oparka, S. S. Cruz, B. Martin, A. Betteridge and C. Hawes (1998). "Stacks on tracks: the plant Golgi apparatus traffics on an actin/ER network." Plant J. **15**(3): 441-447.

Boevink, P., S. Santa Cruz, C. Hawes, N. Harris and K. J. Oparka (1996). "Virus-mediated delivery of the green fluorescent protein to the endoplasmic reticulum of plant cells." Plant J. **10**: 935-941.

Bonifacino, J. S. and E. C. Dell'Angelica (1999). "Molecular Bases for the Recognition of Tyrosine-based Sorting Signals." J. Cell Biol. **145**(5): 923-926.

Bonifacino, J. S. and B. S. Glick (2004). "The Mechanisms of Vesicle Budding and Fusion." Cell **116**(2): 153-166.

Bonifacino, J. S. and J. Lippincott-Schwartz (2003). "Coat proteins: shaping membrane transport." Nat Rev Mol Cell Biol **4**(5): 409-14.

Bonifacino, J. S. and L. M. Traub (2003). "SIGNALS FOR SORTING OF TRANSMEMBRANE PROTEINS TO ENDOSOMES AND LYSOSOMES." Annu. Rev. Biochem. **72**(1): 395-447.

Bonifacino, J. S. and A. M. Weissman (1998). "Ubiquitin and the control of protein fate in the secretory and endocytic pathways." Annu. Rev. Cell Dev. Biol. **14**: 19-57.

Boyce, J. M., D. Coates, M. D. Fricker and D. E. Evans (1994). "Genomic sequence of a calnexin homolog from Arabidopsis thaliana." Plant Physiol. **106**(4): 1691.

Braakman, I. (2001). "A novel lectin in the secretory pathway

An elegant mechanism for glycoprotein elimination
" EMBO J. **2**(8): 666–668.

Brodsky, J. L., E. D. Werner, M. E. Dubas, J. L. Goeckeler, K. B. Kruse and A. A. McCracken (1999). "The Requirement for Molecular Chaperones during Endoplasmic Reticulum-associated Protein Degradation Demonstrates That Protein Export and Import Are Mechanistically Distinct." J. Biol. Chem. **274**(6): 3453-3460.

Bryant, N. J. and T. H. Stevens (1998). "Vacuole biogenesis in *Saccharomyces cerevisiae* - protein transport pathways to the yeast vacuole." Microbiol. Mol. Biol. Rev. **62**(1): 230 ff.

Bukau, B. and A. L. Horwich (1998). "The Hsp70 and Hsp60 chaperone machines." Cell **92**(3): 351-66.

Cao, X., S. W. Rogers, J. Butler, L. Beevers and J. C. Rogers (2000). "Structural requirements for ligand binding by a probable plant vacuolar sorting receptor." Plant Cell **12**(4): 493-506.

Castelli, S. and A. Vitale (2005). "The phaseolin vacuolar sorting signal promotes transient, strong membrane association and aggregation of the bean storage protein in transgenic tobacco." J. Exp. Bot. **56**(415): 1379-1387.

Cereghino, J. L., E. G. Marcusson and S. D. Emr (1995). "The cytoplasmic tail domain of the vacuolar protein sorting receptor Vps10p and a subset of VPS gene products regulate receptor stability, function, and localization." Mol. Biol. Cell **6**(9): 1089-1102.

Chatre, L., F. Brandizzi, A. Hocquellet, C. Hawes and P. Moreau (2005). "Sec22 and Memb11 Are v-SNAREs of the Anterograde Endoplasmic Reticulum-Golgi Pathway in Tobacco Leaf Epidermal Cells." Plant Physiol. **139**(3): 1244-1254.

Cole, N. B., N. Sciaky, A. Marotta, J. Song and J. Lippincott-Schwartz (1996). "Golgi dispersal during microtubule disruption: regeneration of Golgi stacks at peripheral endoplasmic reticulum exit sites." Mol. Biol. Cell **7**(4): 631-650.

Contreras, I., Y. Yang, D. G. Robinson and F. Aniento (2004). "Sorting signals in the cytosolic tail of plant p24 proteins involved in the interaction with the COPII coat." Plant Cell Physiol. **45**(12): 1779-86.

Cooper, A. and H. Bussey (1992). "Yeast Kex1p is a Golgi-associated membrane protein: deletions in a cytoplasmic targeting domain result in mislocalization to the vacuolar membrane." J. Cell Biol. **119**(6): 1459-1468.

Cooper, A. A. and T. H. Stevens (1996). "Vps10p cycles between the late-Golgi and prevacuolar compartments in its function as the sorting receptor for multiple yeast vacuolar hydrolases." J. Cell Biol. **133**(3): 529-41.

Cosson, P. and F. Letourneur (1994). "Coatomer interaction with di-lysine endoplasmic reticulum retention motifs." Science **263**(5153): 1629-31.

Costaguta, G., C. J. Stefan, E. S. Bensen, S. D. Emr and G. S. Payne (2001). "Yeast Gga coat proteins function with clathrin in Golgi to endosome transport." Mol. Biol. Cell **12**(6): 1885-96.

Culianez-Macia, F. A. and C. Martin (1993). "DIP: a member of the MIP family of membrane proteins that is expressed in mature seeds and Dark-Grown seedlings of Antirrhinum majus." Plant J. **4**(4): 717-725.

d'Enfert, C., M. Gensse and C. Gaillardin (1992). "Fission yeast and a plant have functional homologues of the Sar1 and Sec12 proteins involved in ER to Golgi traffic in budding yeast." EMBO J. **11**(11): 4205-11.

Danilczyk, U. G., M. F. Cohen-Doyle and D. B. Williams (2000). "Functional relationship between calreticulin, calnexin, and the endoplasmic reticulum luminal domain of calnexin." J. Biol. Chem. **275**(17): 13089-13097.

daSilva, L. L., J. P. Taylor, J. L. Hadlington, S. L. Hanton, C. J. Snowden, S. J. Fox, O. Foresti, F. Brandizzi and J. Denecke (2005). "Receptor salvage from the prevacuolar compartment is essential for efficient vacuolar protein targeting." Plant Cell **17**(1): 132-48.

daSilva, L. L. P., O. Foresti and J. Denecke (2006). "Targeting of the Plant Vacuolar Sorting Receptor BP80 Is Dependent on Multiple Sorting Signals in the Cytosolic Tail." Plant Cell **18**(6): 1477-1497.

de Figueiredo, P., R. S. Polizotto, D. Drecktrah and W. J. Brown (1999). "Membrane tubule-mediated reassembly and maintenance of the Golgi complex is disrupted by phospholipase A2 antagonists." Mol. Biol. Cell **10**(6): 1763-82.

Debeaujon, I., K. M. Leon-Kloosterziel and M. Koornneef (2000). "Influence of the Testa on Seed Dormancy, Germination, and Longevity in Arabidopsis." Plant Physiol. **122**(2): 403-414.

Dell'Angelica, E. C., C. Mullins, S. Caplan and J. S. Bonifacino (2000). "Lysosome-related organelles." FASEB J. **14**(10): 1265-1278.

Denecke, J., R. De Rycke and J. Botterman (1992). "Plant and mammalian sorting signals for protein retention in the endoplasmic reticulum contain a conserved epitope." EMBO J. **11**(6): 2345-55.

Deshaies, R. J. and R. Schekman (1987). "A yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum." J. Cell Biol. **105**(2): 633-645.

Di Sansebastiano, G. P., N. Paris, S. Marc-Martin and J. M. Neuhaus (1998). "Specific accumulation of GFP in a non-acidic vacuolar compartment via a C-terminal propeptide-mediated sorting pathway." Plant J. **15**(4): 449-57.

Di Sansebastiano, G. P., N. Paris, S. Marc-Martin and J. M. Neuhaus (2001). "Regeneration of a lytic central vacuole and of neutral peripheral vacuoles can be

visualized by green fluorescent proteins targeted to either type of vacuoles." Plant Physiol. **126**(1): 78-86.

Dittmer, F., E. J. Ulbrich, A. Hafner, W. Schmahl, T. Meister, R. Pohlmann and K. von Figura (1999). "Alternative mechanisms for trafficking of lysosomal enzymes in mannose 6-phosphate receptor-deficient mice are cell type-specific." J. Cell Sci. **112**(10): 1591-1597.

Dombrovski, J. E., M. R. Schroeder, S. Y. Bednarek and N. V. Raikhel (1993). "Determination of the functional elements within the vacuolar targeting signal of barley lectin." Plant Cell **5**: 587-596.

Duden, R. (2003). "ER-to-Golgi transport: COP I and COP II function (Review)." Mol. Membr. Biol. **20**(3): 197-207.

Dunkley, T. P. J., S. Hester, I. P. Shadforth, J. Runions, T. Weimar, S. L. Hanton, J. L. Griffin, C. Bessant, F. Brandizzi, C. Hawes, R. B. Watson, P. Dupree and K. S. Lilley (2006). "Mapping the Arabidopsis organelle proteome." Proc. Natl. Acad. Sci. U.S.A **103**(17): 6518-6523.

Ellgaard, L. and A. Helenius (2003). "Quality control in the endoplasmic reticulum." Nat Rev Mol Cell Biol **4**(3): 181-91.

Ellgaard, L., M. Molinari and A. Helenius (1999). "Setting the Standards: Quality Control in the Secretory Pathway." Science **286**(5446): 1882-1888.

Fewell, S. W., K. J. Travers, J. S. Weissman and J. L. Brodsky (2001). "The action of molecular chaperones in the early secretory pathway." Annu. Rev. Genet. **35**(1): 149-191.

Flanagan, J. J. and C. Barlowe (2006). "Cysteine-Disulfide Cross-linking to Monitor SNARE Complex Assembly during Endoplasmic Reticulum-Golgi Transport." J. Biol. Chem. **281**(4): 2281-2288.

Flückiger, R., M. De Caroli, G. Piro, G. Dalessandro, J. M. Neuhaus and G. P. Di Sansebastiano (2003). "Vacuolar system distribution in Arabidopsis tissues, visualized using GFP fusion proteins." J. Exp. Bot. **54**(387): 1577-84.

Fotin, A., Y. Cheng, P. Sliz, N. Grigorieff, S. C. Harrison, T. Kirchhausen and T. Walz (2004). "Molecular model for a complete clathrin lattice from electron cryomicroscopy." Nature **432**(7017): 573-9.

Frand, A. R., J. W. Cuozzo and C. A. Kaiser (2000). "Pathways for protein disulphide bond formation." Trends Cell Biol. **10**(5): 203-10.

Frigerio, L., M. De Virgilio, A. Prada, F. Faoro and A. Vitale (1998). "Sorting of phaseolin to the vacuole is saturable and requires a short C-terminal peptide." Plant Cell **10**(6): 1031-1042.

Frigerio, L., O. Foresti, D. Hernández Felipe, N. Paris, J.-M. Neuhaus and A. Vitale (2001a). "The C-terminal tetrapeptide of phaseolin is sufficient to target green fluorescent protein to the vacuole." Journal of Plant Physiology **158**: 499-503.

Frigerio, L., N. A. Jolliffe, A. Di Cola, D. H. Felipe, N. Paris, J. M. Neuhaus, J. M. Lord, A. Ceriotti and L. M. Roberts (2001b). "The internal propeptide of the ricin precursor carries a sequence-specific determinant for vacuolar sorting." Plant Physiol. **126**(1): 167-75.

Galili, G., C. Sengupta-Gopalan and A. Ceriotti (1998). "The endoplasmic reticulum of plant cells and its role in protein maturation and biogenesis of oil bodies." Plant Mol. Biol. **38**(1-2): 1-29.

Gaynor, E. C., C. Y. Chen, S. D. Emr and T. R. Graham (1998a). "ARF is required for maintenance of yeast Golgi and endosome structure and function." Mol. Biol. Cell **9**(3): 653-70.

Gaynor, E. C., T. R. Graham and S. D. Emr (1998b). "COPI in ER/Golgi and intra-Golgi transport: do yeast COPI mutants point the way?" Biochim. Biophys. Acta **1404**(1-2): 33-51.

Gaynor, E. C., S. te Heesen, T. R. Graham, M. Aebi and S. D. Emr (1994). "Signal-mediated retrieval of a membrane protein from the Golgi to the ER in yeast." J. Cell Biol. **127**(3): 653-65.

Ghosh, P., N. M. Dahms and S. Kornfeld (2003). "Mannose 6-phosphate receptors: new twists in the tale." Nature Reviews Molecular Cell Biology **4**(3): 202-213.

Glick, B. S. and V. Malhotra (1998). "The Curious Status of the Golgi Apparatus." Cell **95**(7): 883-889.

Glickman, M. H. and A. Ciechanover (2002). "The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction." Physiol. Rev. **82**(2): 373-428.

Goldberg, J. (1998). "Structural Basis for Activation of ARF GTPase: Mechanisms of Guanine Nucleotide Exchange and GTP-Myristoyl Switching." Cell **95**(2): 237-248.

Gomord, V., L. A. Denmat, A. C. Fitchette-Laine, B. Satiat-Jeunemaitre, C. Hawes and L. Faye (1997). "The C-terminal HDEL sequence is sufficient for retention of secretory proteins in the endoplasmic reticulum (ER) but promotes vacuolar targeting of proteins that escape the ER." Plant J. **11**(2): 313-25.

Gomord, V. and L. Faye (1996). "Signals and mechanisms involved in intracellular transport of secreted proteins in plants." Plant Physiol. Biochem. **34**(2): 165-181.

Görlich, D. and T. A. Rapoport (1993). "Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane." Cell **75**(4): 615-30.

Gottesman, S., S. Wickner and M. R. Maurizi (1997). "Protein quality control: triage by chaperones and proteases." Genes Dev. **11**(7): 815-23.

Groenendyk, J. and M. Michalak (2005). "Endoplasmic reticulum quality control and apoptosis." Acta Biochim Pol **52**(2): 381-95.

Gu, F., C. M. Crump and G. Thomas (2001). "Trans-Golgi network sorting." Cell. Mol.Life Sc. (CMLS) **58**(8): 1067-1084.

Gu, F. and J. Gruenberg (1999). "Biogenesis of transport intermediates in the endocytic pathway." FEBS Lett. **452**(1-2): 61-66.

Guivarch, A., J. C. Caissard, A. Azmi, T. Elmayan, D. Chriqui and M. Tepfer (1996). "In situ detection of expression of the gus reporter gene transgenic plants - ten years of blue genes." Transgenic Res. **5**(5): 281-288.

Haas, I. G. and M. Wabl (1983). "Immunoglobulin heavy chain binding protein." Nature **306**(5941): 387-9.

Hadlington, J. L. and J. Denecke (2000). "Sorting of soluble proteins in the secretory pathway of plants." Curr. Opin. Plant Biol. **3**(6): 461-468.

Hammond, C., I. Braakman and A. Helenius (1994). "Role of N-Linked Oligosaccharide Recognition, Glucose Trimming, and Calnexin in Glycoprotein Folding and Quality Control." Proc. Natl. Acad. Sci. U.S.A **91**(3): 913-917.

Hanley-Bowdoin, L., S. B. Settlage , B. M. Orozco and S. Nagar (1999). "Geminiviruses: Models for Plant DNA Replication, Transcription, and Cell Cycle Regulation." Critical Reviews in Plant Sciences
18(1): 71-106.

Hannon, G. J. (2002). "RNA interference." Nature **418**(6894): 244-51.

Hanton, S. L., L. E. Bortolotti, L. Renna, G. Stefano and F. Brandizzi (2005). "Crossing the divide--transport between the endoplasmic reticulum and Golgi apparatus in plants." Traffic **6**(4): 267-77.

Happel, N., S. Honing, J.-M. Neuhaus, N. Paris, D. G. Robinson and S. E. H. Holstein (2004). "ArabidopsisµA-adaptin interacts with the tyrosine motif of the vacuolar sorting receptor VSR-PS1." Plant J. **37**(5): 678-693.

Hara-Nishimura, I., T. Shimada, K. Hatano, Y. Takeuchi and M. Nishimura (1998). "Transport of Storage Proteins to Protein Storage Vacuoles Is Mediated by Large Precursor-Accumulating Vesicles." Plant Cell **10**(5): 825-836.

Harris, N. (1986). "Organization of the endomembrane system." Ann Rev Plant Physiol **37**: 73-92.

Hartmann, E., T. A. Rapoport and H. F. Lodish (1989). "Predicting the Orientation of Eukaryotic Membrane-Spanning Proteins." PNAS **86**(15): 5786-5790.

Hasenfratz, M. P., J. M. Jetsch, M. Michalak and F. Durst (1997). "Cloning and characterization of a wounding-induced analog of the chaperone calnexin from Helianthus tuberosus. ." Plant Physiol. Biochem. **35**: 553-564.

Hawes, C. (2005). "Cell biology of the plant Golgi apparatus." New Phyt. **165**(1): 29-44.

Hawes, C. and B. Satiat-Jeunemaitre (2005). "The plant Golgi apparatus--Going with the flow." Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1744**(2): 93-107.

Helenius, A. and M. Aebi (2004). "Roles of N-linked glycans in the endoplasmic reticulum." Annu Rev Biochem **73**: 1019-49.

Herman, E. M. and B. A. Larkins (1999). "Protein storage bodies and vacuoles." Plant Cell **11**(4): 601-14.

High, S. and B. Dobberstein (1991). "The signal sequence interacts with the methionine-rich domain of the 54-kD protein of signal recognition particle." J. Cell Biol. **113**(2): 229-33.

Hiller, M. M., A. Finger, M. Schweiger and D. H. Wolf (1996). "ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway." Science **273**(5282): 1725-8.

Hillmer, S., A. Movafeghi, D. G. Robinson and G. Hinz (2001). "Vacuolar Storage Proteins Are Sorted in the cis-Cisternae of the Pea Cotyledon Golgi Apparatus." J. Cell Biol. **152**(1): 41-50.

Hinz, G., S. Hillmer, M. Baumer and I. Hohl (1999). "Vacuolar Storage Proteins and the Putative Vacuolar Sorting Receptor BP-80 Exit the Golgi Apparatus of Developing Pea Cotyledons in Different Transport Vesicles." Plant Cell **11**(8): 1509-1524.

Hinz, G., A. Menze, I. Hohl and D. Vaux (1997). "Isolation of prolegumin from developing pea seeds - its binding to endomembranes and assembly into prolegumin hexamers in the protein storage vacuole." J. Exp. Bot. **48**(306): 139-149.

Hirsch, C., E. Jarosch, T. Sommer and D. H. Wolf (2004). "Endoplasmic reticulum-associated protein degradation--one model fits all?" Biochim. Biophys. Acta **1695**(1-3): 215-23.

Hirst, J., W. W. Lui, N. A. Bright, N. Totty, M. N. Seaman and M. S. Robinson (2000). "A family of proteins with gamma-adaptin and VHS domains that facilitate trafficking between the trans-Golgi network and the vacuole/lysosome." J. Cell Biol. **149**(1): 67-80.

Höfte, H. and M. J. Chrispeels (1992). "Protein sorting to the vacuolar membrane." Plant Cell **4**: 995-1004.

Hoh, B., G. Hinz, B. K. Jeong and D. G. Robinson (1995). "Protein storage vacuoles form de novo during pea cotyledon development." J. Cell Sci. **108**(1): 299-310.

Holwerda, B. C., N. J. Galvin, T. J. Baranski and J. C. Rogers (1990). "In Vitro Processing of Aleurain, a Barley Vacuolar Thiol Protease." Plant Cell **2**(11): 1091-1106.

Holwerda, B. C., H. S. Padgett and J. C. Rogers (1992). "Proaleurain Vacuolar Targeting Is Mediated by Short Contiguous Peptide Interactions." Plant Cell **4**(3): 307-318.

Hong, W. (2005). "SNAREs and traffic." Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1744**(2): 120-144.

Horazdovsky, B. F., D. B. DeWald and S. D. Emr (1995). "Protein transport to the yeast vacuole." Curr. Opin. Cell Biol. **7**(4): 544-51.

Huang, L., A. E. Franklin and N. E. Hoffman (1993). "Primary structure and characterization of an Arabidopsis thaliana calnexin-like protein." J. Biol. Chem. **268**(9): 6560-6.

Ibba, M. and D. Söll (1999). "Quality Control Mechanisms During Translation." Science **286**(5446): 1893-1897.

Jackson, D. (1991). In-situ hybridization in plants. in Molecular Plant Pathology: A Practical Approach oxford.

Jackson, M. R., T. Nilsson and P. A. Peterson (1993). "Retrieval of transmembrane proteins to the endoplasmic reticulum." J. Cell Biol. **121**(2): 317-333.

Jakob, U., H. Lilie, I. Meyer and J. Buchner (1995). "Transient interaction of Hsp90 with early unfolding intermediates of citrate synthase. Implications for heat shock in vivo." J. Biol. Chem. **270**(13): 7288-94.

Jauh, G.-Y., A. M. Fischer, H. D. Grimes, C. A. Ryan, Jr. and J. C. Rogers (1998). "delta -Tonoplast intrinsic protein defines unique plant vacuole functions." Proc. Natl. Acad. Sci. U.S.A **95**(22): 12995-12999.

Jauh, G.-Y., T. E. Phillips and J. C. Rogers (1999). "Tonoplast Intrinsic Protein Isoforms as Markers for Vacuolar Functions." Plant Cell **11**(10): 1867-1882.

Jiang, L., T. E. Phillips, C. A. Hamm, Y. M. Drozdowicz, P. A. Rea, M. Maeshima, S. W. Rogers and J. C. Rogers (2001). "The protein storage vacuole: a unique compound organelle." J. Cell Biol. **155**(6): 991-1002.

Jiang, L., T. E. Phillips, S. W. Rogers and J. C. Rogers (2000). "Biogenesis of the Protein Storage Vacuole Crystalloid." J. Cell Biol. **150**(4): 755-770.

Jiang, L. and J. C. Rogers (1999). "Sorting of membrane proteins to vacuoles in plant cells." Plant Cell **146**(2): 55-67.

Johnson, K. F. and S. Kornfeld (1992). "The cytoplasmic tail of the mannose 6-phosphate/insulin-like growth factor-II receptor has two signals for lysosomal enzyme sorting in the Golgi." J. Cell Biol. **119**(2): 249-57.

Johnson, L. M., V. A. Bankaitis and S. D. Emr (1987). "Distinct sequence determinants direct intracellular sorting and modification of a yeast vacuolar protease." Cell **48**(5): 875-885.

Jolliffe, N. A., J. C. Brown, U. Neumann, M. Vicre, A. Bachi, C. Hawes, A. Ceriotti, L. M. Roberts and L. Frigerio (2004). "Transport of ricin and 2S albumin precursors to the storage vacuoles of Ricinus communis endosperm involves the Golgi and VSR-like receptors." Plant J. **39**(6): 821-33.

Jolliffe, N. A., C. P. Craddock and L. Frigerio (2005). "Pathways for protein transport to seed storage vacuoles." Biochem. Soc. Trans. **33**(Pt 5): 1016-8.

Jones, E. W. (1977). "Proteinase mutants of Saccharomyces cerevisae." Genetics **85**(1): 23-33.

Jones, E. W., G. S. Zubenko and R. R. Parker (1982). "PEP4 gene function is required for expression of several vacuolar hydrolases in saccharomyces cerevisiae." Genetics **102**(4): 665-677.

Jurgens, G. (2004). "Membrane trafficking in plants." Annu. Rev. Cell Dev. Biol. **20**(1): 481-504.

Kirchhausen, T. (1999). "Adaptors for clathrin-mediated traffic." Annu. Rev. Cell Dev. Biol. **15**(1): 705-732.

Kirchhausen, T. (2000a). "Clathrin." Annu. Rev. Biochem. **69**: 699-727.

Kirchhausen, T. (2000b). "Three ways to make a vesicle." Nat Rev Mol Cell Biol **1**(3): 187-98.

Kirsch, T., N. Paris, J. M. Butler, L. Beevers and J. C. Rogers (1994). "Purification and Initial Characterization of a Potential Plant Vacuolar Targeting Receptor." Proc. Natl. Acad. Sci. U.S.A **91**(8): 3403-3407.

Kirsch, T., G. Saalbach, N. V. Raikhel and L. Beevers (1996). "Interaction of a potential vacuolar targeting receptor with amino- and carboxyl-terminal targeting determinants." Plant Physiol. **111**(2): 469-474.

Kjemtrup, S., K. S. Sampson, C. G. Peele, L. V. Nguyen, M. A. Conkling, W. F. Thompson and D. Robertson (1998). "Gene silencing from plant DNA carried by a Geminivirus." Plant J. **14**(1): 91-100.

Klionsky, D. J., P. K. Herman and S. D. Emr (1990). "The fungal vacuole: composition, function, and biogenesis." Microbiol. Mol. Biol. Rev. **54**(3): 266-292.

Koide, Y., H. Hirano, K. Matsuoka and K. Nakamura (1997). "The N-terminal propeptide of the precursor to sporamion acts as a vacuole-targeting signal even at the C-terminus of the mature part in tobacco cells." Plant Physiol. **114**: 863-870.

Krause, K. H. and M. Michalak (1997). "Calreticulin." Cell **88**(4): 439-43.

Kuehn, M. J. and R. Schekman (1997). "COPII and secretory cargo capture into transport vesicles." Curr. Opin. Cell Biol. **9**(4): 477-483.

Ladinsky, M. S., D. N. Mastronarde, J. R. McIntosh, K. E. Howell and L. A. Staehelin (1999). "Golgi structure in three dimensions: functional insights from the normal rat kidney cell." J. Cell Biol. **144**(6): 1135-49.

Laemmli, U. K. (1972). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature **277**: 680-685.

Lanoix, J., J. Ouwendijk, A. Stark, E. Szafer, D. Cassel, K. Dejgaard, M. Weiss and T. Nilsson (2001). "Sorting of Golgi resident proteins into different subpopulations of COPI vesicles: a role for ArfGAP1." J. Cell Biol. **155**(7): 1199-1212.

Laufs, J., I. Jupin, C. David, S. Schumacher, F. Heyraud-Nitschke and B. Gronenborn (1995). "Geminivirus replication: genetic and biochemical characterization of Rep protein function, a review." Biochimie **77**(10): 765-73.

Laval, V., M. Chabannes, M. Carriere, H. Canut, A. Barre, P. Rouge, R. Pont-Lezica and J. Galaud (1999). "A family of Arabidopsis plasma membrane receptors presenting animal beta-integrin domains." Biochim. Biophys. Acta **1435**(1-2): 61-70.

Laval, V., F. Masclaux, A. Serin, M. Carriere, C. Roldan, M. Devic, R. F. Pont-Lezica and J.-P. Galaud (2003). "Seed germination is blocked in Arabidopsis putative vacuolar sorting receptor (atbp80) antisense transformants." J. Exp. Bot. **54**(381): 213-221.

Lefrancois, S., Canuel M., Zeng J., and Morales C. R. (2005). "Inactivation of sortilin (a novel lysosomal sorting receptor) by dominant negative competition and RNA interference." Biol. Proced. Online **7**(1): 17-25.

Letourneur, F., E. C. Gaynor, S. Hennecke, C. Demolliere, R. Duden, S. D. Emr, H. Riezman and P. Cosson (1994). "Coatomer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum." Cell **79**(7): 1199-1207.

Levine, T. and C. Rabouille (2005). "Endoplasmic reticulum: one continuous network compartmentalized by extrinsic cues." Curr. Opin. Cell Biol. **17**(4): 362-368.

Li, Y.-B., S. W. Rogers, Y. C. Tse, S. W. Lo, S. S. M. Sun, G.-Y. Jauh and L. Jiang (2002). "BP-80 and Homologs are Concentrated on Post-Golgi, Probable Lytic Prevacuolar Compartments." Plant Cell Physiol. **43**(7): 726-742.

Lindahl, T. and R. D. Wood (1999). "Quality Control by DNA Repair." Science **286**(5446): 1897-1905.

Lodish, H., A. Berk, P. Matsudaira, C. A. Kaiser, M. Krieger, M. Scott, P., S. L. Zipursky and J. Darnell (2004). Molecular cell Biology. New York, W.H. Freemann and compagny.

Lord, M., J., J. Davey, L. Frigerio and L. M. Roberts (2000). "Endoplasmic reticulum-associated protein degradation." Semin. Cell Dev. Biol. **11**(3): 159-164.

Losev, E., C. A. Reinke, J. Jellen, D. E. Strongin, B. J. Bevis and B. S. Glick (2006). "Golgi maturation visualized in living yeast." Nature **441**(7096): 1002-6.

Malhotra, V. and M. P. Yaffe (2005). "Membranes and organelles: Regulating the size, shape, and plasticity of cellular compartments." Curr. Opin. Cell Biol. **17**(4): 343-344.

Marcusson, E. G., B. F. Horazdovsky, J. L. Cereghino, E. Gharakhanian and S. D. Emr (1994). "The sorting receptor for yeast vacuolar carboxypeptidase Y is encoded by the VPS10 gene." Cell **77**(4): 579-86.

Marra, P., T. Maffucci, T. Daniele, G. D. Tullio, Y. Ikehara, E. K. L. Chan, A. Luini, G. Beznoussenko, A. Mironov and M. A. De Matteis (2001). "The GM130 and GRASP65 Golgi proteins cycle through and define a subdomain of the intermediate compartment." Nat Cell Biol **3**(12): 1101-1113.

Marsh, B. J. and K. E. Howell (2002). "The mammalian Golgi--complex debates." Nat Rev Mol Cell Biol **3**(10): 789-95.

Martinez-Menarguez, J. A., R. Prekeris, V. M. J. Oorschot, R. Scheller, J. W. Slot, H. J. Geuze and J. Klumperman (2001). "Peri-Golgi vesicles contain retrograde but not anterograde proteins consistent with the cisternal progression model of intra-Golgi transport." J. Cell Biol. **155**(7): 1213-1224.

Marty-Mazars, D., M.-C. Clémencet, P. Cozolme and F. Marty (1995). "Antibodies to the tonoplast from the storage parenchyma cells of beetroot recognize a major intrinsic protein related to TIPs." Eur. J. Cell Biol. **66**: 106-118.

Marty, F. (1999). "Plant vacuoles." Plant Cell **11**(4): 587-600.

Maruyama, N., L. C. Mun, M. Tatsuhara, M. Sawada, M. Ishimoto and S. Utsumi (2006). "Multiple Vacuolar Sorting Determinants Exist in Soybean 11S Globulin." Plant Cell **18**(5): 1253-1273.

Matsuoka, K. (2000). "C-terminal propeptides and vacuolar sorting by BP-80-type proteins: not all C-terminal propeptides are equal." Plant Cell **12**(2): 181-2.

Matsuoka, K., D. C. Bassham, N. V. Raikhel and K. Nakamura (1995). "Different sensitivity to wortmannin of two vacuolar sorting signals indicates the presence of distinct sorting machineries in tobacco cells." J. Cell Biol. **130**(6): 1307-1318.

Matsuoka, K., S. Matsumoto, T. Hattori, Y. Machida and K. Nakamura (1990). "Vacuolar targeting and posttranslational processing of the precursor to the sweet potato tuberous root storage protein in heterologous plant cells." J. Biol. Chem. **265**(32): 19750-19757.

Matsuoka, K. and K. Nakamura (1991). "Propeptide of a precursor to a plant vacuolar protein required for vacuolar targeting." Proc.Natl. Acad. Sci.U.S.A. **88**(3): 834-838.

Matsuoka, K. and J.-M. Neuhaus (1999). "Cis-elements of protein transport to the plant vacuoles." J. Exp.Bot. **50**(331): 165-174.

Matsuura-Tokita, K., M. Takeuchi, A. Ichihara, K. Mikuriya and A. Nakano (2006). "Live imaging of yeast Golgi cisternal maturation." Nature **441**(7096): 1007-10.

McCarty, J. S., A. Buchberger, J. Reinstein and B. Bukau (1995). "The Role of ATP in the Functional Cycle of the DnaK Chaperone System." J. Mol. Biol. **249**(1): 126-137.

McClellan, A. J., M. D. Scott and J. Frydman (2005). "Folding and Quality Control of the VHL Tumor Suppressor Proceed through Distinct Chaperone Pathways." Cell **121**(5): 739-748.

Mellman, I. and K. Simons (1992). "The Golgi complex - In vitro veritas?" Cell **68**(5): 829-840.

Miller, E. A., M. C. S. Lee and M. A. Anderson (1999). "Identification and characterization of a prevacuolar compartment in stigmas of Nicotiana alata." Plant Cell **11**(8): 1499-1508.

Mironov, A. A., G. V. Beznoussenko, P. Nicoziani, O. Martella, A. Trucco, H.-S. Kweon, D. Di Giandomenico, R. S. Polishchuk, A. Fusella, P. Lupetti, E. G. Berger, W. J. C. Geerts, A. J. Koster, K. N. J. Burger and A. Luini (2001). "Small cargo proteins and large aggregates can traverse the Golgi by a common mechanism without leaving the lumen of cisternae." J. Cell Biol. **155**(7): 1225-1238.

Mironov, A. A., G. V. Beznoussenko, R. S. Polishchuk and A. Trucco (2005). "Intra-Golgi transport: A way to a new paradigm?" Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1744**(3): 340-350.

Mironov, A. A., P. Weidman and A. Luini (1997). "Variations on the intracellular transport theme - maturing cisternae and trafficking tubules." J. Cell Biol. **138**(3): 481-484.

Munro, S. (2005). "The Golgi apparatus: defining the identity of Golgi membranes." Curr. Opin. Cell Biol. **17**(4): 395-401.

Munro, S. and H. R. Pelham (1986). "An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein." Cell **46**(2): 291-300.

Müntz, K. (1998). "Deposition of storage proteins." Plant Mol. Biol. **38**(1-2): 77-99.

Murphy, K. A., R. A. Kuhle, A. M. Fischer, A. M. Anterola and H. D. Grimes (2005). "The functional status of paraveinal mesophyll vacuoles changes in response to altered metabolic conditions in soybean leaves." Functional Plant Biology **32**(4): 335-344.

Nagai, M., T. Meerloo, T. Takeda and M. G. Farquhar (2003). "The Adaptor Protein ARH Escorts Megalin to and through Endosomes." Mol. Biol. Cell **14**(12): 4984-4996.

Nagar, S., T. J. Pedersen, K. M. Carrick, L. Hanley-Bowdoin and D. Robertson (1995). "A geminivirus induces expression of a host DNA synthesis protein in terminally differentiated plant cells." Plant Cell **7**(6): 705-19.

Nakamura, K., K. Matsuoka, F. Mukumoto and N. Watanabe (1993). "Processing and transport to the vacuole of a precursor to sweet potato sporamin in transformed tobacco cell line BY-2." J. Exp. Bot. **44**(Suppl.): 331-338.

Nakayama, K. and S. Wakatsuki (2003). "The structure and function of GGAs, the traffic controllers at the TGN sorting crossroads." Cell Struct. Funct. **28**(5): 431-42.

Napier, R. M., L. C. Fowke, C. Hawes, M. Lewis and H. R. Pelham (1992). "Immunological evidence that plants use both HDEL and KDEL for targeting proteins to the endoplasmic reticulum." J. Cell Sci. **102**(2): 261-271.

Nebenführ, A. (2002). "Vesicle traffic in the endomembrane system: a tale of COPs, Rabs and SNAREs." Curr. Opin. Plant Biol. **5**(6): 507-12.

Nebenführ, A. and L. A. Staehelin (2001). "Mobile factories: Golgi dynamics in plant cells." Trends Plant Sc. **6**(4): 160-167.

Neuhaus, J.-M. and N. Paris (2005). Plant vacuoles: from biogenesis to function. Plant endocytosis. J. Samaj, F. Baluska and D. Menzel. Berlin, Springer Verlag. **1:** 63-82.

Neuhaus, J.-M. and J. C. Rogers (1998). "Sorting of proteins to vacuoles in plant cells." Plant Mol. Biol. **38**(1 - 2): 127-144.

Neuhaus, J.-M., L. Sticher, F. Meins, Jr. and T. Boller (1991a). "A short C-terminal sequence is necessary and sufficient for the targeting of chitinases to the plant

vacuole." Proceedings of the National Academy of Sciences of the United States of America **88**(22): 10362-10366.

Neuhaus, J. M., M. Pietrzak and T. Boller (1994). "Mutation analysis of the C-terminal vacuolar targeting peptide of tobacco chitinase: low specificity of the sorting system, and gradual transition between intracellular retention and secretion into the extracellular space." Plant J. **5**(1): 45-54.

Neuhaus, J. M., L. Sticher, F. Meins, Jr. and T. Boller (1991b). "A short C-terminal sequence is necessary and sufficient for the targeting of chitinases to the plant vacuole." Proc. Natl. Acad. Sci. U. S. A. **88**(22): 10362-6.

Neumann, U., F. Brandizzi and C. Hawes (2003). "Protein transport in plant cells: in and out of the Golgi." Ann. Bot. (Lond.) **92**(2): 167-80.

Nickel, W. and F. T. Wieland (1998). "Biosynthetic protein transport through the early secretory pathway." Histochem. Cell Biol. **109**(5 - 6): 477-486.

Nielsen, M. S., P. Madsen, E. I. Christensen, A. Nykjaer, J. Gliemann, D. Kasper, R. Pohlmann and C. M. Petersen (2001). "The sortilin cytoplasmic tail conveys Golgi-endosome transport and binds the VHS domain of the GGA2 sorting protein." EMBO J. **20**(9): 2180-90.

Nothwehr, S. F., C. J. Roberts and T. H. Stevens (1993). "Membrane protein retention in the yeast Golgi apparatus: dipeptidyl aminopeptidase A is retained by a cytoplasmic signal containing aromatic residues." J. Cell Biol. **121**(6): 1197-1209.

Nothwehr, S. F. and T. H. Stevens (1994). "Sorting of membrane proteins in the yeast secretory pathway." J. Biol. Chem. **269**(14): 10185-10188.

Oh, H. J., X. Chen and J. R. Subjeck (1997). "hsp110 Protects Heat-denatured Proteins and Confers Cellular Thermoresistance." J. Biol. Chem. **272**(50): 31636-31640.

Oh, H. J., D. Easton, M. Murawski, Y. Kaneko and J. R. Subjeck (1999). "The Chaperoning Activity of hsp110. Identification of functional domains by use of targeted deletions." J. Biol. Chem. **274**(22): 15712-15718.

Okita, T. W. and J. C. Rogers (1996). "Compartmentation of Proteins in the Endomembrane System of Plant Cells." Annu Rev Plant Physiol Plant Mol Biol **47**: 327-350.

Oliver, J. D., H. L. Roderick, D. H. Llewellyn and S. High (1999). "ERp57 Functions as a Subunit of Specific Complexes Formed with the ER Lectins Calreticulin and Calnexin." Mol. Biol. Cell **10**(8): 2573-2582.

Olsen, G. J., H. Matsuda, R. Hagstrom and R. Overbeek (1994). "fastDNAmL: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood." Comput Appl Biosci **10**(1): 41-8.

Orci, L., D. J. Palmer, M. Ravazzola, A. Perrelet, M. Amherdt and J. E. Rothman (1993). "Budding from Golgi membranes requires the coatomer complex of non-clathrin coat proteins." Nature **362**(6421): 648-52.

Orci, L., M. Stamnes, M. Ravazzola, M. Amherdt, A. Perrelet, T. H. Sollner and J. E. Rothman (1997). "Bidirectional transport by distinct populations of copi-coated vesicles." Cell **90**(2): 335-349.

Osowska-Rogers, S., E. Swiezewska, B. Andersson and G. Dallner (1994). "The endoplasmic reticulum-Golgi system is a major site of plastoquinone synthesis in spinach leaves." Biochem. Biophys. Res. Commun. **205**(1): 714-21.

Otegui, M. S., Y. S. Noh, D. E. Martinez, M. G. Vila Petroff, L. A. Staehelin, R. M. Amasino and J. J. Guiamet (2005). "Senescence-associated vacuoles with intense proteolytic activity develop in leaves of Arabidopsis and soybean." Plant J. **41**(6): 831-44.

Paris, N. and J. M. Neuhaus (2002). "BP-80 as a vacuolar sorting receptor." Plant Mol. Biol. **50**(6): 903-14.

Paris, N. and J. C. Rogers (1996). "The role of receptors in targeting soluble proteins from the secretory pathway to the vacuole." Plant Physiology and Biochemistry **34**(2): 223-227.

Paris, N., S. W. Rogers, L. Jiang, T. Kirsch, L. Beevers, T. E. Phillips and J. C. Rogers (1997). "Molecular Cloning and Further Characterization of a Probable Plant Vacuolar Sorting Receptor." Plant Physiol. **115**(1): 29-39.

Paris, N., C. M. Stanley, R. L. Jones and J. C. Rogers (1996). "Plant Cells Contain Two Functionally Distinct Vacuolar Compartments." Cell **85**(4): 563-572.

Park, M., S. J. Kim, A. Vitale and I. Hwang (2004). "Identification of the Protein Storage Vacuole and Protein Targeting to the Vacuole in Leaf Cells of Three Plant Species." Plant Physiol. **134**(2): 625-639.

Park, M., D. Lee, G. J. Lee and I. Hwang (2005). "AtRMR1 functions as a cargo receptor for protein trafficking to the protein storage vacuole." J. Cell Biol. **170**(5): 757-67.

Parodi, A. J. (2000a). "Protein glucosylation and its role in protein folding." Annu. Rev. Biochem. **69**: 69-93.

Parodi, A. J. (2000b). "Role of N-oligosaccharide endoplasmic reticulum processing reactions in glycoprotein folding and degradation." Biochem. J. **348**(Part 1): 1-13.

Pearse, B. M. (1975). "Coated vesicles from pig brain: purification and biochemical characterization." J. Mol. Biol **97**: 93-98.

Pearse, B. M. F. and M. S. Robinson (1990). "Clathrin, Adaptors, and Sorting." Annu. Rev. Cell Biol. **6**(1): 151-171.

Peele, C., C. V. Jordan, N. Muangsan, M. Turnage, E. Egelkrout, P. Eagle, L. Hanley-Bowdoin and D. Robertson (2001). "Silencing of a meristematic gene using geminivirus-derived vectors." Plant J. **27**(4): 357-366.

Pelham, H. R. (1998). "Getting through the Golgi complex." Trends Cell Biol. **8**(1): 45-9.

Pelham, H. R., K. G. Hardwick and M. J. Lewis (1988). "Sorting of soluble ER proteins in yeast." EMBO J. **7**(6): 1757-62.

Pelham, H. R. B. (2001). "Traffic through the Golgi apparatus." J. Cell Biol. **155**(7): 1099-1102.

Pelham, H. R. B. and J. E. Rothman (2000). "The Debate about Transport in the Golgi--Two Sides of the Same Coin?" Cell **102**(6): 713-719.

Phillipson, B. A. and J. Denecke (1997). "Transport of type I and II membrane spanning proteins in the plant endomembrane system, in: conference on The plant secretory system." In Mechanism, Pathways and Applications in Biotechnology (University York).

Pimpl, P., A. Movafeghi, S. Coughlan, J. Denecke, S. Hillmer and D. G. Robinson (2000). "In Situ Localization and in Vitro Induction of Plant COPI-Coated Vesicles." Plant Cell **12**(11): 2219-2236.

Pimpl, P., J. P. Taylor, C. Snowden, S. Hillmer, D. G. Robinson and J. Denecke (2006). "Golgi-Mediated Vacuolar Sorting of the Endoplasmic Reticulum Chaperone BiP May Play an Active Role in Quality Control within the Secretory Pathway." Plant Cell **18**(1): 198-211.

Piper, R. C., A. A. Cooper, H. Yang and T. H. Stevens (1995). "VPS27 controls vacuolar and endocytic traffic through a prevacuolar compartment in Saccharomyces cerevisiae." J. Cell Biol. **131**(3): 603-617.

Plemper, R. K., S. Bohmler, J. Bordallo, T. Sommer and D. H. Wolf (1997). "Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation." Nature **388**(6645): 891-5.

Plemper, R. K. and D. H. Wolf (1999). "Retrograde protein translocation: ERADication of secretory proteins in health and disease." Trends Biochem. Sci. **24**(7): 266-70.

Preuss, M. L., J. Serna, T. G. Falbel, S. Y. Bednarek and E. Nielsen (2004). "The Arabidopsis Rab GTPase RabA4b Localizes to the Tips of Growing Root Hair Cells." Plant Cell **16**(6): 1589-1603.

Rambourg, A. and Y. Clermont (1990). "Three-dimensional electron microscopy: structure of the Golgi apparatus." Eur. J. Cell Biol. **51**(2): 189-200.

Rapiejko, P. J. and R. Gilmore (1997). "Empty site forms of the SRP54 and SR alpha GTPases mediate targeting of ribosome-nascent chain complexes to the endoplasmic reticulum." Cell **89**(5): 703-13.

Raymond, C. K., I. Howald-Stevenson, C. A. Vater and T. H. Stevens (1992). "Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E vps mutants." Mol. Biol. Cell **3**(12): 1389-1402.

Roberts, C. J., S. F. Nothwehr and T. H. Stevens (1992). "Membrane protein sorting in the yeast secretory pathway: evidence that the vacuole may be the default compartment." J. Cell Biol. **119**(1): 69-83.

Robinson, D. G., M. Baumer, G. Hinz and I. Hohl (1998a). "Vesicle transfer of storage proteins to the vacuole - the role of the Golgi apparatus and multivesicular bodies." J. Plant Physiol. **152**(6): 659-667.

Robinson, D. G., G. Hinz and S. E. H. Holstein (1998b). "The molecular characterization of transport vesicles." Plant Mol. Biol. **38**(1 - 2): 49-76.

Robinson, M. S. and J. S. Bonifacino (2001). "Adaptor-related proteins." Curr. Opin. Cell Biol. **13**(4): 444-453.

Rossanese, O. W., J. Soderholm, B. J. Bevis, I. B. Sears, J. O'Connor, E. K. Williamson and B. S. Glick (1999). "Golgi structure correlates with transitional endoplasmic reticulum organization in Pichia pastoris and Saccharomyces cerevisiae." J. Cell Biol. **145**(1): 69-81.

Rothman, J. E. (1994). "Mechanism of intracellular protein transport." Nature **372**(6501): 55-63.

Rothman, J. H. and T. H. Stevens (1986). "Protein sorting in yeast: Mutants defective in vacuole biogenesis mislocalize vacuolar proteins into the late secretory pathway." Cell **47**(6): 1041-1051.

Rothman, J. H., C. T. Yamashiro, P. M. Kane and T. H. Stevens (1989). "Protein targeting to the yeast vacuole." Trends Biochem. Sci. **14**(8): 347-350.

Rüdiger, S., A. Buchberger and B. Bukau (1997). "Interaction of Hsp70 chaperones with substrates." Nat. Struct. Biol. **4**(5): 342-9.

Rüdiger, S., J. Schneider-Mergener and B. Bukau (2001). "Its substrate specificity characterizes the DnaJ co-chaperone as a scanning factor for the DnaK chaperone." EMBO J. **20**(5): 1042-50.

Saalbach, G., R. Jung, G. Kunze, I. Saalbach, K. Adler and K. Muntz (1991). "Different legumin protein domains act as vacuolar targeting signals." Plant Cell **3**(7): 695-708.

Saalbach, G., M. Rosso and U. Schumann (1996). "The vacuolar targeting signal of the 2S albumin from Brazil nut resides at the C terminus and involves the C-terminal propeptide as an essential element." Plant Physiol. **112**(3): 975-985.

Sanderfoot, A. A., S. U. Ahmed, D. Marty-Mazars, I. Rapoport, T. Kirchhausen, F. Marty and N. V. Raikhel (1998). "A putative vacuolar cargo receptor partially colocalizes with atPEP12p on a prevacuolar compartment in *Arabidopsis* roots." Proc.Natl. Acad. Sci. U. S. A. **95**(17): 9920-9925.

Sanderfoot, A. A., F. F. Assaad and N. V. Raikhel (2000). "The Arabidopsis genome. An abundance of soluble N-ethylmaleimide-sensitive factor adaptor protein receptors." Plant Physiol. **124**(4): 1558-69.

Sanderfoot, A. A., V. Kovaleva, D. C. Bassham and N. V. Raikhel (2001). "Interactions between Syntaxins Identify at Least Five SNARE Complexes within the Golgi/Prevacuolar System of the Arabidopsis Cell." Mol. Biol. Cell **12**(12): 3733-3743.

Sanderfoot, A. A. and N. V. Raikhel (1999). "The specificity of vesicle trafficking: Coat proteins and SNAREs." Plant Cell **11**(4): 629-641.

Sanger, F., S. Nicklen and A. R. Coulson (1977). "DNA Sequencing with Chain-Terminating Inhibitors." PNAS **74**(12): 5463-5467.

Satiat-Jeunemaitre, B., L. Cole, T. Bourett, R. Howard and C. Hawes (1996). "Brefeldin A effects in plant and fungal cells: something new about vesicle trafficking?" J. Microsc. **181**(Pt 2): 162-77.

Scales, S. J., M Gomez, and T E Kreis (2000). "Coat proteins regulating membrane traffic." Int. Rev. Cytol. **195:**: 67–144.

Schekman, R., C. Barlowe, S. Bednarek, J. Campbell, T. Doering, R. Duden, M. Kuehn, M. Rexach, T. Yeung and L. Orci (1995). "Coat proteins and selective protein packaging into transport vesicles." Cold Spring Harb. Symp. Quant. Biol. **60**: 11-21.

Schekman, R. and L. Orci (1996). "Coat proteins and vesicle budding." Science **271**: 1526-1533.

Schmid, D., A. Baici, H. Gehring and P. Christen (1994). "Kinetics of molecular chaperone action." Science **263**(5149): 971-3.

Schrag, J. D., J. J. Bergeron, Y. Li, S. Borisova, M. Hahn, D. Y. Thomas and M. Cygler (2001). "The Structure of calnexin, an ER chaperone involved in quality control of protein folding." Mol. Cell **8**(3): 633-44.

Schutze, M. P., P. A. Peterson and M. R. Jackson (1994). "An N-terminal double-arginine motif maintains type II membrane proteins in the endoplasmic reticulum." EMBO J. **13**(7): 1696-705.

Shelness, G. S. and G. Blobel (1990). "Two subunits of the canine signal peptidase complex are homologous to yeast SEC11 protein." J. Biol. Chem. **265**(16): 9512-9519.

Shimada, T., M. Kuroyanagi, M. Nishimura and I. Hara-Nishimura (1997). "A pumpkin 72-kDa membrane protein of precursor-accumulating vesicles has characteristics of a vacuolar sorting receptor." Plant Cell Physiol. **38**(12): 1414-1420.

Shimada, T., E. Watanabe, K. Tamura, Y. Hayashi, M. Nishimura and I. Hara-Nishimura (2002). "A Vacuolar Sorting Receptor PV72 on the Membrane of Vesicles that Accumulate Precursors of Seed Storage Proteins (PAC Vesicles)." Plant Cell Physiol. **43**(10): 1086-1095.

Shimada, T., K. Yamada, M. Kataoka, S. Nakaune, Y. Koumoto, M. Kuroyanagi, S. Tabata, T. Kato, K. Shinozaki, M. Seki, M. Kobayashi, M. Kondo, M. Nishimura and I. Hara-Nishimura (2003). "Vacuolar Processing Enzymes Are Essential for Proper Processing of Seed Storage Proteins in Arabidopsis thaliana." J. Biol. Chem. **278**(34): 32292-32299.

Spang, A. (2002). "ARF1 regulatory factors and COPI vesicle formation." Curr. Opin. Cell Biol. **14**(4): 423-427.

Stack, J. H., B. Horazdovsky and S. D. Emr (1995). "Receptor-mediated protein sorting to the vacuole in yeast: roles for a protein kinase, a lipid kinase and GTP-binding proteins." Annu. Rev. Cell Dev. Biol. **11**: 1-33.

Staehelin, L. A. and I. Moore (1995). "The plant Golgi apparatus: Structure, functional organization and trafficking mechanisms." Annual Review of Plant Physiology and Plant Molecular Biology **46**(288): 261-288.

Steele, C., D. E. Evans, B. Satiat-Jeunemaitre and C. Hawes (1995). "The elucidation of intra-Golgi transport in plant cells by the application of a variety of Golgi-disrupting drugs." Proceeding RMS **30**: 36.

Stevens, T., B. Esmon and R. Schekman (1982). "Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole." Cell **30**(2): 439-448.

Sticher, L., U. Hinz, A. D. Meyer and F. Meins (1992). "Intracellular transport and processing of a tobacco vacuolar β-1,3-glucanase." Planta **188**(4): 559-565.

Storrie, B. and T. Nilsson (2002). "The Golgi apparatus: balancing new with old." Traffic **3**(8): 521-9.

Swanson, S. J., P. C. Bethke and R. L. Jones (1998). "Barley Aleurone Cells Contain Two Types of Vacuoles: Characterization of Lytic Organelles by Use of Fluorescent Probes." Plant Cell **10**(5): 685-698.

Swiezewska, E., G. Dallner, B. Andersson and L. Ernster (1993). "Biosynthesis of ubiquinone and plastoquinone in the endoplasmic reticulum-Golgi membranes of spinach leaves." J. Biol. Chem. **268**(2): 1494-9.

Tague, B. W., C. D. Dickinson and M. J. Chrispeels (1990). "A short domain of the plant vacuolar protein phytohemagglutinin targets invertase to the yeast vacuole." Plant Cell **2**(6): 533-46.

Taiz, L. (1992). "The Plant Vacuole." J. Exp. Biol. **172**(Pt 1): 113-122.

Takeuchi, M., T. Ueda, K. Sato, H. Abe, T. Nagata and A. Nakano (2000). "A dominant negative mutant of sar1 GTPase inhibits protein transport from the endoplasmic reticulum to the Golgi apparatus in tobacco and Arabidopsis cultured cells." Plant J. **23**(4): 517-25.

Tijsterman, M., R. F. Ketting and R. H. A. Plasterk (2002). "THE GENETICS OF RNA SILENCING." Annu. Rev. Genet. **36**(1): 489-519.

Traub, L. M. (2003). "Sorting it out: AP-2 and alternate clathrin adaptors in endocytic cargo selection." J. Cell Biol. **163**(2): 203-208.

Traub, L. M. (2005). "Common principles in clathrin-mediated sorting at the Golgi and the plasma membrane." Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1744**(3): 415-437.

Traub, L. M. and S. Kornfeld (1997). "The trans-Golgi network: a late secretory sorting station." Curr. Opin. Cell Biol. **9**(4): 527-533.

Tse, Y. C., B. Mo, S. Hillmer, M. Zhao, S. W. Lo, D. G. Robinson and L. Jiang (2004). "Identification of Multivesicular Bodies as Prevacuolar Compartments in Nicotiana tabacum BY-2 Cells." Plant Cell **16**(3): 672-693.

Turnage, M. A., N. Muangsan, C. G. Peele and D. Robertson (2002). "Geminivirus-based vectors for gene silencing in Arabidopsis." Plant J. **30**(1): 107-114.

Ueda, T., Yamaguchi M., Uchimiya H., and Nakano A. (2001). "Ara6, a plant-unique novel type Rab GTPase, functions in the endocytic pathway of Arabidopsis thaliana." EMBO J. **20**: 4730–4741.

Uemura, T., Takashi Ueda, Ryosuke L. Ohniwa, Akihiko Nakano, Kunio Takeyasu and Masa H. Sato (2004). "Systematic Analysis of SNARE Molecules in Arabidopsis: Dissection of the post-Golgi Network in Plant Cells." Cell Struct. Funct. **29**(2): 49-65

Valls, L. A., C. P. Hunter, J. H. Rothman and T. H. Stevens (1987). "Protein sorting in yeast: The localization determinant of yeast vacuolar carboxypeptidase Y resides in the propeptide." Cell **48**(5): 887-897.

Valls, L. A., J. R. Winther and T. H. Stevens (1990). "Yeast carboxypeptidase Y vacuolar targeting signal is defined by four propeptide amino acids." J. Cell Biol. **111**(2): 361-368.

Vernoud, V., A. C. Horton, Z. Yang and E. Nielsen (2003). "Analysis of the Small GTPase Gene Superfamily of Arabidopsis." Plant Physiol. **131**(3): 1191-1208.

Vertel, B. M., L. M. Walters and D. Mills (1992). "Subcompartments of the endoplasmic reticulum." Semin. Cell Biol. **3**(5): 325-41.

Vida, T. A., G. Huyer and S. D. Emr (1993). "Yeast vacuolar proenzymes are sorted in the late Golgi complex and transported to the vacuole via a prevacuolar endosome-like compartment." J. Cell Biol. **121**(6): 1245-1256.

Vitale, A., A. Ceriotti and J. Denecke (1993). "The role of the endoplasmic reticulum in protein synthesis, modification and intracellular transport." J Exp Bot **44**: 1417-1444.

Vitale, A. and M. J. Chrispeels (1992). "Sorting of Proteins to the Vacuoles of Plant Cells." Bioessays **14**(3): 151-160.

Vitale, A. and N. V. Raikhel (1999). "What do proteins need to reach different vacuoles?" Trends in Plant Science **4**(4): 149-155.

Voinnet, O. and D. C. Baulcombe (1997). "Systemic signalling in gene silencing." Nature **389**: 553.

von Heijne, G. (1986). "A new method for predicting signal sequence cleavage sites." Nucleic Acids Res. **14**(11): 4683-4690.

Von Heijne, G. (1988). "Transcending the impenetrable: how protein come to terms with membranes." Biochim. Biophys. Acta **947**: 307-333.

Von Schaaewen, A. and M. J. Chrispeels (1993). "Identification of vacuolar sorting information in phytohemagglutinin, an unprocessed vacuolar protein." J Exp Bot **44**: 339-342.

Wahlberg, J. M. and M. Spiess (1997). "Multiple determinants direct the orientation of signal-anchor proteins: the topogenic role of the hydrophobic signal domain." J. Cell Biol. **137**(3): 555-62.

Wang, T. F., J. H. Chang and C. Wang (1993). "Identification of the peptide binding domain of hsc70. 18-Kilodalton fragment located immediately after ATPase domain is sufficient for high affinity binding." J. Biol. Chem. **268**(35): 26049-26051.

Watanabe, E., T. Shimada, K. Tamura, R. Matsushima, Y. Koumoto, M. Nishimura and I. Hara-Nishimura (2004). "An ER-localized form of PV72, a seed-specific vacuolar sorting receptor, interferes the transport of an NPIR-containing proteinase in Arabidopsis leaves." Plant Cell Physiol. **45**(1): 9-17.

Waters, M. G., T. Serafini and J. E. Rothman (1991). "'Coatomer': a cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles." Nature **349**(6306): 248-251.

Watson, P. and D. J. Stephens (2005). "ER-to-Golgi transport: Form and formation of vesicular and tubular carriers." Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1744**(3): 304-315.

Weber, T., F. Parlati, J. A. McNew, R. J. Johnston, B. Westermann, T. H. Sollner and J. E. Rothman (2000). "SNAREpins are functionally resistant to disruption by NSF and alphaSNAP." J. Cell Biol. **149**(5): 1063-72.

Weigert, R., M. G. Silletta, S. Spano, G. Turacchio, C. Cericola, A. Colanzi, S. Senatore, R. Mancini, E. V. Polishchuk, M. Salmona, F. Facchiano, K. N. Burger, A. Mironov, A. Luini and D. Corda (1999). "CtBP/BARS induces fission of Golgi membranes by acylating lysophosphatidic acid." Nature **402**(6760): 429-33.

Weinberger, A., F. Kamena, R. Kama, A. Spang and J. E. Gerst (2005). "Control of Golgi morphology and function by Sed5 t-SNARE phosphorylation." Mol. Biol. Cell **16**(10): 4918-30.

Western, T. L., D. J. Skinner and G. W. Haughn (2000). "Differentiation of mucilage secretory cells of the Arabidopsis seed coat." Plant Physiology **122**(2): 345-355.

Wickner, S., M. R. Maurizi and S. Gottesman (1999). "Posttranslational Quality Control: Folding, Refolding, and Degrading Proteins." Science **286**(5446): 1888-1893.

Wiertz, E. J., D. Tortorella, M. Bogyo, J. Yu, W. Mothes, T. R. Jones, T. A. Rapoport and H. L. Ploegh (1996). "Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction." Nature **384**(6608): 432-8.

Wilcox, C. A., K. Redding, R. Wright and R. S. Fuller (1992). "Mutation of a tyrosine localization signal in the cytosolic tail of yeast Kex2 protease disrupts Golgi retention and results in default transport to the vacuole." Mol. Biol. Cell **3**(12): 1353-1371.

Wilsbach, K. and G. S. Payne (1993). "Dynamic retention of TGN membrane proteins in Saccharomyces cerevisiae." Trends Cell Biol. **3**(12): 426-432.

Wink, M. (1993). "The plant vacuole - A multifunctional compartment." J.Exp.Bot. **44**(Suppl.): 231-246.

Zeghouf, M., B. Guibert, J. C. Zeeh and J. Cherfils (2005). "Arf, Sec7 and Brefeldin A: a model towards the therapeutic inhibition of guanine nucleotide-exchange factors." Biochem. Soc. Trans. **33**(Pt 6): 1265-8.

Zhang, J.-X., I. Braakman, K. E. S. Matlack and A. Helenius (1997). "Quality Control in the Secretory Pathway: The Role of Calreticulin, Calnexin and BiP in the Retention of Glycoproteins with C-Terminal Truncations." Mol. Biol. Cell **8**(10): 1943-1954.

Zimmermann, P., M. Hirsch-Hoffmann, L. Hennig and W. Gruissem (2004). "GENEVESTIGATOR. Arabidopsis Microarray Database and Analysis Toolbox." Plant Physiol. **136**(1): 2621-2632.

ANNEX

1)Sequence of AtVSR1;1 (AT3g52850)

ATGAAGCT
TGGGCTTTTC ACTCTCTCGT TTCTTCTGAT CTTGAATCTA GCAATGGGTA GATTCGTTGT
TGAGAAGAAC AATCTCAAAG TTACATCACC TGATTCGATC AAAGGTATTT ACGAATGTGC
CATTGGTAAT TTCGGAGTTC CTCAATACGG TGGTACTTTA GTCGGCACCG TCGTCTATCC
TAAATCCAAT CAGAAAGCTT GTAAAAGCTA CTCCGATTTC GATATCTCCT TCAAATCCAA
ACCTGGACGA TTACCAACTT TTGTCCTTAT CGATCGTGGA GATTGTTACT TCACTTTGAA
AGCATGGATA GCTCAACAAG CTGGAGCAGC AGCGATACTT GTAGCTGATA GTAAAGCTGA
GCCATTGATT ACAATGGATA CACCTGAAGA AGATAAATCT GATGCGGATT ATCTTCAGAA
CATTACCATT CCTTCTGCTC TCATTACTAA AACATTGGGA GACAGTATAA AGTCTGCTCT
TTCCGGTGGT GATATGGTTA ACATGAAGCT AGATTGGACT GAGTCGGTTC CACATCCTGA
TGAGCGAGTA GAGTATGAAC TGTGGACTAA TAGCAATGAC GAGTGTGGGA AGAAGTGTGA
TACTCAGATT GAGTTTCTCA AGAATTTTAA AGGAGCTGCT CAGATTCTTG AGAAAGGTGG
GCATACTCAG TTCACGCCAC ATTATATTAC TTGGTACTGT CCTGAAGCGT TTACGTTGAG
TAAACAGTGT AAGTCTCAGT GCATCAACCA TGGAAGGTAT TGTGCGCCTG ATCCTGAGCA
GGATTTTACG AAAGGGTATG ATGGAAAGGA TGTTGTTGTT CAGAATCTAC GTCAGGCTTG
TGTCTACAGA GTGATGAATG ATACCGGTAA GCCGTGGGTC TGGTGGGACT ATGTGACTGA
CTTTGCTATC CGGTGTCCAA TGAAGGAGAA GAAGTACACC AAGGAATGCG CAGATGGAAT
TATTAAGTCC CTTGGCATTG ATCTCAAAAA GGTGGACAAG TGTATCGGAG ACCCTGAAGC
AGATGTGGAG AACCCAGTTC TTAAAGCAGA GCAGGAGTCA CAGATAGGCA AAGGTTCCCG
TGGAGACGTG ACTATACTCC CGGCTCTTGT CGTGAACAAC AGACAATACA GAGGTAAATT
GGAAAAGGGG GCAGTGCTTA AGGCTATGTG TTCGGGTTTT CAAGAGTCAA CGGAACCAGC
TATCTGTCTT ACTGAAGATT TGGAAACTAA TGAATGTTTG GAAAACAACG GTGGATGCTG
GCAAGACAAA GCTGCCAACA TTACTGCATG CAGGGATACT TTTAGGGGAA GATTGTGTGA
GTGCCCTACT GTTCAAGGTG TTAAATTCGT TGGTGACGGT TACACTCACT GCAAAGCCTC
TGGAGCTTTG CATTGTGGTA TCAACAATGG AGGATGCTGG AGAGAATCCC GAGGTGGCTT
CACTTACTCT GCTTGCGTAG ATGATCATTC AAAGGATTGC AAATGCCCAC TTGGGTTCAA
GGGCGATGGA GTGAAGAACT GTGAAGATGT GGACGAGTGC AAAGAAAAAA CGGTGTGCCA
GTGCCCAGAG TGTAAATGTA AAAACACTTG GGGAAGTTAT GAATGCAGCT GCAGCAACGG
TTTGCTTTAC ATGCGTGAGC ACGACACTTG CATAGGTTCA GGCAAAGTTG GAACCACAAA
ACTCAGCTGG AGCTTTTTGT GGATCCTTAT AATCGGGGTG GGTGTTGCAG GTCTTTCTGG
ATATGCAGTC TACAAATACA GAATCAGGAG TTACATGGAT GCGGAAATTA GAGGGATCAT
GGCACAGTAC ATGCCATTGG AAAGTCAACC ACCCAACACA AGTGGTCACC ATATGGATAT
ATGA

2) Sequence of AtVSR 1;2 (AT2g30290)

ATGAGGACGA CGAATGTATG GTTAGTTGTA ATAGTATGGG TAACGGTGGG GTGGAGTTCA
TGCACAGGGA GGTTCGTTGT GGAGAAGAAC AACCTCCGAG TGACTTCGCC GGAGTCCATC
AGAGGAGTCT ATGAATGTGC CCTCGGAAAT TTTGGCGTCC CTCAATACGG CGGAAGCATG
TCCGGTGCGG TGGTTTATCC TAAAACTAAT CAGAAAGCTT GCAAGAACTT TGACGATTTT
GAGATTTCTT TCAGATCCAG AGTCGCTGGA TTGCCCACAT TCGTTCTTGT GGATCGAGGA
GATTGTTACT TTACTTTGAA GGCGTGGAAT GCGCAACGAG CTGGTGCCGC AACCATCTTG
GTGGCGGATA ACAGACCCGA GCAACTCATC ACCATGGACG CACCAGAGGA TGAGACGTCA
GATGCAGATT ACCTACAAAA TATCACAATT CCTTCAGCAT TAGTGAGCAG ATCTCTAGGG
AGTGCCATCA AAACGGCCAT AGCTCATGGC GATCCCGTTC ATATAAGTCT AGACTGGCGG
GAGGCTCTTC CACATCCAAA CGATCGAGTA GCTTACGAGT TATGGACCAA CAGTAATGAT
GAATGCGGAT CCAAATGTGA TGCACAGATC CGGTTTCTTA AGAGGTTTAA AGGAGCTGCT
CAGATTCTTG AGAAAGGAGG CTACACTCGC TTCACTCCCC ATTACATCAC CTGGTATTGT
CCTGAAGCGT TTCTGGCAAG TAGACAATGT AAAACACAAT GCATTAATGG TGGAAGGTAT
TGTGCTCCGG ACCCTGAGCA AGATTTCTCC AGAGGATACA ATGGAAAAGA CGTAATTATT
CAGAATTTAC GCCAAGCTTG CTTCTTTAGA GTGACTAATG AAAGTGGAAA GCCTTGGCTT
TGGTGGGATT ATGTCACCGA CTTCGCCATT CGTTGTCCCA TGAAAGAGGA GAAGTACAAC
AAGAAATGTG CTGATCAAGT CATTCAATCT CTTGGAGTTG ATGTGAAGAA AATTGACAAA
TGCATCGGAG ACATTGACGC AAATGCTGAA AATCCTGTTC TTAAAGAAGA ACAAGTTGCA

CAAGTTGGGA AAGGCTCGAG AGGAGATGTG ACGATACTAC CAACTATTGT GATAAACAAC
AGACAATATA GAGGGAAATT GCAAAGATCG GCCGTGCTTA AGGCCCTTTG CTCAGGGTTT
CGTGAGACGA CGGAGCCACC CATTTGTTTA ACCGAAGACA TAGAAACCAA TGAGTGTTTA
CAAAACAATG GAGGGTGTTG GGAAGATAAA ACAACCAACA TTACAGCTTG CAGGGACACT
TTCAGAGGAA GAGTATGTCA ATGTCCCATT GTTCAAGGTG TCAAGTTTCT CGGTGACGGT
TATACACATT GTGAAGCTTC GGGGGCACTA CGTTGTGGCA TAAACAATGG AGGATGTTGG
AAACAAACTC AAATGGGAAA AACATATTCC GCTTGCCGTG ATGATCATTC GAAAGGCTGC
AAATGTCCTC CTGGATTCAT AGGGGATGGA CTCAAAGAGT GCAAAGATGT GAATGAGTGT
GAGGAGAAAA CAGCGTGCCA ATGTCGCGAT TGCAAATGCA AAAACACATG GGGAAGCTAT
GAATGTAGTT GCAGCGGAAG CTTGCTTTAC ATAAGAGAAC ACGACATTTG CATAAATAGA
GATGCAAGAG GAGATTTCAG TTGGGGAGTG ATATGGATAA TAATAATGGG ATTAGGTGCA
GCTGCTTTAG GAGCTTACAC TGTTTATAAA TACAGAATTC GGACATATAT GGACTCAGAG
ATAAGAGCTA TAATGGCACA ATACATGCCT CTTGATAATA ATCCCAATAC TCAACTTTCT
TCTCAACTAG AGTTGTAA

3) Sequence of AtVSR2 ;1 (AT2g14720)

ATGAAGCA GCTTCTGTGT TATCTTCCAT GGCTGCTTCT TCTCTCTCTT
GTGGTTTCCC CTTTTAGCGA GGCTAGATTC GTTGTGAGTA ATGAGAAGAA TAGTTTGTCG
GTGACGTCGC CGGAGAGTAT AAAAGGAACA CATGATAGTG CAATTGGTAA CTTCGGGATT
CCTCAATACG GTGGAAGTAT GGCTGGTACG GTGGTTTATC CGAAAGAGAA TCAGAAATCG
TGTAAGGAAT TTAGCGATTT CTCGATTTCG TTCAAGTCTC AGCCTGGTGC TTTACCTACT
TTCCTCTTAG TTGATCGTGG AGATTGTTTC TTCGCTTTGA AGGTATGGAA CGCACAGAAA
GCAGGTGCTT CTGCTGTTCT TGTGGCTGAT AATGTTGATG AGCCTTTGAT TACAATGGAT
ACACCTGAAG AAGATGTTTC TTCTGCAAAG TATATCGAGA ATATTACTAT ACCTTCTGCT
CTTGTTACTA AAGGTTTTGG TGAAAAGCTG AAGCAAGCTA TTAGTGGAGG TGATATGGTT
AACTTGAATC TTGACTGGAG AGAGGCTGTT CCACATCCTG ATGACCGTGT TGAGTATGAG
TTGTGGACTA ATAGTAATGA TGAATGTGGG GTTAAGTGTG ATATGTTGAT GGAGTTTGTG
AAGGATTTTA AGGGAGCGGC GCAGATTCTT GAGAAAGGCG GTTTTACGCA GTTTAGGCCT
CATTATATTA CTTGGTATTG TCCTCATGCT TTCACGTTGA GTCGACAGTG TAAGTCTCAG
TGTATCAATA AAGGAAGGTA CTGTGCTCCT GATCCAGAGC AGGACTTTAG CTCGGGATAC
GATGGAAAAG ACGTGGTTGT GGAAAATTTG AGACAGCTTT GTGTTTACAA GGTGGCGAAT
GAAACCGGCA AACCTTGGGT CTGGTGGGAT TATGTTACTG ATTTCCAGAT CAGATGTCCA
ATGAAGGAGA AAAAATACAA CAAAGATTGT GCTGAGTCTG TAATCAAATC TCTTGGAATC
GATAGCAGAA AAATTGACAA GTGTATGGGA GACCCTGATG CTGACTTGGA CAATCCAGTT
TTAAAGGAAG AACAAGATGC TCAAGTTGGC AAGGGTACAA GGGGTGATGT TACCATATTG
CCTACCTTAG TTGTCAACAA CAGACAGTAC CGAGGCAAGT TGGAGAAGAG TGCAGTACTC
AAGGCTCTAT GCTCTGGTTT TGAGGAGTCA ACTGAACCAG CTATATGCCT CAGCACAGAT
ATGGAGACAA ACGAGTGCTT AGATAACAAT GGCGGTTGTT GGCAAGATAA ATCAGCCAAC
ATAACTGCTT GCAAGGATAC GTTTCGTGGA AAAGTATGCG TGTGTCCTAT AGTTGATGGT
GTGCGATTCA AAGGAGATGG TTACAGCCAC TGTGAGCCAA GCGGGCCAGG GAGATGTACA
ATCAACAATG GAGGTTGTTG GCATGAAGAG AGAGATGGAC ATGCGTTCTC TGCTTGTGTG
GACAAGGACA GTGTGAAATG CGAGTGTCCT CCAGGATTTA AAGGAGACGG TGTTAAGAAA
TGTGAAGACA TCAATGAGTG CAAAGAGAAG AAAGCATGTC AGTGCCCGGA ATGTAGCTGT
AAGAACACCT GGGGAAGCTA TGAGTGCTCT TGTAGCGGGG ACCTTCTCTA CATGAGAGAC
CATGACACTT GCATCAGCAA GACGGGTTCA CAAGTGAAAT CAGCGTGGGC GGGCGTTTGG
CTTATAATGT TATCATTGGG ACTTGCAGCT GCTGGTGCAT ACCTCGTTTA CAAATATAGA
TTGAGGCAAT ACATGGACTC AGAGATCAGA GCCATAATGG CACAGTACAT GCCACTGGAC
AGCCAACCCG AGGTCCCGAA CCACACGAAT GATGAACGTG CCTAA

4) Sequence of AtVSR2;2 (AT2g14740)

ATGAAGCAGC TTCTGTGTTA CCTTCCATGG CTGCTTCTCC TCACTCTTCT GGTTTCCCCT
TTAAACGACG CTCGATTCGT GGTGGAGAAG AACAGTTTGT CCGTGACGTC GCCGGAGAGT
ATAAAAGGAA CTCATGATAG TGCAATTGGT AACTTCGGGA TTCCTCAATA CGGTGGAAGT
ATGGCTGGTA CGGTGGTTTA TCCGAAAGAG AATCAGAAAT CGTGTAAGGA ATTTAGCGAT
TTCTCGATTT CGTTCAAGTC TCAGCCTGGT GCTTTACCTA CTTTCCTCTT AGTTGATCGT
GGAGATTGTT TCTTCGCTTT GAAGGTATGG AACGCACAGA AAGCAGGTGC TTCTGCTGTT
CTTGTAGCTG ATAATGTTGA TGAACCTTTG ATTACAATGG ATACACCTGA AGAAGATGTT
TCTTCTGCAA AGTATATTGA GAATATTACT ATACCTTCTG CTCTTGTTAC TAAAGGTTTT
GGTGAAAAGC TGAAGAAAGC TATTAGTGGA GGAGATATGG TTAACTTGAA TCTTGACTGG
AGAGAGGCTG TTCCGCATCC TGATGACCGT GTTGAGTATG AGTTGTGGAC TAATAGTAAT
GATGAATGTG GGGTTAAGTG TGATATGTTG ATGGAGTTTG TGAAAGATTT TAAGGGAGCG
GCGCAGATTC TTGAGAAAGG CGGGTTTACG CAGTTTAGAC CTCATTATAT TACTTGGTAT
TGTCCTCATG CTTTCACGTT GAGTCGACAG TGTAAGTCTC AGTGTATCAA TAAAGGAAGG
TACTGTGCTC CTGATCCAGA GCAGGACTTT AGCTCGGGAT ACGATGGAAA AGATGTGGTC

GTGGAAAACT TGAGACAGCT TTGTGTTTAC AAGGTGGCGA ATGAAACCGG CAAACCTTGG
GTCTGGTGGG ATTATGTTAC TGATTTCCAG ATCAGATGTC CCATGAAGGA GAAGAAATAC
AATAAGGAGT GTGCTGATTC CGTTATCAAA TCTCTTGGAA TTGATAGTAA AAAACTTGAC
AAGTGTATGG GAGACCCTGA TGCTGACTTG GACAATCCAG TTCTTAAGGA AGAACAAGAT
GCTCAAGTTG GCAAGGGTTC AAGGGGTGAT GTTACCATAT TGCCTACCTT GGTTGTCAAC
AACAGACAGT ACCGAGGAAA GTTGGAGAAG AGTGCAGTAC TCAAGGCTTT ATGCTCTGGT
TTTGAGGAGA CCACTGAACC AGCGATATGC CTCAGCACAG ATGTGGAGTC AAACGAGTGC
TTAGATAACA ATGGTGGTTG TTGGCAAGAT AAATCAGCCA ACATAACTGC TTGCAAGGAT
ACTTTTCGTG GAAGAGTATG CGAGTGTCCT ACAGTTGATG GTGTGCAATT CAAAGGGGAT
GGTTACAGTC ACTGTGAACC AAGCGGGCCA GGGAGATGCA CAATCAACAA TGGAGGTTGT
TGGCATGAAG AGCGAGATGG ACATGCGTTC TCTGCTTGTG TGGACAAGGA CAGTGTTAAG
TGCGAGTGTC CTCCAGGATT TAAAGGAGAT GGTACTAAGA AGTGTGAAGA CATTAATGAG
TGCAAAGAGA AGAAAGCATG CCAGTGCCCA GAGTGTAGCT GCAAGAACAC ATGGGGAAGC
TATGAGTGCT CTTGTAGCGG GGACCTTCTC TACATCAGAG ATCATGACAC TTGCATCAGC
AAGACGGGTG CACAAGTGAG ATCAGCATGG GCGGCCGTTT GGCTTATAAT GTTATCATTG
GGACTTGCAG CTGCTGGTGC ATACCTCGTT TACAAATATA GGCTAAGGCA ATACATGGAC
TCAGAGATCA GAGCCATAAT GGCACAGTAC ATGCCATTGG ACAGCCAACC CGAGATCCCG
AACCACGTGA ATGATGAACG CGCCTGA

5) Sequence of AtVSR3;1 (AT4g20110)

ATGGGTTTAG TCAACGGGAG AGCTTCGTTG ACCTTTCTCC TCGCGGCGTT GACCATCATC
GCTATGGTCG TCGAGGCTAG GTTTGTGGTG GAGAAAGAAA GCATAAGCGT GCTGAATCCA
GAGGAGATGA GGTCGAAGCA CGACGGCTCG ATAGCCAATT TCGGTTTACC CGATTACGGT
GGGTTTTTAA TCGGGTCAGT GGTTTATCCG GATAGTAAAA CCGATGGATG CTCTGCTTTT
GGTAAAACCT TCAAGCCCAA GTTTCCTCGT CCCACTATTC TGCTTCTTGA TCGTGGAGGT
TGCTACTTTG CCTTAAAAGC GTGGCACGCG CAGCAAGCAG GCGCGGCTGC AGTTCTTGTG
GCGGATAATG TAGACGAGCC ATTGTTGACA ATGGATTCAC CAGAGGAGAG CAAAGATGCG
GATGGTTTCA TAGAGAAGCT AACAATCCCA TCGGTGTTAA TCGATAAATC ATTTGGAGAT
GACTTAAGAC AAGGGTTTCA GAAAGGGAAA AACATAGTTA TAAAACTAGA TTGGAGAGAG
TCTGTGCCTC ATCCTGATAA GAGAGTAGAA TATGAGCTGT GGACTAATAG CAATGATGAG
TGTGGTGCAC GGTGTGATGA ACAGATGGAC TTTGTCAAGA ACTTTAAAGG TCATGCTCAG
ATACTCGAAA AAGGCGGTTA TACCGCGTTT ACGCCGCATT ATATTACTTG GTTTTGCCCT
TTTCAGTTTA TAAACAGTCC ACATTGTAAG TCTCAGTGTA TAAACCATGG GAGGTATTGT
GCTCCTGACC CTGAGGATAA TTTCAGAGAA GGGTATGAAG GGAAAGATGT TGTGCTTGAG
AATCTGAGAC AGCTTTGTGT GCATAGAGTT GCGAATGAGA GTAGCAGGCC TTGGGTTTGG
TGGGATTATG TTACCGATTT TCATTCTCGA TGTTCGATGA AGGAGAAGAA ATACAGCATA
GATTGTGCTG AGAGTGTCAT CAAATCTCTG AATTTACCTA TTGAGAAGAT CAAGAAATGC
ATTGGTGATC CTGAGGCTGA TACAGAGAAC CAAGTTCTGA GAACTGAGCA AGTATCTCAG
ATTGGCCGAG GAAACCGGGG AGATGTTACG ATATTGCCAA CATTAGTCAT CAATAACGCT
CAATATCGAG GGAGATTGGA GAGAACCGCG GTTTTAAAGG CGATATGCGC TGGTTTTAAT
GAAACATCGG AGCCTGCCAT TTGCTTAAAC ACAGGTCTAG AGACAAATGA GTGCCTTGAA
AACAATGGTG GTTGCTGGCA GGATACAAAA GCAAACATCA CTGCTTGTCA AGACACATTC
AGAGGAAGAC TCTGCGAGTG TCCGGTTGTA AAAGGTGTTC AATATAAAGG AGACGGGTAC
ACTTCATGTA CACCTTATGG GCCTGCGAGG TGTACTATGA ACAATGGAGG TTGCTGGTCT
GACACAAGGA ACGGCTTAAC TTTCTCTGCT TGCTCAGACT CTGTATCTAC TGGCTGCAAA
TGTCCTGAAG GTTTCCAAGG CGACGGTTTG ACGTGTGAAG CAGATATTAA CGAATGTAAA
GAGCGTTCGG TATGTCAATG TAGCGGTTGC AGATGCAAGA ACTCATGGGG TGGATACAAA
TGCAGCTGTT CTGGTGACCG GCTTTACATA AACGATCAAG ATACTTGTAT AGAGAGATAT
GGATCCAAAA CGGCATGGTG GCTCACATTC TTGATACTGG CTATCGTTGC AGTAGCCGGT
TTAGCTGGTT ATATATTCTA CAAATACCGG TTCAGGTCTT ACATGGACTC AGAGATTATG
ACGATCATGT CACAGTATAT GCCACTTGAG AGCCAAAGAG CTCGTGAAGT TCCATCAGAA
GCCGAGCCTT TTACACTCTA A

6) Sequence of AtVSR3;2 (AT2g34940)

ATGTCTCCGA GCAATAAAGG AACCGTCTTG GCTCTGATTC TAGCGTTGAC CATGGTGGTG
GTCAACGGGT TTTCATCGAG ATTCTTCGTG GAGAAAAGCA GCTTGACGGT CCTTAACTCA
TGGGAAATGG GAGCTAAGCA CGACGCGGCC ATAGCAAACT TTGGTCTCCC AAAGTACGGC
GGTTTCATGA TCGGCTCTGT GGTCTACGCA GGCCAAGACG CTTACGGATG CAACTCTTTC
AACAAAACCT TCAATACCAA GTCTCCTTAT CCCAAAATTC TCCTCATTGA TCGTGGAGTG
TGTAACTTTG CTTTGAAGAT ATGGAACGGA CAACAATCCG GCGCAGCGGC TGTTCTTTTA
GCAGATAACA TTGTTGAGCC ATTGATAACA ATGGATACAC CCCAAGATGA AGATCCTGAC
TTTATAGACA AAGTCAAGAT CCCATCAGCT TTAATCCTTC GCTCTTTCGG TGATAGCCTC
AAGAAAGCTC TTAAAAGAGG TGAGGAGGTA ATCTTGAAGA TGGATTGGAG TGAGTCTATA
CCAAACCCTG ATGAGAGAGT TGAGTATGAG CTATGGGCTA ATACTAATGA TGAATGTGGT

GTACACTGCG ATAAACAGAT AGATTTCATT AAGAACTTTA AGGGAATGGC TCAGATTCTT
GAAAAAGGCG GTTATACTTT GTTCAGACCT CACTACATTT CTTGGGTTTG TCCCAAAGAG
CTTCTACTTA GCAAGCAGTG TAGGACTCAG TGTATAAACC AAGGGAGGTA TTGTGCTCTT
GATACTAAGC AAGAATTTGA AGATGGATAT AATGGGAAAG ACGTCGTTTA TGAGAATCTG
AGGCAGTTAT GTGTTCATAA AGTAGCTAAG GAGAAGAACA CTTCTTGGGT TTGGTGGGAC
TATGTGACAG ATTTTAACAT CAGGTGTTCT ATGAAGGAGA AGAAATACAG CAGAGAATGT
GCAGAGACTA TTGTGGAATC TCTCGGGCTG TCTCTTGAGA AGATCAAGAA ATGCATTGGT
GATCCTGATG CTGATGTAGA GAATGAAGTT CTAAAAGCCG AGGAAGCTTT TCAGTTAGGC
CAAGAGAATC GTGGCATTGT TACAATCTTT CCTACATTAA TGATCAACAA TGCTCAATAT
CGCGGTAAAC TGGAGAGAAC CGCGGTGCTG AAGGCTATAT GTTCAGGATT CAAGGAAAGA
ACAGAACCGT CAATATGTTT GAATTCAGAT ATAGAGACCA ATGAATGTCT TATAGAAAAT
GGAGGATGTT GGCAAGACAA AAGATCCAAT GTAACTGCTT GCAAGGACAC ATTTAGGGGA
AGAGTATGTG AGTGCCCGGT TGTCGATGGT GTTCAATATA AAGGAGATGG TTATACCTCC
TGCAAACCTT ATGGACCTGC GAGATGTTCA ATGAACAATG GAGACTGCTG GTCTGAAACC
AGAAAGGGTC TAACTTTCTC TTCTTGTTCA GACTCAGAGA CATCAGGATG TCGTTGCCCT
CTAGGTTTCC TTGGAGATGG TCTAAAATGT GAAGACATTG ATGAATGCAA AGAGAAATCA
GCTTGTAAAT GTGATGGCTG CAAATGCAAG AACAATTGGG GAGGATATGA ATGCAAATGT
TCTAACAATA GTATCTACAT GAAAGAAGAG GACACTTGTA TCGAGAGAAG AAGTGGATCA
AGAAGCAGAG GGTTGTTCAC AATTGTGGTT CTAACCGCCA TCGCGGGTAT CTCTTTAGGT
GCTTATATAT TCTACAAGTA CCATCTTCAG TCATACATGG ATTCAGAGAT CGTGTCCATT
ATGTCTCAGT ACATACCACT CGATAGCCAA AGCATTAACC AAGACTCTTT TAAGTAA

7) Sequence of AtVSR3:3 (AT1g30900)

ATGTCTTTGA TTCATAAAGG AGCCACCTTG GCTCTGTTTC TAGCGTTGAC TATGGTGGTC
AACGGAGTTT TCGGGAGATT CATCGTTGAG AAGAGTAGCG TGACGATTCT AAACCCTTTG
GCAATGCGGT CTAAGCACGA CGCAGCCATT GCTAACTTCG GTGTTCCTAA CTACGGTGGT
TACATGATCG GCTCCGTCGT TTACGCCGGT CAAGGAGCTT ATGGATGTGA CTCTTTTGAC
AAAACTTTCA AACCCAAATT CCCTCGTCCT ACCATTTTGA TCATCGATCG TGGAGAGTGT
TACTTTGCAT TAAAGGTATG GAACGGTCAA CAATCCGGTG TAGCAGCAGT TTTAGTAGCT
GATAACGTCG ATGAGCCATT GATAACAATG GATTCACCTG AGGAATCCAA AGAAGCTGAT
GACTTTATAG AGAAACTCAA CATTCCATCG GCTTTAATAG ACTTTTCTTT CGCCAATACT
CTCAAGCAAG CTCTTAAGAA AGGTGAGGAA GTAGTCCTGA AGATAGACTG GAGTGAGTCA
TTACCTCATC CGGATGAGAG AGTTGAGTAT GAGCTATGGA CTAACACGAA CGATGAGTGT
GGTGCACGGT GTGATGAGCA GATGAATTTC GTAAAAAACT TCAAAGGACA TGCGCAGATT
CTTGAAAAAG GAGGATACTC TTTGTTCACA CCTCATTACA TTACATGGTT TTGTCCTAAA
GATTATGTTT CTAGCAATCA ATGTAAGTCT CAGTGTATAA ACCAAGGGAG GTATTGTGCT
CCTGACCCTG AACAAGACTT TGGTGATGGA TACGATGGTA AAGACATTGT CTTCGAGAAC
TTGAGACAGT TGTGTGTTCA TAAAGTAGCT AAAGAGAATA ACCGGTCTTG GGTTTGGTGG
GACTATGTGA CTGATTTTCA CATTAGATGT TCAATGAAGG AGAAGAAGTA TAGCAAAGAA
TGTGCAGAGA GAGTTGTTGA ATCTCTAGGT TTGCCACTTG ACAAGATCAA GAAATGTATT
GGTGATCCTG ATGCTAATGT GGAGAATGAA GTTTTGAAAG CTGAGCAAGC ACTTCAGGTA
GGACAAGGTG ACCGCGGAGA TGTCACAATC TTGCCAACAT TGATCGTCAA CAATGCTCAA
TACCGCGGTA AACTTGAGAG AAATGCAGTA CTTAAGGCTA TATGTTCTGG ATTCAAGGAA
AGAACCGAAC CCGGGATCTG TCTAAGTGGA GATATTGAAA CAAATGAATG TCTCGAAGCA
AATGGAGGGT GTTGGGAGGA CAAGAAGTCC AATGTAACAG CTTGCAAGGA CACATTTAGA
GGAAGAGTCT GTGAATGCCC TGTTGTGAAG GTGTACAGTA TAAAGGAGAT GGATATACAT
CATGTGAACC TTATGGCCCT GCAAGATGCT CGATTAACCA AGGAGGTTGC TGGTCTGAAA
CCAAAAAGGG CTTAACTTTC TCGGCTTGCT CGAACTTGGA GACATCGGGA TGTCGCTGCC
CTCCAGGGTT TAAAGGAGAT GGTCTTAAAT GTGAAGACAT TGATGAGTGT AAGGAGCAAT
TGTCAATGTG ATGGATGCAA CTGTAAGAAC AAATGGGGAG GCTTTGAATG CAAATGCTCT
GGAAATCGTC TCTACATGAA AGAACAAGAC ACTTGTATTG AGAGAAGCGG ATCAAGAATC
GGATGGTTCC CTACATTTGT GATTCTAGCT GCAGTTGCAA GCATATGTGT AGGTGGTTAC
GTATTCTACA AGTATCGTCT CAGGTCTTAT ATGGATTCAG AAATCATGGC GATTATGTCT
CAGTACATGC CATTAGAGAG CCAAAACACA ACCGATCCAA TGACTGGTGA ATCTCAACAC
CAACAGCTGA GATTAACTTC TGCAGCCTAA

8) Sequence of AtRMR1 (At1g71980)

ATGAATCGTG CTTTGGTCCT ACTTTTATAT GTTTGTACTG TTTCTTGTTT AGCTTCAAGC
AAAGTTATTT TGATGAGGAA TAACATCACT CTCTCTTTTG ATGACATCGA AGCTAACATC
GCTCCGTCAG TGAAGGGTAC AGGTGAAATT GGAGTGGTTT ATGTGGCTGA GCCTCTTGAC
GCTTGTCAAA ATCTTATGAA TAAACCAGAA CAGAGCTCCA ATGAAACTTC TCCTTTTGTG
TTGATTGTTA GAGGAGGCTG TAGTTTTGAA GAGAAAGTTA GAAAAGCTCA GAGAGCTGGT
TTCAAAGCTG CTATTATCTA TGACAATGAA GACCGTGGAA CATTGATAGC AATGGCAGGT
AACTCTGGAG GTATAAGGAT TCATGCGGTC TTTGTTACGA AAGAAACGGG AGAAGTTTTA

AAGGAGTATG CGGGTTTCCC CGATACGAAA GTTTGGTTGA TCCCAAGTTT TGAGAACTCG
GCGTGGTCTA TTATGGCGGT TTCGTTTATC TCGCTGCTTG CAATGTCGGC TGTTCTCGCT
ACTTGTTTCT TTGTGCGTAG GCATCGAATA AGAAGGCGGA CATCTCGGTC CTCTCGAGTG
CGTGAGTTTC ACGGTATGAG CCGCCGCTTG GTGAAAGCAA TGCCGAGTCT TATATTCAGT
TCGTTTCATG AAGATAACAC TACTGCATTC ACTTGTGCTA TTTGCCTTGA AGACTACACT
GTTGGAGACA AGCTCAGGCT CTTACCTTGC TGTCACAAGT TTCATGCTGC GTGTGTTGAC
TCATGGTTAA CCTCTTGGAG AACTTTCTGT CCGGTGTGCA AACGAGATGC AAGAACGAGC
ACGGGAGAGC CTCCAGCTTC AGAGAGCACG CCATTGCTCT CATCTGCTGC ATCGTCTTTC
ACTTCTTCCT CTCTGCACTC TTCAGTCAGA TCATCTGCAC TATTGATTGG TCCTTCCTTG
GGCTCATTAC CAACTTCAAT CTCTTTCTCT CCCGCATACG CAAGCTCATC CTATATTAGA
CAATCATTCC AGTCTTCCTC TAACCGTCGA TCACCTCCAA TAAGCGTAAG TCGAAGCTCA
GTGGATCTCA GACAACAAGC AGCTTCTCCA TCTCCATCAC CATCACAGAG ATCATACATT
TCCCATATGG CTTCTCCACA GTCACTAGGT TACCCAACTA TCTCCCCTTT CAACACGAGG
TACATGTCAC CGTATAGACC TAGCCCGAGC AATGCATCAC CTGCAATGGC TGGATCATCG
AATTATCCGT TGAATCCACT GCGTTACAGT GAATCAGCTG GAACTTTCTC TCCATACGCC
TCTGCAAACT CGCTTCCAGA CTGTTAG

9) Sequence of AtRMR2 (At5g66160)

ATGAGACTCG TCGTCTCAAG CTGTCTACTA GTTGCAGCTC CTTTTCTCTC CTCTCTGTTA
CGAGTCTCAC TCGCCACTGT TGTCCTCAAT TCCATCTCCG CCTCTTTTGC CGATCTCCCA
GCCAAATTTG ACGGCTCCGT GACCAAAAAC GGAATCTGTG GAGCTCTATA CGTCGCAGAT
CCTCTCGACG GTTGCTCACC GCTTCTCCAC GCCGCCGCAT CCAACTGGAC GCAACACAGA
ACTACTAAGT TCGCTTTGAT AATCAGAGGC GAATGTTCTT TTGAGGATAA GCTGCTCAAT
GCCCAGAACT CAGGTTTTCA AGCTGTGATT GTCTATGACA ACATTGACAA CGAAGATCTC
ATCGTCATGA AGGTGAACCC TCAGGACATT ACAGTTGATG CAGTCTTCGT TTCAAATGTC
GCCGGTGAGA TTTTGAGAAA GTACGCGAGA GGCCGAGATG GTGAATGCTG CCTTAATCCG
CCAGACAGAG GGAGCGCTTG GACTGTGTTG GCCATCTCCT TCTTCTCTCT CCTTCTTATA
GTCACTTTCC TGTTGATTGC CTTCTTTGCA CCCAGACACT GGACCCAATG GCGAGGGAGG
CACACCAGGA CCATCAGGTT AGATGCAAAG CTCGTCCACA CACTCCCCTG CTTCACCTTC
ACTGATTCTG CTCACCACAA GGCCGGGGAA ACATGTGCTA TATGTCTCGA GGATTACAGA
TTTGGAGAAA GCCTCAGACT TCTCCCCTGC CAACATGCTT TTCACTTGAA TTGCATCGAC
TCTTGGTTGA CAAAATGGGG TACATCTTGC CCTGTGTGCA AGCATGACAT AAGAACCGAG
ACTATGTCTT CTGAGGTACA TAAACGAGAG AGTCCGAGAA CAGATACAAG TACGAGTAGA
TTTGCCTTTG CCCAATCCAG TCAAAGCCGT TAG

10) Sequence of AtRMR3 (At1g22670)

ATGAATCTTG TTGTTCTGCT AATCCTAACA TTACTCCTTT TCATTGTTTC TTATGTAGTA GACGCAGGC
CAAGTCATTT TGGTTGATTC CAACATAACT CGCTCTTTTG TCGACATGGA AGCTGATTTC TCTCCATCAG
TGACTACGGT GGAAACGGAG TGGTTTATGT AGCTGAGCCT CTCAACGCTT GCCGAAACTT GAGGAATAAA
CCGGAGCAGA GCCCTTATGG TACTTCCCCT CTTGTGTTGA TCATAAGAGG AGGCTGCAGT TTTGAGTACA
AAGTCAGAAA CGCGCAGAGA AGCGGTTTCA AGGCTGCCAT TGTCTATGAC AATGTGGACC GCAACTTCTT
ATCCGCAATG GGGGGAGACT CGGACGGTAT AAAGATTCAA GCGGTTTTTG TGATGAAGAG AGCCGGAGAA
ATGCTCAAGA AGTACGCGGG TTCGGAGGAA ATGGAAGTCA TGTTGGTTCC TCCTAATACA GAGGACTCGG
TGTGGTCATT GTACGCTTCC ATAGCATTGA TCTTGTCGCT GGCTATTTTT TGTGTTATGG TTACTTGTGT
CTTCTTCTAT AGATATTGCT CAACAATTAG AAACTCTACA TCTCAATTCA ATGGGATGTG CCGTAGAACG
GTGAAAGCAA TGCCGAGTGT TACATTCACT TGTGCAAAAA TAGACAACAC TACAGAGTTT CATGTCGCTT
GCGTAGACTC GTGGCTTATA TCATGGAGAA CGTTTTGTCC AGTGTGTAAA CGGGATGCGA GAACGACCGC
AGATGAGCCA CTAGCTACAG AGAGCACACC GTTTCTCAGT TCTTCCATTG CAACATCATC TCTAGTGTGT
ATAGACTCTC CTCCTTTGGG ATCCTCAGTT TCTTTCTCTC CAGCGCATGT GAGCTCGTCC TTCATTCATC
AATTTGTCAG GTCTTCGCCA ATGAATGGCA GCCGTATCTC AGAGAATCTT AGGCGACAAG CCTCACCATT
ACAGTCATCA TCACAGCGAT CACACCTCTC TATGAAGTCT TCCCATTCAC TGGGTTATTC GACCATGTCA
CCTCTCAACG CGATGGGCAT GTCACCATAC CGGCCATACC CAAGCAATGC ATCGCCTGGA TTATTCAGTT
CAACAAATCA TCTGCTTTCC AATTATACAG CAAATACATT CTCTCATTTC GCCTCTGCAC ACTCGCTTCC
GGACTAG

11) Sequence of AtRMR4 (At4g09560)

ATGATCCGTT CTTCGATTG TAATTTTATCT CTGTTACTAA TTTCACACTT GGTTTCTGCA AAAGTTCTGT
TGATCGGTAA CAGCACATC TCTCTCCTTCG ACGACGTCGA AGCCACTTTC ACTCCGATGA TTAAGAGATC
GGATCAAGGC GGTGTGTTG TATGTAAGCAG AGCCACTCGA TGCTTGTTCG GATTTGGTGA ATACGGTGAA
TGTGAAAAAT GGAACTACT GTGTCTCCTCC GTATGTGTTG ATTATCCGCG GTGGTTGTAG TTTCGAAGATA
AGATTAGGAA TGCTCAAAA GGCTGGTTATA AAGCTGCTAT TGTTTATGAC TATGAAGATT TTGGGTTCTT
AGTATCAATG GAGCGAAACC CCTCTGGTGT ACTTATTTAT GGTACGTTTG TCTCCAAAGC AACTGGGGAA

GTACTTAAAG AGTATGCGGG TCGTACCGAT TTTGAAGTGT GGCTCATGCC AAGTTTCGAG ACTTCAGCAT
GGTCAATCAT GGCTATTTCT TTCATATCTC TCCTCGCCAT GTCGGCTGTG CTCGCTACTT GCTTCTTTGT
CCGTAGGCAT CGAGTTAGGC GTCGGCGTAT TCTGGCTCTT AATGGCAATG ACTTTCATCG TATGCCCAAA
AGCATGATAA TACGTATGCC TACTACCATA TTTAACGGTA TTTGTGATGA AGCAACTACA TCTATATTGT
GCTGCATATG CCTTGAGAAT TATGAGAAAG GGGACAAGCT AAGGATCCTA CCTTGCCATC ACAAATTTCA
TGTTGCTTGT GTAGACTTGT GGCTTGGCCA GAGGAAATCC TTTTGCCCGG TTTGCAAACG CGATGCAAGA
AGCATCAGTA CCGACAAGCC CCCATCAGAG CACACACCCT TTCTTTCTCG GACTCCTAGC ATGACCCCGA
CGTCATCGTT TCTCTTATCA TCATCCTCCA CAACCCCATT GCAGTCATCT CATGAGCTAC CAATATCCAT
CAGAGTAGAC CCTTCTTTAC CATCCACCTC AATGCAGCCA CACACAGTTC CTATGTATCT CTCCCACTCT
CGCTCCCACA CAAGCTTCCA AAATGGATCA AACCGATTTT CCCGGCCTAT ACCAGTTAGC CGGAGTTCAG
CAGATCTCAG GAACGCCGTT TCCCAAAGAT CTTACAACTC ACCCCACCAG GTTTCTTTGC CTCGTTTCCT
CCACTCAAGA TATACGCACA TACTTGGCCC GGGAAATGCA TCAAGAAGCC AGGTTGTTGG GTTGTTAACA
AGCCAGCGCG AGCATTCACT TCATCAAAAT GACTCGCGCA GGTCTTTCAT TCACTTTGCA TCTGCGAGCT
CCTTACCAGG CTGGTAA

12) Sequence of AtRMR5 (At1g35630)

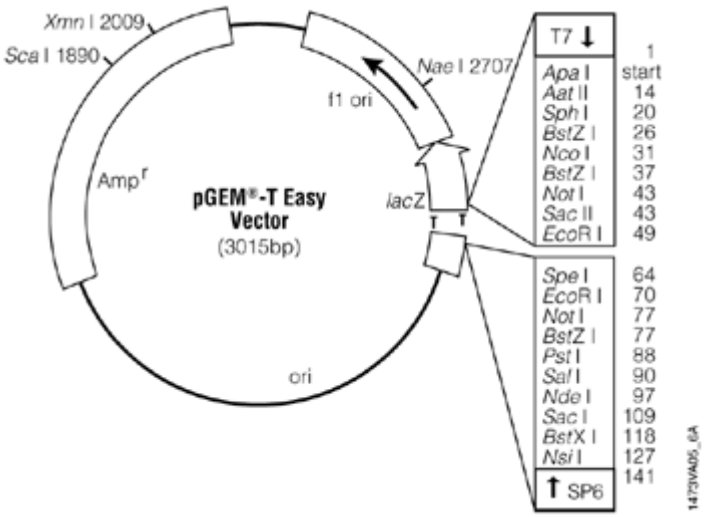
ATGAACTATA GTTGGATTAC AATCATGTCT CTGTTGGTAA TTTGTAAGCT GGCTTCGGCG
AAAGTAGTGT TGATCGGGAA AAACACAATT CTATCCTTTG ATGATGTCGA GGCAACTTTC
ACTCCAATTG TTAGAAACTC GGGGGAATGT GGAATTTTGT ACGTTGCAGA GCCTCTTGAG
GCATGCTCGG ATATAACCAA CATGGCGGAA AAAAGATCAA AGTATAGGTC CTCTTATGTA
TTGATCGTCC TTGGTGGCTG TAGTTTTGAG GAAAAGGTTA GAAAGGCGCA GAAAGCTGGA
TACAAAGCTG CGATTGTCTA TAACGATGGA TATGATGAGC TCTTAGTACC TAGAAATTCA
TCTGGTGTGG ATATACATGG CTTGCTTGTT ACAAGAGCAT CAGGGGAGGT GCTTAAAGGG
TACGCGGATC AAGACGAGAT GAAGCTTTGG CTCATCCCGG GATTCGGGAT TTCATCTTGG
TCCATCATGG GTATTACATT CATATCTTTA CTCGCCATGT CTGCTATTCT AGCCACTTGT
TTCGTTGTCC GTAGGCATCA AATTAGACAG AGTGTGAGGG ATTTACCACA TGGTGGCCAA
GGACTTTCTT GTATGCCAAG AGACTTGTTG CAAAGCATGC CGACTGAAGT ATATAGCGGT
GTTCTTGAAG AAAGTTCAAC TTCGGTTACT TGTGCTATAT GTATCGATGA TTATTGCGTT
GGTGAAAAAC TCCGAATCCT ACCTTGCAAA CACAAATATC ATGCGGTGTG TATCGATTCT
TGGCTCGGAC GTTGTAGATC CTTTTGTCCG GTTTGTAAAC AAAATCCAAG AACAGGAAAT
GATGTTCCAC CAGCATCAGA AACAACACCT CTGATTTCTC CTAGCCCGAA CTCTATTACT
TCACTACAAT CGTTTTATGA TCTACCAATA GTTGTCAGAG TATATCTGTAA

13) Sequence of AtRMR6 (At1g35625)

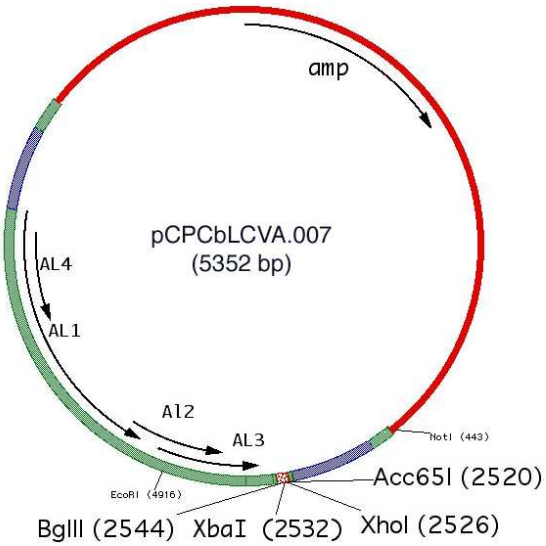
ATGAACGGTA GTTGGATTAC AATCCTCTCT TTGTTGGTAA TTTCTCAGCT GGCTTCTTCG
AAAGTAACGT TGATCGGGAA AAACACATTT CTCTCATTTG ATGATGTCGA AGCAAATTTC
ACACCAGTTG TTAGAAGATC GGGAGAATAC GGATTGTTGT ACGCTGCAGA GCCTCTTGAT
GCGTGCTCGT ACTTAACAAA CATGGCGGAA AAAGGTTCGA AATTTAGGCC CTCGTATGTA
TTGATCGTCC GTGGTGGCTG TAGTTTTGAG GAAAAAATAA GAAATGCGCA GGAAGCTGGA
TACAAAGCTG CGATCGTCTA TAACGATAGA TATGAGGAGC TCTTAGTACG TAGAAATTCA
TCTGGTGTCT ATATACATGG TGTGCTTGTT ACAAGAACAT CAGGGGAGGT ACTTAAAGAG
TATACCAGTC GAGCTGAGAT GGAGCTCTTG CTCATCCCGG GATTCGGGAT TTCATCTTGG
TCAATCATGG CTATCACTTT CGTATCGTTA CTCGTCATTT CTGCCGTCCT AGCCTCTTAT
TTCTCTGTCC GTAGGCATCG AATTAGACAG CATGTGAGGG ATTTACATCA TGGTGGCCAA
GGACATTCTC GTATGCCAAA AGACTTGTTG CAAAGCATGC CGACTGAAGT ATATACCGGT
GTTCTTGAAG AAGGTTCGAC TTCTGTTACT TGTGCTATAT GTATTGATGA TTATCGCGTT
GGTGAAATAC TCAGGATCCT ACCTTGCAAA CACAAATATC ATGCGGTGTG TATCGATTCT
TGGCTCGGAC GTTGTAGATC CTTTTGTCCG GTTTGTAAAC AAAATCCAAG AACAGGAAAT
GATGTACCAC CAGCATCAGA AACAACACCT TTGATTTCTC CTGGTCCGAA CTCTATTACT
TCACTACAAT CGTTTTATGA TCTACCAATA GTTGTCAGAG TATATCTGTAA

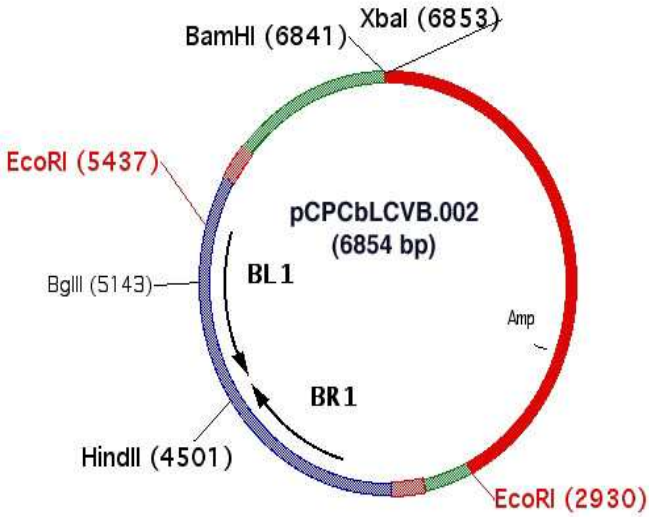
Plasmids and vectors

Vector for subcloning

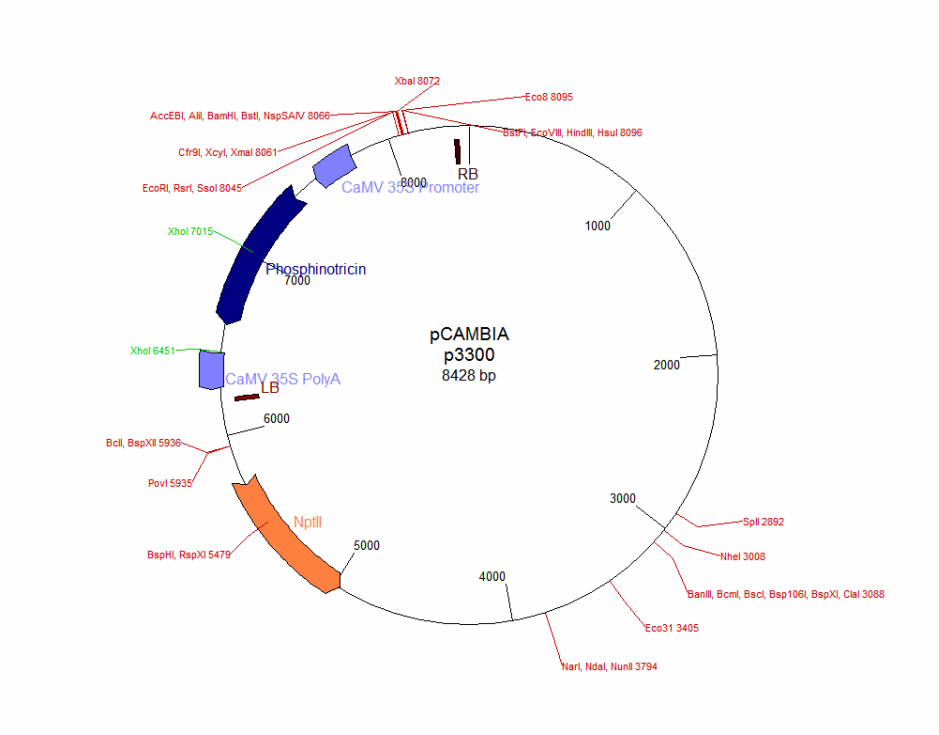

Xmn I 2009
Sca I 1890
Nae I 2707
f1 ori
Amp r
pGEM®-T Easy
Vector
(3015bp)
lacZ
ori
T7
Apa I 1 start
Aat II 14
Sph I 20
BstZ I 26
Nco I 31
BstZ I 37
Not I 43
Sac II 43
EcoR I 49
Spe I 64
EcoR I 70
Not I 77
BstZ I 77
Pst I 88
Sal I 90
Nde I 97
Sac I 109
BstX I 118
Nsi I 127
SP6 141
1473VA05_6A

Vectors for transient silencing





Vector for stable silencing



Acknowledgement

First I want to thank my family for their continued support during my studies and especially during my Ph.D. Specially my husband who helped me a lot from the beginning to the end of my thesis.

I express my gratitude to Pr. Jean-Marc Neuhaus to accept me in his research team, for supervising this work, for his advices and guidance.

I thank Prof. Dominique Robertson for providing the geminiviral vector. The particle delivery system was kindly provided by Prof. Chris Kuhlemeier (Berne) and Therese Mandel (Berne) helped with the *in situ* hybridisation.

Many thanks to Marc Creus, Anne Gouget, Guillaume Gouzerh for their collaboration and active discussions of results. I will like to thank also Sophie Marc-martin for her contribution in some cloning experiments, to Ricardo Flückiger for his friendship and advices on confocal microscopy.

I want to thank all members of the Molecular and cell Biology Laboratory for their friendship.