University of Neuchâtel
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# Functional Characterisation of AtRMR Proteins in *Arabidopsis thaliana*

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# IMPRIMATUR POUR LA THESE

# Functional characterisation of AtRMR proteins in *Arabidopsis thaliana*

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FACULTE DES SCIENCES

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# Abstract

**Keywords :** Vacuolar sorting; RMR; VSR; amylase secretion assay; GFP; dominant negative.

**Mots-clés :** Tri vacuolaire; RMR; VSR; test de secretion amylase; GFP; dominant négatif.

Plant cells contain two or even three types of vacuoles: the lytic, the (seed) protein storage and vegetative storage vacuoles. Soluble vacuolar proteins are sorted through the secretory pathway to these vacuoles by three different routes, depending on different types of Vacuolar Sorting Determinants (VSD) and involving several types of receptors and vesicles. The AtRMR1 protein has been identified in cellular structures associated with the seed storage vacuole pathway (Jiang et al. 2000). Based on its localisation and homology to a known vacuolar receptor, it has been hypothesised to be a receptor protein for the C-terminal type of VSD (CtVSD) involved in sorting to the storage vacuole. The genome of *Arabidopsis thaliana* contains 5 genes homologous to *AtRMR1*. The main goal of this study was to test the involvement of AtRMR1 in vacuolar sorting and to test the specificity of the different AtRMR proteins for different known CtVSDs.

To test the involvement of AtRMR proteins in vacuolar sorting we studied the effects of manipulations of these genes on targeting of vacuolar reporter proteins. I used two different models: *A. thaliana* leaf protoplasts and whole plants of insertional mutants from the SALK Institute collection.

The protoplast model allowed me to study *in vivo* the effects on vacuolar sorting of versions of AtRMR1 with loss of function deletions or modified functions. I obtained interesting results with two of these constructs: one lacking the luminal VSD-binding domain (RMRΔlum) and one consisting of this soluble luminal domain retained in the ER by the addition of a HDEL peptide signal (RMRlumER). When overexpressed with GFP fused to the ssVSD of barley aleurain, the RMRΔlum construct showed a different pattern of fluorescence compared with the control: in RMRΔlum, a significant proportion of protoplasts showed a dot-like fluorescent pattern, whereas the fluorescence was more often found in the central vacuole or ER in the control. When I used either the CtVSD of tobacco chitinase or a short version of the barley aleurain ssVSD, I didn't see a different fluorescent pattern with or without the dominant negative construct. To better estimate the effects of the dominant negative constructs, the level of secretion of enzymatic reporters was investigated. The reporters were α-amylase fused either to the CtVSD of tobacco chitinase, the CtVSD of barley lectin or the ssVSD of sweet potato sporamin. For these three different reporters, overexpression of RMRΔlum induced an increased secretion of the reporter, whereas a simple fractionation showed that the RMRlumER construct provoked their accumulation in microsomal compartments, presumably ER. Two other constructs, where either the luminal domain of AtRMR1 was redirected to the PM (RMRlumPM) or the soluble cytosolic domain was overexpressed (RMRcyt) didn't have any effect on the sorting of enzymatic reporters, suggesting that the transmembrane and cytosolic domains are both important for the VSD binding and

that the cytosolic domain alone is not able to interfere with the vacuolar sorting machinery.

I completed these results with a study of vacuolar sorting in gene knockout (KO) mutants. I carried out observations on whole plants containing single KO mutations for *AtRMR1, AtRMR3* and *AtRMR4* genes, where the fluorescent reporters GFPchi or $Aleu_{143}$GFP had been introduced by crossing or by agro-infiltration. In these plants, the fluorescence pattern of vacuolar reporters showed a striking difference compared with reporter plants: they mostly appeared in punctate, peripheral structures and tended to accumulate in the corners of the cells.

Taken together these results give new insights in the receptor-mediated protein vacuolar sorting: (1) AtRMR1 is important for both the CtVSD and ssVSD pathways, (2) three single KO for three AtRMR genes show similar impairment in vacuolar sorting. There are at least two possible explanations that help to define a new model for RMR function: first, RMR could be a “general purpose” receptor that discriminates as early as in the ER/Golgi the proteins to be sorted to plant vacuoles (CtVSD and ssVSD proteins), whereas receptors of the VSR family would act more specifically in a later intermediate sorting compartment; second, AtRMR action could be regulated through the formation of receptor complexes, as at least three of them seem to be needed simultaneously for a proper sorting of vacuolar reporters.

# Abbreviations

**BP-80**: Binding Protein of 80 kDa.

**CCV**: Clathrin-Coated Vesicle.

**CD-MPR**: Calcium-Dependent Mannose-6-Phosphate Receptor.

**CI-MPR**: Calcium-Independent Mannose-6-Phosphate Receptor.

**CMA**: Chaperone-Mediated Autophagy.

**conVSD**: condensation-dependent VSD.

**COP**: Coat Protein.

**CtVSD**: C-terminal VSD

**Cvt**: Cytoplasme-to-Vacuole Transport.

**DIP**: Dark-Induced Protein.

**DNA**: DeoxyriboNucleic Acid.

**DV**: Dense Vesicle.

**EGF**: Epidermal Growth Factor.

**ER**: Endoplasmic Reticulum.

**FPE**: Fluid Phase Endocytosis.

**GA**: Golgi Apparatus.

**GFP**: Green Fluorescent Protein.

**GGA**: Golgi-located γ-adaptin ear homology domain ARF binding protein.

**LV**: Lytic Vacuole.

**NV**: Neutral Vacuole.

**PAC**: Precursor Accumulating Vesicle.

**PM**: Plasma Membrane.

**PSV**: Protein Storage Vacuole.

**PV72**: PAC Vesicle protein of 72 kDa.

**RME**: Receptor-Mediated Endocytosis.

**RMR**: Receptor-Membrane-RingH2.

**RNA**: RiboNucleic Acid.

**SNARE**: Soluble N-ethylmaleimide-sensitive protein Attachement Protein Receptor.

**ssVSD**: sequence-specific VSD.

**TGN**: Trans-Golgi Network.

**TIP**: Tonoplast Intrinsic Protein.

**VSD**: Vacuolar Sorting Determinant.

**VSR**: Vacuolar Sorting Receptor.

**VSV**: Vegetative Storage Vacuole.

# Introduction

Eukaryotic cells contain different types of organelles. These specialised compartments within the cell are limited by biological membranes and assume specific functions that are necessary for the life of the cell. Some are present in all eukaryotic cells: the nucleus, which contains the genetic information; the mitochondria, which are important for the cell respiration and lipid metabolism; the endoplasmic reticulum and the Golgi apparatus, which are important for the proper synthesis of certain proteins, lipids and polysaccharides; and the vacuoles or lysosomes that assume osmoregulatory, solute storage and degradative functions. While of similar origin, plant cells contain additional organelles, the plastids. Additionally, the plant vacuole system is more complex than the yeast vacuole and the animal lysosomes.

# I. Overview of the endomembrane system

In eukaryotic cells, the system of internal membranes that delimitate subcompartments within the cell, plastids and mitochondria excluded, is often named endomembrane system. The endomembrane system is made of specific organelles, which are mainly specialised in production and maturation of macromolecules, their storage, degradation and recycling. These organelles are the nuclear envelope, the endoplasmic reticulum, the Golgi apparatus, various endosomes and the vacuole.

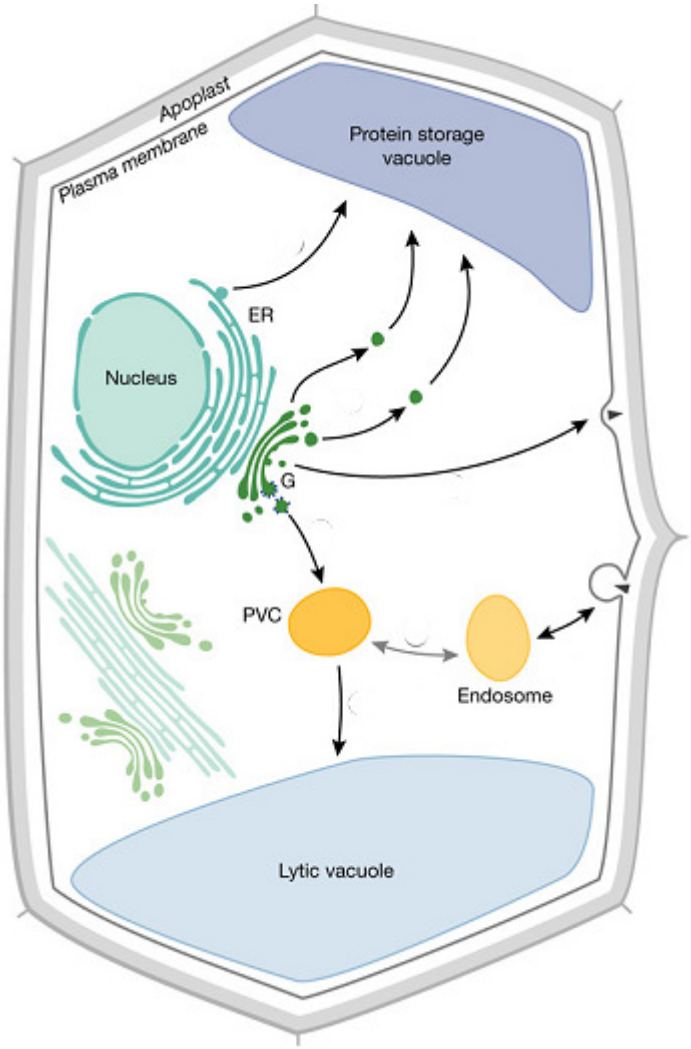



**Fig 1: scheme of the plant endomembrane system (modified from Brandizzi and Hawes 2004).**
In this scheme are represented the main compartments of the plant secretory pathway: Nucleus, Endoplasmic Reticulum (ER), Golgi apparatus (G), a prevacuole (PVC), an endosome and two vacuolar compartments (Protein Storage Vacuole and Lytic Vacuole).

## *A. The Endoplasmic reticulum*

The ER can be divided in three functional parts: the smooth ER, the rough ER (RER) and the nuclear envelope. The smooth ER is specialised in the synthesis of lipids, detoxification of xenobiotics and calcium regulation. The rough ER (RER) is named so due to the presence on its surface of ribosomes which give it a rough appearance in electron microscopy. The RER is specialised in the synthesis of proteins of the secretory pathway. The nuclear envelope delimits the borders of the nucleus but has also a role in the regulation of mRNA metabolism (Vertel et al. 1992; Gomord and Faye 1996).

## *B. The Golgi Apparatus*

After synthesis in the ER, most secretory proteins are transported to the Golgi Apparatus (GA). This organelle is made of flattened cisternae. With the exception of

*Saccharomyces cerevisiae*, where they are dispersed in the cytosol, cisternae are stacked and sometimes interconnected by tubular elements, forming Golgi stacks. In mammalian cells, these Golgi stacks are concentrated in a juxtanuclear area, whereas in plant cells they are dispersed in the cytosol, where they can rapidly move along actin filaments. The GA is made of four subcompartments: the *cis*-, the *medial*-, the *trans*-Golgi and the *trans*-Golgi network (TGN).

The GA plays a particularly important role in plant cells for the maturation of proteins and the synthesis of lipids (especially ubiquinone and plastoquinone) and polysaccharides (hemicellulose and pectins but not cellulose).

Most of the secretory proteins leave the GA at the *trans*-Golgi, where they are packaged into vesicles prior to their transport to either the plasma membrane *via* the endosomes or to the vacuolar/lysosomal system.

### *C. The endosomes*

Endosomes are small organelles, with no well defined shape, found in all eukaryotic cells. It is an intermediate compartment that takes up solutes from the PM via endocytosis. Endosomes are also thought to be intermediate compartments of the secretory pathway (see page 26).

### *D. Presentation of the plant vacuolar system*

The vacuoles are widespread organelles among Eukaryotes; they nevertheless differ largely in their functions and importance between the kingdoms.

In some protists, a specialised vacuole plays a central role during phagocytosis, as a digestive compartment (“storage sacs”).

Protozoans have contractile vacuoles, first reported in the 1700s by Spallanzani. The contractile vacuole network of *Dictyostelium discoideum* consists of tubes and bladders. It is thought to balance the water content of the cell by accumulating and expelling excess water from the cell's cytosol (Allen 2000). The contractile vacuole is involved also in Ca2+ transport and pH regulation (Malchow et al. 2006). In the protist *Giardia lamblia*, lysosome-like peripheral vacuoles accumulate endocytosed exogenous macromolecules and soluble hydrolases (Touz et al. 2004). In *D. discoideum* the two types of vacuoles can coexist in a single cell (Temesvari et al. 1996).

**Fig. 2: Photograph of a *Paramecium* cell showing two contractile vacuoles (taken from Allen 2000).**
Each contractile vacuole is made of a central vacuole and 5 to 10 radial arms. The CV on the left is in a late filling phase (with an expanded central vacuole), the other is expelling its content (radial arms form ampullae, arrowheads). Scale bar 20 µm.

In yeast, the vacuole is used in detoxification, storage of amino acids and autophagy (a process initiated upon nutrient starvation by which proteins are degraded; cf page 23). The vacuole is the most prominent organelle in a yeast cell, and it plays a central role in cellular physiology. The vacuole is involved in cytosolic ion and pH homeostasis and is the main storage site for calcium and other divalent cations. Metabolites such as basic amino acids and polyphosphate, as well as toxic substances, are also stored within this organelle. In addition, the vacuole is important in osmoregulation, sporulation, and regulatory processes that require degradation. The best known role for this organelle is in protein turnover; the vacuole contains a large number of membrane-bound and soluble hydrolases (Klionsky 1998).

In animals, vacuoles are intermediate compartments in the endocytosis and exocytosis pathways. Lysosomes are present at different levels in different tissues and cell types. Lysosomes are the terminal destination for many endocytic, autophagic and secretory materials targeted for destruction. In addition lysosomes play critical roles in metal ion homeostasis and plasma membrane repair (Mullins and Bonifacino 2001). They vary in morphology and regulation of their activities (Raposo, 2002). Lysosomal compartments also function in some cells as secretory compartments (secretory lysosomes). Lysosomes are defined by the existence of a limiting membrane and a high content of acid hydrolases. Interestingly lysosomes lack Calcium-Dependent and Calcium-Independent Mannose-6 Phosphate Receptors (CD- and CI-MPRs see page 40), which distinguishes them from endocytic intermediate compartments (Mullins and Bonifacino 2001).

Plants have the most prominent and versatile vacuolar system. The vacuoles usually account for 30 to 90 % of the volume of mature plant cells. Plant vacuoles are involved in homeostasis, detoxification, storage of various compounds (Taiz 1992; Winkeler et al. 2003), degradation of xenobiotics, recycling of macromolecules, cell turgor, cell pH maintenance, cell growth, cell metabolism. These cellular functions also include the plant defence against pathogens and herbivores (through the controlled release of toxic compounds stored in vacuoles), plant movements (Ward and Schroeder 1994; Fleurat-Lessard et al. 1997) and soil detoxification.

## 1. Plants have different vacuoles

Early microscopic studies have shown that plant cells can harbour vacuoles which are widely diverse in form, size, content and functional dynamics. Furthermore, a single cell may contain more than one kind of vacuole. Electron microscopic observation clearly show the existence of two vacuolar compartments with different electron densities in a single cell (Michel et al. 1992). Dyes, such as Neutral Red (Ehara et al. 1996; Di Sansebastiano et al. 1998; Epimashko et al. 2004), also allowed to detect vacuolar compartments with different pH within single cells. Neutral Red is a membrane-permeant solute when unprotonated but membrane-impermeant when protonated, which causes it to get trapped in acidic compartments. These observations established the presence in plant cells of different types of vacuoles. Later studies permitted to better describe the organisation, specialisation and plasticity of the plant vacuole endomembrane system.

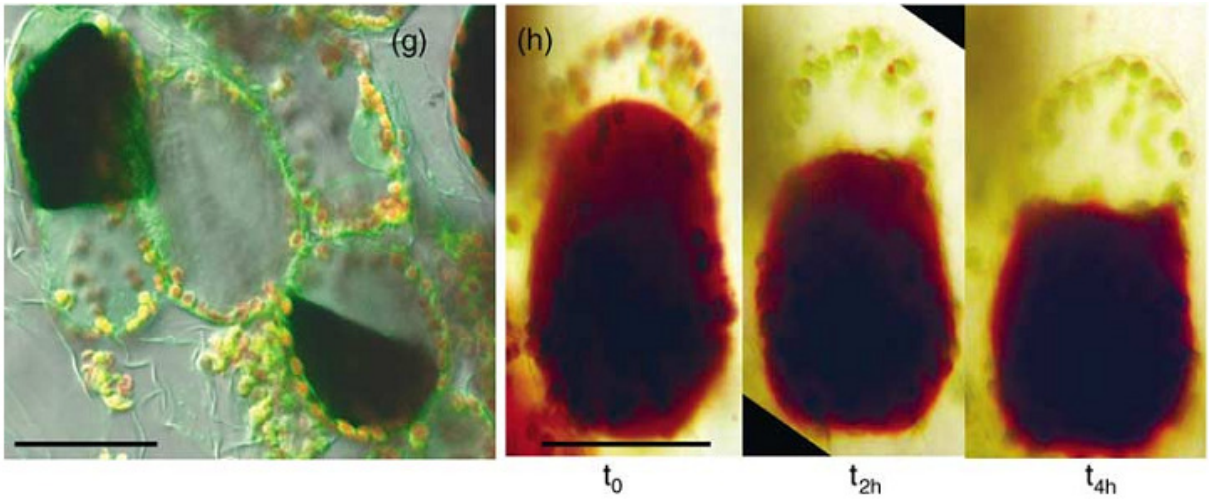



**Fig 3: Plant cells can have simultaneously two distinct vacuolar compartments (taken from Epimashko et al. 2004). Mesophyll cells of *Mesembryanthemum. crystallinum* have two types of vacuoles with different acidity.**
(g) Confocal picture of NR-stained mesophyll cells of salt-stressed plants with two distinct vacuoles.
(h) Bright field microscopic pictures showing a single mesophyll cell with an acidic (NR positive) and a neutral vacuole. The acidic vacuole has accumulated malate in the dark. Upon illumination for 2 hours and 4 hours, the NR positive vacuole releases malate for photosynthesis and its volume decreases accordingly, while the neutral vacuole enlarges in compensation.

## 2. The use of reporter proteins

The first clear molecular evidence for the existence of different vacuoles in plants was brought by the observation of two members of a family of vacuolar aquaporins called Tonoplast Intrinsic Proteins (TIPs) that did not colocalise in pea root tip cells (Paris et al. 1996). Later, immunolocalisation experiments using antipeptide antibodies specific for the α-, δ- and γ-TIP showed that these tonoplast proteins do not fully colocalise at least in some plant cells (Jauh et al. 1999). TIPs belong to the superfamily of the Major Intrinsic Proteins, known to facilitate the passive transport of water and other small neutral solutes across membranes in a wide range of

organisms (Delisle and Bansal 1996; Murata et al. 2000). 10 TIPs have been identified in *A. thaliana* (Wallace and Roberts 2004).

Other studies using soluble reporters also suggest the existence of these different vacuoles (Di Sansebastiano et al. 2001). It is now thought that plant cells have two or even three different types of vacuoles: the lytic vacuole, the seed protein storage vacuole and the neutral vacuole (or vegetative storage vacuole).

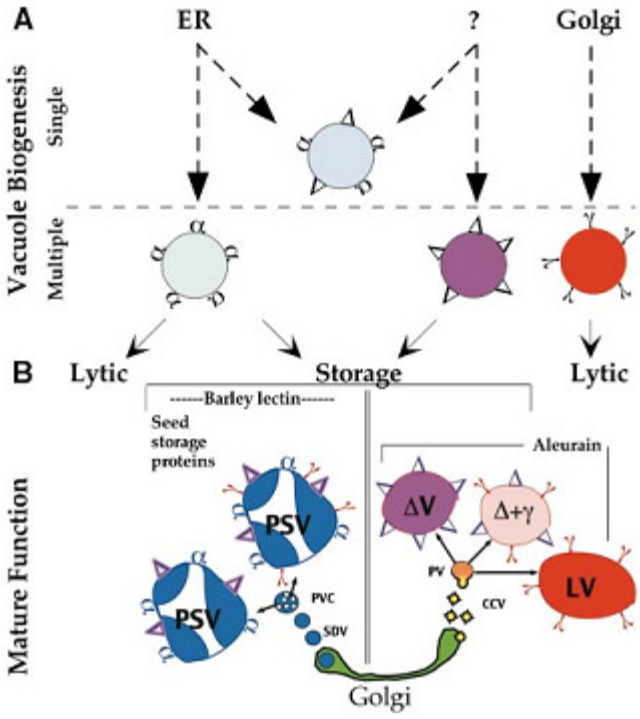



**Fig 4: antibodies raised against different TIP isoforms label different sorts of vacuoles (taken from Jauh et al. 1999).**
(A) During vacuole biogenesis: in meristematic cells, a single small vacuole forms that is labelled by α- and δ-TIPs. Other relatively undifferenciated cells can have different vacuoles labelled by either α-, γ- (for lytic vacuoles) or δ-TIPs (for storage vacuoles).
(B) When the vacuole matures: α-, δ- and γ-TIPs label the PSV, whereas other storage vacuoles accumulate δ-TIP (δV) or δ- and γ-TIP. The LV contains γ-TIP.

## 3. The Lytic Vacuole

The LV is an acidic compartment, rich in enzymes (proteases, lipases, glycosidases) analogous to those of the animal lysosome. This compartment is mainly involved in the maintenance of the cell turgor pressure, the degradation of macromolecules and toxic compounds, as well as the storage of ions and metabolites and the sequestration of xenobiotics. The tonoplast of this vacuole is labelled mainly by γ-TIP and one typical soluble reporter for this vacuole is the barley cysteine protease aleurain (Paris et al. 1996; Di Sansebastiano et al. 1998; Flückiger 1999).

## 4. The Protein Storage Vacuole

The PSV compartment is unique to the plant kingdom and is specialised mainly in the storage of proteins (Okita and Rogers 1996; Shy et al. 2001; Muntz and Shutov

2002). PSV have been characterised in various plants and cell types. In seeds of some plants, the PSV is a complex structure with internal compartments (Jiang et al. 2000). When expressed in leaf protoplasts of *A. thaliana*, tobacco and common bean, the vacuolar storage protein phaseolin is accumulated in small vacuoles (Park et al. 2004). However, in this case phaseolin is ectopically expressed and may therefore not fully prove the existence of a PSV in leaves.

### 5. The Vegetative Storage Vacuole

The VSV is also called neutral vacuole due to the fact that it is not labelled by Neutral Red dye. In previously evacuolated protoplasts, while the newly forming large central vacuole is labelled by the LV reporter $Aleu_{143}GFP$, the reporter for neutral vacuole, the GFPchi, is localised in small peripheral vacuoles. Later the two compartments seem to fuse, as GFPchi labels the central vacuole (Di Sansebastiano et al. 2001). The tonoplasts of neutral vacuoles are characterised by the presence of δ-TIP (Jauh et al. 1998).

### 6. The plant vacuolar system is highly versatile

Some plant cells can harbour more than one type of vacuoles (Epimashko et al. 2004) but under certain circumstances hybrid vacuoles can also be found, as tonoplast membranes of big central vacuoles can be labelled by two different TIPs (Paris et al. 1996; Epimashko et al. 2004). Moreover, the vacuole inner morphology can be variable and harbour complex structures. For example, in seed cells of tobacco, the vacuole is a multivesicular compartment made of globoid and crystalloid subcompartments delimited by membranes and surrounded by a matrix within the external tonoplast boundaries (Jiang et al. 2000). During plant development, in elongating cells, the large central vacuole is labelled by the LV reporter $Aleu_{143}GFP$ and the storage vacuole reporter GFPchi labels small compartments; when the cell has reached its final size, the GFPchi starts to label the large central vacuole, indicating that in this physiological state, the previously lytic and storage compartments have merged into a hybrid vacuole (Fluckiger et al. 2003). The paraveinal mesophyll cells (PVM) of soybean leaves form a tissue that is specialised in nitrogen storage and remobilisation. Especially, the removal of the flower shoot tip induces a sink-limited physiological condition that causes the accumulation of vegetative lipoxygenases and vegetative storage proteins that serve as transitory nitrogen stores in the vacuoles of PVM. When plants were subjected to this change in physiological conditions, the γ-TIP marker decreased in these vacuoles, whereas labelling with the δ-TIP marker increased, indicating the conversion of a lytic vacuole to a vegetative storage vacuole. This was reversed when the shoot tips were allowed to regrow (Murphy et al. 2005).

## II. Proteins reach the vacuoles *via* various pathways

The contents of the vacuolar system originate from both endogenous biogenesis and controlled endocytosis (Marty 1999). There are three distinct pathways to the vacuoles/lysosomes (fig. 5): internalisation of regions of the cytoplasm in the vacuole via autophagy, endocytosis from the plasma membrane and sorting mechanisms in the secretory pathway that discriminate which proteins must be secreted and which are destined to the vacuole.

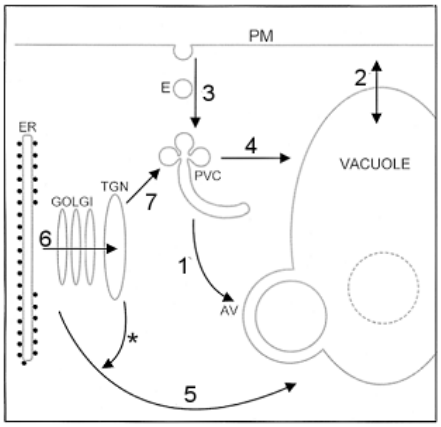



**Fig 5: the transport pathways to the plant vacuole, (modified from Marty 1999).**
The autophagy pathway: cytosolic compounds are either engulfed in evaginations of the PVC (1) or directly taken up by the vacuole (2).
The endocytic pathway: PM cargo is internalised to the PVC (3) and finally reaches the vacuole (4).
The biosynthetic pathway: proteins synthesised in the ER are either directly sent to the vacuole (5) or pass through the Golgi (6), the PVC (7) and finally the vacuole (4)

## *A. The pathways from the cytosol to the vacuoles*

Autophagy is a cellular process that is found in yeast, animal and plant cells. This pathway is mediated by cellular mechanisms common to the different kingdoms (non-selective mechanism), but also by mechanisms specific for yeast (Cvt pathway) or animal cells (CMA).

### 1. Autophagy in yeasts

Autophagy has been best described in yeast cells, where a selective (Cytoplasm to Vacuole Transport pathway or Cvt) and a non-selective (autophagy) mechanism are distinguished (for review see Nair and Klionsky 2005; Bassham et al. 2006). These mechanisms are morphologically very similar, but differ in their function, autophagy being a degradative process induced upon starvation (Chen et al. 1994; Aubert et al. 1996; Moriyasu and Ohsumi 1996) while Cvt is important in biosynthesis - notably in the maturation of the vacuolar protein aminopeptidase I (Scott et al. 1997). The events taking place in these two pathways are similar. Double membrane vesicles form tubular structures that surround the cargo in the cytosol to be delivered to the vacuole, forming double-spanned organelles called autophagosomes and Cvt vesicles for the autophagy and Cvt pathway respectively (Baba et al. 1994). The outer membrane of these organelles fuses with the tonoplast, releasing their content, still trapped in the inner membrane, into the lumen of the vacuole. Finally the inner membrane is broken down and the cytosolic content is released in the lumen of the vacuole.

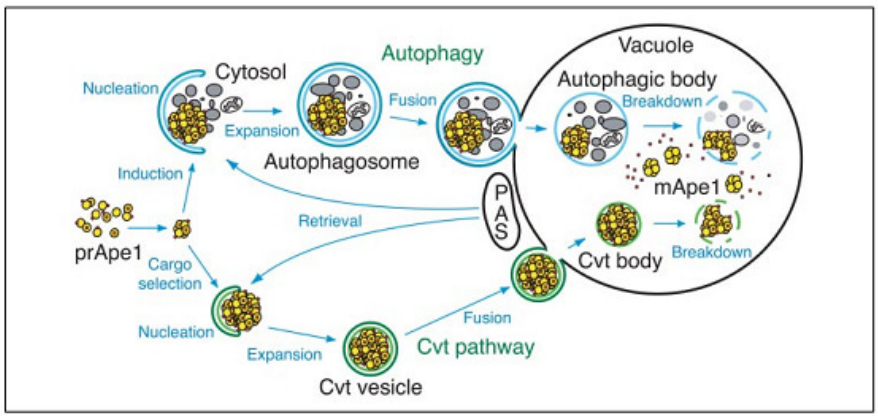



**Fig 6: autophagy in yeasts, taken from (Nair and Klionsky 2005).**
In both autophagy and Cvt pathway, cytosol cargoes are engulfed by double-spanned membrane vesicles. In the case of autophagy, the vesicles engulf various cytoplasmic components, whereas the vesicles formed by Cvt appear to exclude bulk cytosol and to incorporate only the protein aggregate of prApeI.
In both cases, the vesicle outer membrane fuses to the tonoplast, and then the inner membrane is processed and releases its content in the lumen of the vacuole. The prApe1 hydrolase is processed and activated in the vacuolar lumen.

### 2. Autophagy in animal cells

In mammalian cells, the Cvt pathway has not yet been described, but another alternative autophagy-like pathway is involved in the specific delivery of cytosolic compounds to the lysosome, the Chaperone-Mediated Autophagy (CMA). In the

CMA, particular cytosolic proteins are specifically recognized by chaperone complexes present in the cytosol and in the lumen of the lysosome (Cuervo and Dice 1998).

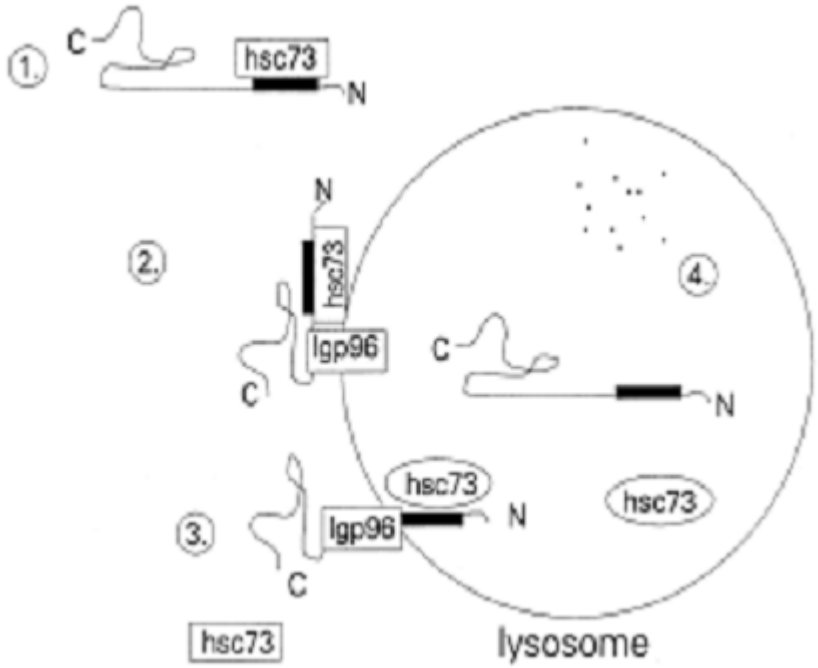



**Fig 7: Chaperone-Mediated Autophagy in animal cells (taken from Cuervo and Dice 1998).**
(1) The cytosolic chaperone hsc73 specifically binds to a target protein in the cytosol.
(2) The hsc73-protein complex then binds to the lysosomal membrane protein lgp96.
(3) The cytosolic protein is translocated across the lysosomal membrane, probably through a proteinaceous channel. The translocation of the protein is thought to be helped by the action of a lysosomal hsc73.
(4) Finally the cytosolic protein is released and degraded into the lumen of the lysosome.

## 3. Autophagy in plants

In plants, while no Cvt-specific or CMA-like pathways have been discovered yet, autophagy has been well documented (Bassham et al. 2006).

In unvacuolated meristematic cells (Marty 1978), autophagic vacuoles originate from provacuoles that are formed by a yet undiscovered mechanism either by fusion of ER stacks or by vesicles that bud from the trans-Golgi. Provacuoles form tubules that ultimately enclose discrete portions of cytoplasmic material. Tubular prevacuoles form cagelike traps that fuse in a zipperlike fashion and build a continuous cavity around the segregated region of the cytoplasm, called autophagosome. These autophagosomes are thus made of two concentric endomembranes that separate a central compartment made of the trapped cytoplasm from a peripheral compartment, derived from the prevacuole, acidic in nature and containing acid hydrolases. The digestive enzymes are thought to be released into the central lumen as the inner boundary membrane deteriorates. The outer membrane remains insensitive to the hydrolases and becomes the tonoplast of the forming lytic vacuole.

Due to its degradative function, autophagy is an important phenomenon in protein and organelle turnover. It is implicated in vacuole biogenesis, cell differentiation and recycling of cytoplasmic content during plant development. Notably, in developing pea cotyledons, the vegetative storage vacuole is replaced by a protein storage vacuole by a mechanism that looks like autophagy: ultrastructural and immunocytochemical data show that the vegetative vacuole is surrounded by a cisternal tubular compartment containing storage proteins (Hoh et al. 1995).

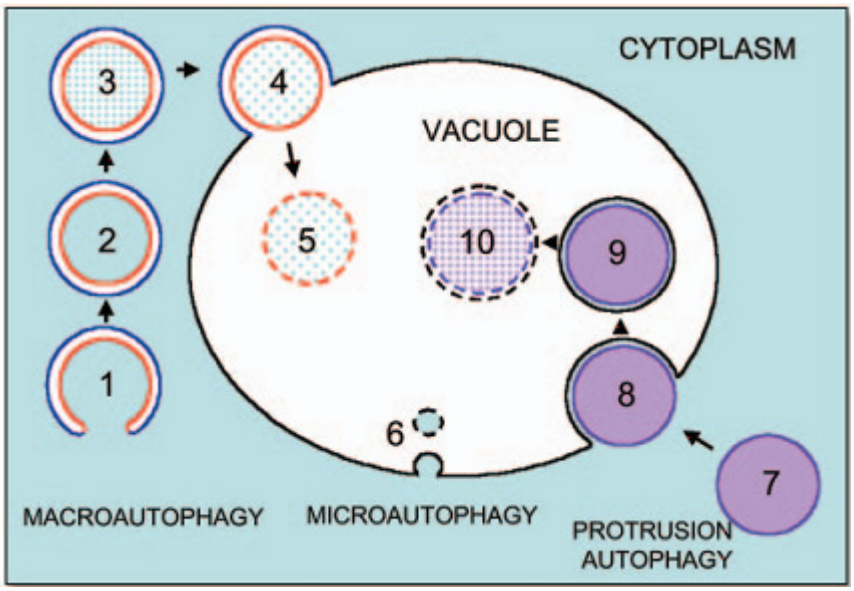



**Fig 8: autophagy in plant cells, (taken from Bassham et al. 2006).**
Macroautophagy: preautophagosomal structures (1) that emerge from prevacuoles engulf a part of the cytosol, forming phagosomes (2). Phagosomes later mature (3) and fuse to the vacuole (4) where their contents are released and degraded (5).
Microautophagy: a small vesicle (6) invaginates directly from the tonoplast and is released to the vacuole lumen where it is degraded.
Protrusion autophagy: whole organelles (7) are delivered to the vacuole lumen by direct protrusion (8) and further engulfment (9) to be finally processed by hydrolases (10).

## *B. The endocytic pathway*

Endocytosis is a cellular event common to all cell types. It consists in the internalisation of material present in the cell exterior. Here we will discuss the nature of endocytosis-specific cellular compartments – the endosomes – in Eukaryotes, the pathways through which external solutes can be taken up by the cells, and the place taken by endocytosis in the intracellular trafficking events.

### 1. Endosomes

In yeast and animal cells early and late endosomes are well defined by biochemical markers and functional features, whereas the endosomal compartments are more elusive in plant cells, appearing as Partialy Coated Reticulum, TGN or endosomes.

The studies of endosomes and more generally endocytosis-related structures have been helped by the use of chemical dyes, biotinylated proteins, pheronomes or hormones, PM receptors.

In plants, the study of endocytosis has long been challenged by the lack of clear reporters (Aniento and Robinson 2005). Nevertheless, several endocytic cargo molecules have now been clearly identified, such as auxin efflux carrier (Geldner et al. 2003), plasma membrane ATPase (Geldner et al. 2001), a receptor kinase (Shah et al. 2002) and cell wall pectins (Baluska et al. 2002; Baluska et al. 2005).

## 2. Fluid phase / Receptor-mediated endocytosis

Two mechanisms are distinguished: the Fluid Phase Endocytosis (FPE) and the Receptor-Mediated Endocytosis (RME).

The FPE is also named bulk flow endocytosis or constitutive endocytosis, is not saturable, which means that the uptake kinetics does not depend on the concentration of cargo molecule. Lucifer Yellow is a membrane-impermeant fluorescent molecule that has been used widely as an FPE reporter in animal, yeast and plant cells (Stewart, 2001, for review see also Aniento and Robinson, 2005). FM4-64 is an amphiphilic styryl dye (fig. 9) that has been used widely to study endocytic events in eukaryotic cells. It belongs to a family of dyes, developep by Betz et al. (1992) that fluoresce only when in a hydrophobic environment (lipid-rich membrane). FM4-64 is thought to enter the cell by endocytosis; therefore it has been helpful to dissect this cellular event.

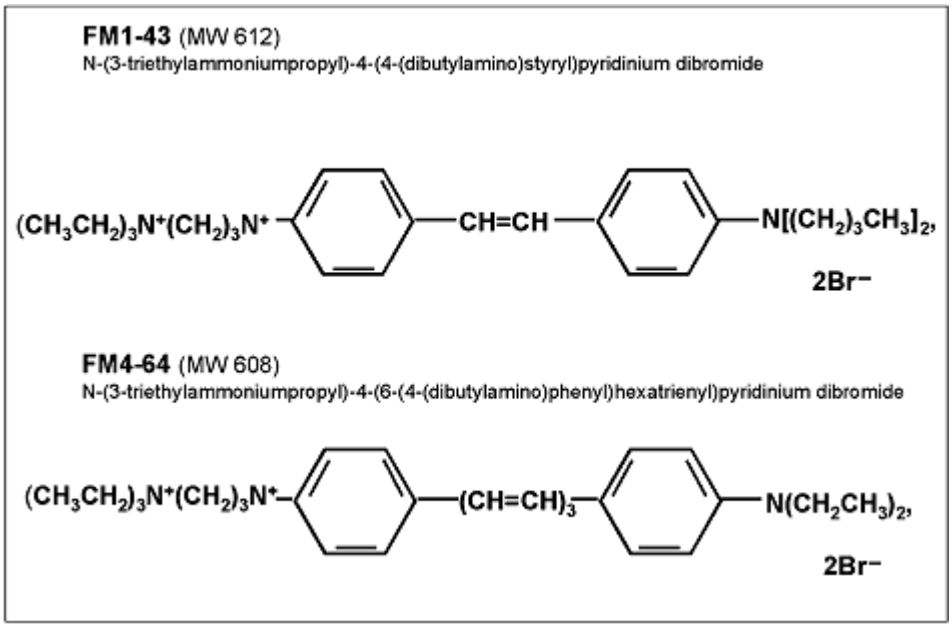



**Fig 9: chemical formulae of two FM dyes used for the study of endocytosis (taken from Bolte et al. 2004).**

The solutes can be internalised in clathrin-coated pits or from sterol-rich domains of the plasma membrane. Clathrin-coated pits are membrane patches decorated by clathrin polymers at their cytosolic face (Fan et al. 1982). Clathrin-coated vesicles (CCVs) invaginate from the plasma membrane and later fuse to the endosomal compartments to deliver their cargo. In the RME, the solutes are first bound to specific PM receptors, the complex is then internalised and the receptor-ligand complex is dissociated in endosomes (van Deurs et al. 1982; Engqvist-Goldstein and Drubin 2003; Ehrlich et al. 2004). Additionally, non CCV-dependent endocytic

pathways have been described. This mechanism involves lipid rafts, membrane sub domains enriched in specific sterols and sphingolipids. It is best described in animal cells, where specific plasma membrane invaginations called caveolae have been identified as early as in the 1950s. In plants, structural sterol-enriched domains present in the plasma membrane have been shown to be distributed in the endocytic network (Grebe, 2003), arguing in favour of an involvement of lipid rafts in plant endocytosis. Nevertheless, both in plant and animal cells, the exact role of lipid rafts in endocytosis is still a matter of debate (Samaj, 2005; Lajoie, 2007).

Genetic studies have shown the involvement of specific lipids (Kobayashi et al. 1998), vesicular trafficking components (cf page 33), proton pumps that control the pH of endosomal compartments (Sun-Wada et al. 2003) and endosomal proteases - such as papain (Yamada et al. 2005).

## 3. Endosomal compartments are linked to the biosynthetic pathway

It is now well documented that in yeast, animal and plant cells, endocytosis, exocytosis and the biosynthesis pathways have crossroads.

Interestingly, FM4-64 progressively stains the tonoplast when internalised, suggesting an endocytic pathway to the plant vacuole. There is a close interconnection of endocytosis with the biosynthetic pathways, for instance the PVC compartment can be both described as an intermediate between Golgi and vacuoles in the secretory pathway and/or an intermediate between plasma membrane and vacuole in the endocytic pathway. For example, an antibody raised against a vacuolar sorting receptor (BP80), known to traffic together with vacuole proteins from the trans-Golgi network to the prevacuole (see chapter IV-B), has been found to colocalise partly with the endocytosis reporter FM4-64 (Bolte et al. 2004). The *trans*-golgi network (see page 33) is thought to be the link between these three vacuolar pathways.

Two models have been proposed to explain how the endosomal and lysosomal compartments are related to each other. The first proposes maturation of early endosomes to late endosomes and ultimately lysosomes, each step being defined by a set of specific molecular markers (Murphy 1991). The other model proposes fixed compartments connected via vesicular shuttles (Le Borgne et al. 1996; Sun-Wada et al. 2003).

In plants, the balance between exocytosis and endocytosis of cell wall components, such as pectins, is crucial for the growth of the plant (Baluska et al. 2005), such as the oriented growth of the pollen tube (Wang et al. 2005), some specific cell movements, aperture of the stomata (Kubitscheck et al. 2000; Homann and Thiel 2002) and cell division (Dhonukshe et al. 2006).

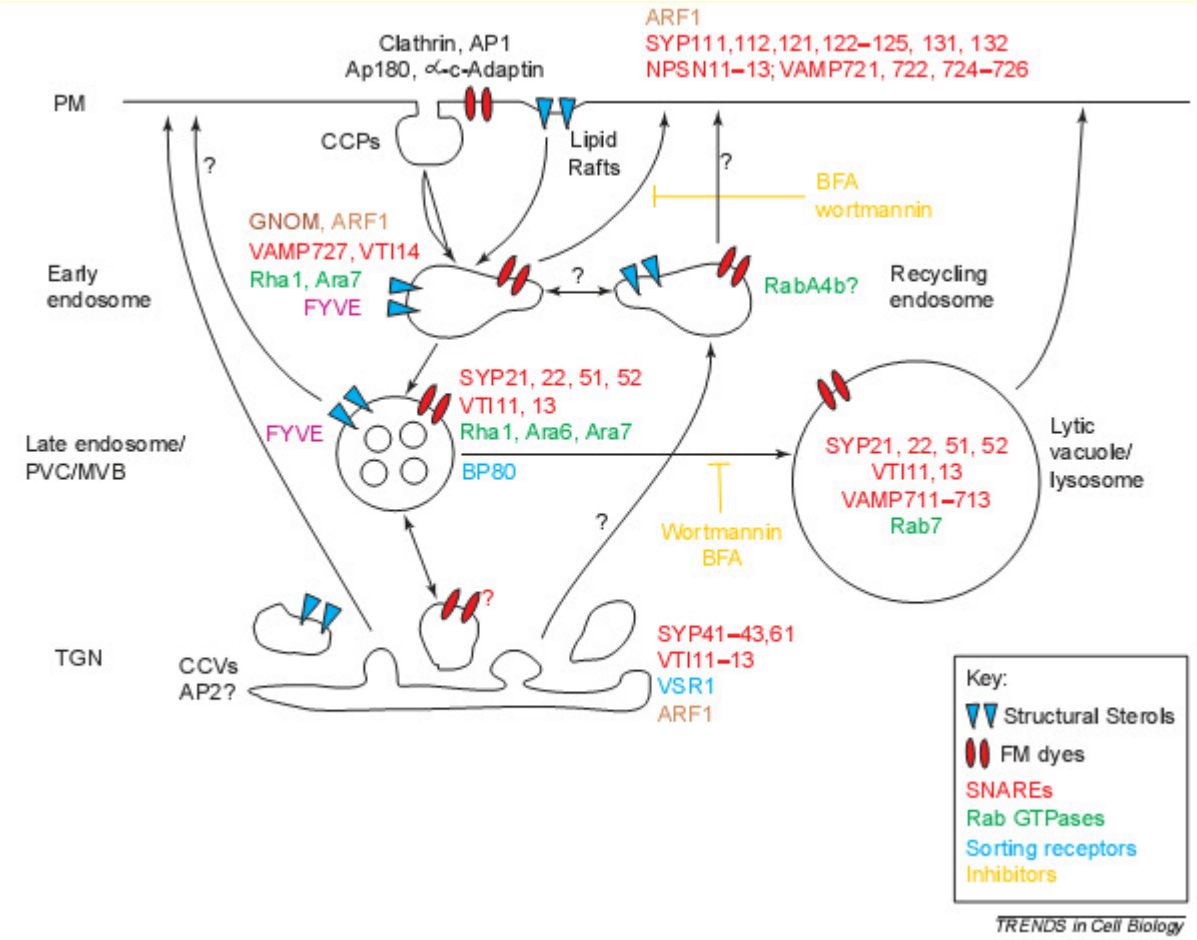



**Fig 10 : Endocytosis machinery in plant cells (taken from Samaj et al. 2005).**
Plasma membrane molecules and cargo are internalised firstly by Clathrin-Coated Vesicles.
These internalised molecules are sorted by the TGN and two populations of endosomes: the early/recycling endosome and the late endosome/PVC. From there, the cargo molecules are either recycled back to the PM or sent to the Lytic Vacuole for degradation.

## *C. The biosynthetic pathway*

The plant secretory pathway is schematised in fig. 1. It is a complex machinery composed of various organelles: the nuclear envelope, the endoplasmic reticulum, the Golgi apparatus, the vacuoles, intermediate compartments (such as the endosomal/prevacuolar compartments) and the plasma membrane.

This biosynthetic pathway is by far the major route to the plant vacuoles and has already been extensively studied. In the following section I will concentrate on the description of the fate of soluble proteins through this pathway, since it is the topic of this thesis.

## 1. Protein synthesis and import in the rough Endoplasmic Reticulum

Proteins are imported in the RER in a cotranslational manner. The newly synthesised polypeptide chain is translocated through the membrane of the ER while being synthesised by a ribosome, which is attached to its cytosolic side.

Soluble proteins are translocated to the lumen of the RER only if they carry a specific signal peptide. The peptide sequence required for a proper entry in the secretory pathway is located at the N-terminal end of the protein precursor and is usually about 10 to 50 amino acids long (Von Heijne 1988) . This sequence begins with 1 to 5 positively charged residues at its N-terminus and ends with a polar C-terminus containing a proteolytic cleavage site. These two elements are separated by the H-region, mostly made of hydrophobic amino acids and 7 to 16 residues long (von Heijne 1986; Gomord and Faye 1996). As soon as this sequence is synthesised and emerges from the ribosomes, it is recognised and bound by a cytosolic ribonucleoprotein complex called the Signal Recognition Particle (SRP), composed of a 7S RNA and six protein subunits. The nascent signal peptide and the SRP form a complex which stops the translation (High and Dobberstein 1991). This complex also targets the ribosome-nascent protein complex to the SRP receptor in a GTP-dependent manner. The SRP-ribosome-preprotein complex is then recognised by the Docking Protein, anchored in the membrane of the ER (Rapiejko and Gilmore 1997; Nagai et al. 2003). The SRP complex is then released and the ribosome-preprotein complex is delivered to the translocation complex or translocon. The translocon is made of the Sec61 protein, the signal peptidase, the oligosaccharyltransferase (OST) and the translocating chain-associated membrane protein (TRAM). Sec61 forms the translocation pore and a conformational change of Sec61 and TRAM is thought to cause the pore to open, thus allowing the entry of the nascent chain. The signal peptidase cleaves the signal peptide after its translocation (Deshaies and Schekman 1987; Shelness and Blobel 1990; Gorlich and Rapoport 1993). However, at least in yeast, some proteins are transported to the ER in a SRP-independent manner: they are translated in the cytosol and then targeted and translocated post-translationaly to the ER through the same translocon.

Several types of membrane proteins can be distinguished. The type I integral membrane proteins have a cleavable signal peptide like soluble proteins, whereas type II or type III integral membrane proteins have one or several (respectively) internal non-cleavable signal sequence which functions as a membrane domain (Spiess 1995). The orientation of their insertion in the ER is based on the ionic charges of the residues flanking their membrane domains: positively charged side chains tend to stay on the cytosolic face of the membrane (Hartmann et al. 1989; Wahlberg and Spiess 1997).

## 2. Fate of the proteins in the Endoplasmic Reticulum

### a) Protein quality control

The protein quality control (QC) takes place in the cytosol, or after the translocation into the ER lumen, during the folding and assembly of proteins (Bukau et al. 2006).

It has been estimated that in animal cells, about a third of the total proteins does not pass the quality control and is therefore degraded (Schubert, 2000). Protein quality control is a process through which the cell controls the folding of newly synthesised proteins, helps them to acquire their native structure and target the ones unable to fold to degradation. Improperly folded polypeptides are recognised by helper proteins that bind to typical features of non-native structures. These chaperones assist misfolded proteins or divert them to degradation. Several chaperones use ATP during this step (Gottesman et al. 1997).

The protein QC in the ER includes four major mechanisms : retention of malfolded proteins in the ER, refolding, ER-Associated Degradation (ERAD) of proteins that fail to fold and retrieval to the ER from other organelles or trafficking via the Golgi for degradation in the lysosome or the vacuole (Ellgaard et al. 1999).

The first mechanism of retention in the ER and folding of misfolded proteins depends on chaperones of the Hsp70 type, with the help of cofactors such as Hsp40. Contrary to correctly folded proteins, misfolded proteins present exposed hydrophobic domains and possibly also wrongly paired cysteines in disulfide bonds and they tend to aggregate (Ellgaard and Helenius 2003). Chaperones recognise these features (Wickner et al. 1999). When bound to ATP, Hsp70 proteins are in an open conformation in which a transiently exposed hydrophobic pocket binds to the exposed non-native structures of the misfolded proteins. Hydrolysis of ATP makes the chaperone undergo a conformational change to a closed form that helps the target protein to fold (Schmid et al. 1994; Fewell et al. 2001). Exchange of ADP for ATP leads to the release of the properly folded protein (McCarty et al. 1995). Moreover, binding of chaperones and their stimulation of refolding prevents aggregation of misfolded proteins in the ER (Groenendyk 2005).

Calnexin and calreticulin are two ER chaperones that play a crucial role in the folding of N-glycosylated proteins. These chaperones bind to the monoglycosylated glycan linked to unfolded proteins. The complex formed by calnexin or calreticulin and the unfolded glycosylated protein undergo a cycle of release and attachment of glucose catalysed by glucosidase II and glycoprotein glucosyltransferase. This cycle is thought to help the formation of a folded protein, which contains a $Man_9GlcNac_2$ glycosylation motif. If during the calnexin/calreticulin cycles the protein fails to fold or necessitates too many cycles, a terminal mannose can be cut off by a ER mannosidase I or II and the unfolded protein degraded (Spiro 2004).

At this step various other factors such as thiol-disulfide oxidoreductase, PDI, calreticulin, calnexin and ERP57 contribute to the proper formation of disulfide bonds (Kang and Cresswell 2002).

When a protein fails to fold in the ER, it undergoes ER-Associated Degradation (Bonifacino and Weissman 1998; Plemper and Wolf 1999; Lord et al. 2000). This process is initiated by the binding of chaperones to the misfolded protein, but instead of assisting it to refold, the chaperones help its retrotranslocation through the Sec61 channel to the cytoplasm. Once in the cytoplasm, the misfolded ER protein is deglycosylated, polyubiquitinated and finally taken up by a 26S proteasome for degradation (Hiller et al. 1996; Brodsky et al. 1999; Plemper and Wolf 1999). However, a recent report shows that the BiP chaperone can be also sent to the lytic vacuole in tobacco along with a subset of its ER ligands (Pimpl et al. 2006).

### b) Retention in the ER

Newly synthesised proteins need to be properly folded before leaving the ER to their final destination. This folding is assisted by the proteins PDI (Protein Disulfide Isomerase), BiP (chaperone of the HsP70 family), calnexin and calreticulin.

The above mentioned proteins, whose function is restricted to the ER are retained in this organelle, while their substrates, once properly folded, leave the organelle. Their retention is dependent on the presence of a retention signal at the C-terminus of the protein, which is usually HDEL in yeast and KDEL in mammalian cells. In plants, both tetrapeptides seem to be efficient retention signals (Denecke et al. 1992; Gomord and Faye 1996; Gomord et al. 1997). HDEL proteins show a characteristic ER location, whereas KDEL proteins are immunodetected in discrete parts of the ER (Napier et al. 1992). Normally secreted proteins are retained in the ER in animal cells when fused to KDEL. In plant cells reporter proteins fused to H/KDEL are at least partly retained in the ER (Denecke et al. 1992; Napier et al. 1992; Boevink et al. 1996; Gomord et al. 1997).

A similar mechanism allows the retention or retrieval of integral proteins in the ER. Many ER resident type I integral membrane proteins carry a di-lysine motif (i.e KKXX or KXKXX) at their C-terminus that has been shown to be necessary and sufficient for their retrieval (Jackson et al. 1993; Gaynor et al. 1994; Benghezal et al. 2000). In most type II integral membrane proteins, a di-arginine motif present at the N-terminal end has a similar function (Schutze et al. 1994).

## 3. Transport of solutes through the Golgi Apparatus

The GA is the main site of post-translational modifications of polypeptides, such as remodelling of the N-linked oligosaccharide chains and the synthesis of the O-linked oligosaccharide chains of the glycoproteins. Notably, N-glycans are very similar in the plant and animal kingdoms, differing only in terminal residues: plant N-glycans often have an α-1,3-fucose and β-1,2-xylose instead of the α-1,6-fucose found in mammals, while animal proteins often have a terminal N-acetylneuraminic acid. These modifications occur sequentially while secretory proteins are transported through the Golgi stacks and are processed by Golgi enzymes (glycosidases and glycosyltransferases). Proteins enter the GA at its cis-face and move through the medial-Golgi.

Two models have been elaborated to describe the way secreted proteins travel through the Golgi.

### a) The vesicle shuttle model

This model postulates that Golgi cisternae are fixed and assumes the anterograde transport of proteins from the *cis*- to the *trans*-Golgi and the retrograde sorting to the *cis*-Golgi are mediated by COPI vesicles. This theory is in agreement with EM data showing that the anterograde cargo proinsulin and the retrograde cargo KDEL receptor locate in different sets of COPI vesicles that bud from the Golgi (Orci et al. 1997).

### b) The cisternal maturation model

In this model, the secretory proteins are transported through the GA without the help of vesicles. Instead the anterograde transport is a passive maturation of the Golgi cisternae. In this system, *de novo* formed cisternae push older cisternae and their contents to the *trans*-Golgi. The enzymatic content of these cisternae evolves due to a retrograde transport of Golgi enzymes mediated by COPI vesicles (cisternae mature by retrieving "early" cisternal enzymes to younger cisternae and receiving "late" Golgi enzymes from more mature cisternae). The *trans*-cisterna detaches to become the TGN and eventually disintegrates (Glick et al. 1997).

Most recently, studies focused on the colocalisation of fluorescently tagged Golgi proteins and their dynamics showed that in yeast cells expressing the cis-Golgi marker Sys1-GFP and the trans-Golgi marker Sec7-DsRed, Golgi cisternae, firstly labelled in green (SyS1-GFP) underwent a fast green-to-red shift meaning an enrichment in the late Golgi marker. They also showed that the kinetics of this maturation of the cisternal contents matched the intra-Golgi velocity of secretory proteins and concluded that cisternal maturation therefore accounts for most of the secretory protein traffic (Losev et al. 2006).

Notably, this is the only model compatible with the mechanism of sorting of algal scales or procollagen complexes too bulky to be transported by COPII vesicles (Melkonian et al. 1991; Bonfanti et al. 1998).

## 4. Exit from the Golgi

For secretory proteins the export site from the Golgi is located in the *trans*-Golgi Network (TGN). This compartment has been best described in animal and yeast cells, where it is a sacculo-tubular endomembrane structure at the trans-face of the GA. In these cells, the TGN seems to be more than an extension of the trans-face of the GA: upon BFA treatment, specific markers of the TGN are found in the lysosome, whereas markers of the GA localise in the ER (Lippincott-Schwartz et al. 1991).

In plant cells, the identity of the TGN, or even its very existence is still a matter of debate. In most of the studies where TGN is mentioned, no clear colocalisation with tested TGN markers are presented and the TGN is invoked to describe intermediate compartments that are neither endosomes nor prevacuoles. Nevertheless, good candidates for TGN markers have been identified. The syntaxins – classes of SNARE proteins - Syp51, AtVPS45 and Syp61 have been shown in EM studies to localise in what looks like tubulo-reticular structures (Bassham et al. 2000; Sanderfoot et al. 2001).

The TGN is more than the exit point of proteins from the GA: it is at the crossroads of the biosynthetic, endocytic and autophagic pathways.

# *D. Vesicular trafficking*

## 1. General features

Several different vesicle-mediated transports take place in a living cell. Despite their differences, a common functional model has been proposed (Kuehn and Schekman 1997; Aridor et al. 1999; Bonifacino and Glick 2004).

A vesicle buds from the donor membrane ("vesicle budding"), selectively recruiting cargo proteins while leaving the resident proteins in the origin organelle ("protein sorting"). The vesicles are subsequently targeted to an acceptor membrane ("vesicle targeting"), their membrane fuse and the vesicles release their content into the acceptor compartment ("vesicle fusion"). To balance the forward movement of membranes, the vesicle transport machinery, as well as resident proteins that have escaped the donor organelle, needs to be recycled. This is also achieved by a vesicular transport (retrograde transport).

Genetic studies have allowed to finely dissecting the components required for the formation, transport, recycling of vesicles. The elements required for a proper vesicular traffic are coat proteins, a donor membrane-associated GTPase (or a specific phosphoinositide), SNAREs, accessory proteins for fission, uncoating enzymes, cytoskeleton, Rab GTPases at the acceptor membrane and tethering factors (Bonifacino and Glick 2004).

## 2. Vesicle budding from the donor membrane

The formation of a vesicle at the donor membrane is due to the controlled assembly of a structural proteic backbone that shapes locally the membrane into a bud and finally a vesicle. It is initiated by the membrane recruitment of a small GTPase or the concentration of a specific phosphoinositide at the donor membrane. The small GTPase is membrane-associated in its GTP bound form and factors called Guanosine Exchange Factors (GEF) specifically exchange GDP for GTP in the catalytic site of the GTPase, thus favoring the association of GTP-bound GTPase with the donor membrane. The GTPase recruits to the membrane the coat components. The coat components have a double action: first they shape the membrane, secondly they recruit cargo proteins either directly (Kuehn and Schekman 1997; Aridor et al. 1999) or bound to transmembrane receptors (Griffis et al. 2002; Happel et al. 2004). The further assembly of the coat complexes and their polymerisation forces the local bending of the membrane that ends up in the formation of a spherical cage. The scission of the vesicle could be due to the action of the coat assembly on the closing cage or to the action of specific factors such as dynamins.

## 3. Composition of the main types of vesicles

Vesicular trafficking involves small GTPase that regulate the assembly of coats, protein complexes, and which activities are modified by various factors (GTP-GDP Exchange Factors, GTPase-Activating Proteins).

The COPI coat is formed of seven subunits called α-, β-, β'-, γ-, δ-, ε- and σ-COPs (for coat proteins). The assembly of the coatomer is initiated by the membrane-bound small GTPase ARF1-GTP that recruits the coat via the β- and γ-COPs. The disassembly of the coatomer is ensured by the hydrolysis of the GTP by ARF1, stimulated by a cytosolic GAP factor.

Dissociation of the COPI coat is initiated by the hydrolysis of the GTP bound to ARF1. This hydrolysis is catalysed by an ARF-GAP which is stimulated by membrane curvature, ensuring a rapid uncoating after vesicle budding (Bigay et al. 2003)

The assembly of COPI vesicles is prevented by brefeldin A. This drug prevents the activation of ARF1 by its ARF-GEF, blocking the recruitment of coat proteins to the membrane (Robineau et al. 2000).

The formation of the COPII complex in yeast, mammal and plant cells is initiated by a small GTPase Sar1p. Six homologues of Sar1p, as well as other components of the yeast and mammal COPII complexes have been identified in plants. The mechanism by which COPII coats form is similar as for the COPI coat. The cytosolic GDP-bound Sar1p is activated by Sec12p, its GTP-GDP Exchange Factor (GEF), converting it to a Sar1p-GTP complex attached to the membrane. Sar1p-GTP recruits a Sec23p-Sec24p complex through direct interaction with Sec23p, forming the pre-budding complex. Then a Sec13p-Sec31p complex polymerises onto the Sec23p-Sec24p complex, allowing cross-linking of the pre-budding complex. Dissociation of the coat requires the hydrolysis of the GTP bound to Sar1p by the Sec23p (a GTPase Activating Protein GAP), leading to a destabilisation of the coat.

Formation of the clathrin coat is regulated by Arf1 in the Golgi, but by phosphatidyl inositol 4,5 diphosphate at the PM. Adaptor proteins, such as adaptins, GGA or Hrs, link the clathrin triskelion to membrane proteins anchored in the donor membrane. There are four kinds of adaptins. The AP-2 adaptin is incorporated in vesicles that bud from the plasma membrane. It is a protein complex made of an adaptin α, an adaptin β, a polypeptide of approximately 50 kDa (AP50 or $\mu_2$) and a polypeptide of approximately 20 kDa (AP17 or $\sigma_2$). Another adaptin, AP1, is associated with vesicles budding from the TGN and the endosome. It is made of an adaptin γ, an adaptin β', a polypeptide of approximately 50 kDa (AP47 or μ1) and a polypeptide of approximately 20 kDa (AP19 or σ1). The polypeptides μ1 and μ2 (respectively from the adaptin complexes AP1 and AP2) have been shown to interact with a tyrosine motif (YXXΦ where Y represents a tyrosine and Φ an amino acid with a hydrophobic chain) or a di-leucine signal ([DE]XXXL[LI]) present in some transmembrane proteins (Ohno et al. 1995; Peden et al. 2001; Happel et al. 2004).

GGAs (Golgi-located γ-adaptin ear homology domain ARF binding protein) are another family of adaptor proteins. Here the adaptor is a monomer with adaptin-related domains. It has been shown that GGA bind to a di-leucine motif present in the cytoplasmic tail of the transmembrane cargo receptor mannose-6-phosphate receptor that sorts proteins to the animal lysosome (Dell'Angelica et al. 2000; Zhu et al. 2001). So far, GGAs have been described in mammalian cells and in yeasts but no GGA has been detected in plants.

## 4. Docking and tethering

The specificity of vesicle targeting is vastly relying on the interaction of specific Rab proteins and tethering factors.

Rabs are small GTPases ubiquitiously found in the eukaryotic cells: 11 Rab proteins have been found in yeast, animal cells have more than 60 and the genome of *A. thaliana* contains 57 different Rabs. These proteins are implicated in all the steps of vesicular trafficking, from cargo selection and vesicle formation to fusion of the vesicle to the acceptor membrane and further recycling of the vesicle. Rabs cycle between a soluble GDP-bound form and a GTP-bound membrane-attached form. Prior to membrane tethering, a specific GEF exchange the GDP bound to soluble Rab for GTP, leading to the attachment of the Rab-GTP to the acceptor membrane. Rabs recruit their effectors (tethering complexes, SNAREs, motor proteins). Among

these effectors, a GAP protein activates the Rab GTPase activity, Rab hydrolyses its bound GTP to GDP and is released to the cytosol, supported by GDI (a GDP-dissociation inhibitor), ready for a new round of membrane fusion.

Tethering factors can be divided into two groups: long rod-like coiled-coil proteins and large multisubunit complexes (reviewed in Whyte and Munro 2002). The large multisubunit complexes form a more heterogenous familly. They can act prior to Rab activation (TRAPPI), or as pure Rab effectors (COG, GARP, exocyst complexes), or both (class C Vpc complex).

## 5. Fusion of the vesicles with the target compartment

The membrane fusion events that drive the release of the vesicular cargo in the target compartment are regulated by various factors.

The attachment of vesicle membranes to their target is mediated by a class of transmembrane proteins called SNAREs for Small NSF (N-ethylmaleimide Sensitive Factor) Attachment protein Receptors. SNAREs together with cytosolic factors form complexes that specifically allow fusion of the vesicle membrane with the target organelle. Many SNARE proteins (at least 54 different SNAREs in the genome of *Arabidopsis thaliana*) have been identified in plants.

The combinations of the SNAREs located in the membrane of the donor vesicle and in the target compartment ensure the specificity of the fusion. There are two classes of SNAREs: v-SNAREs are located in the vesicle membrane, t-SNAREs (now renamed Q- or R-SNAREs depending on the presence of a conserved Arg or Gln in a central position in the main helix) in the target organelle. The formation of a SNARE-complex due to interactions between the coiled-coiled domains of v-SNAREs and t-SNAREs leads to the membrane fusion. The SNARE complex is made of four components: a v-SNARE, a t-SNARE, SNAP (soluble NSF attachment protein) and NSF. SNAREs are regulated by cytosolic factors such as Rab small GTPases. Once a v-SNARE present at the surface of the vesicle interacts with the t-SNARE present at the surface of the acceptor membrane (forming the *trans*-SNARE complex), SNAP binds to the complex and then recruits NSF. Once fusion is achieved, ATP hydrolysis by NSF forces the disassembly of the *cis*-SNARE complex, the v-SNAREs can then be recycled to the donor compartment by retrograde transport, while t-SNAREs are available for an other fusion event.

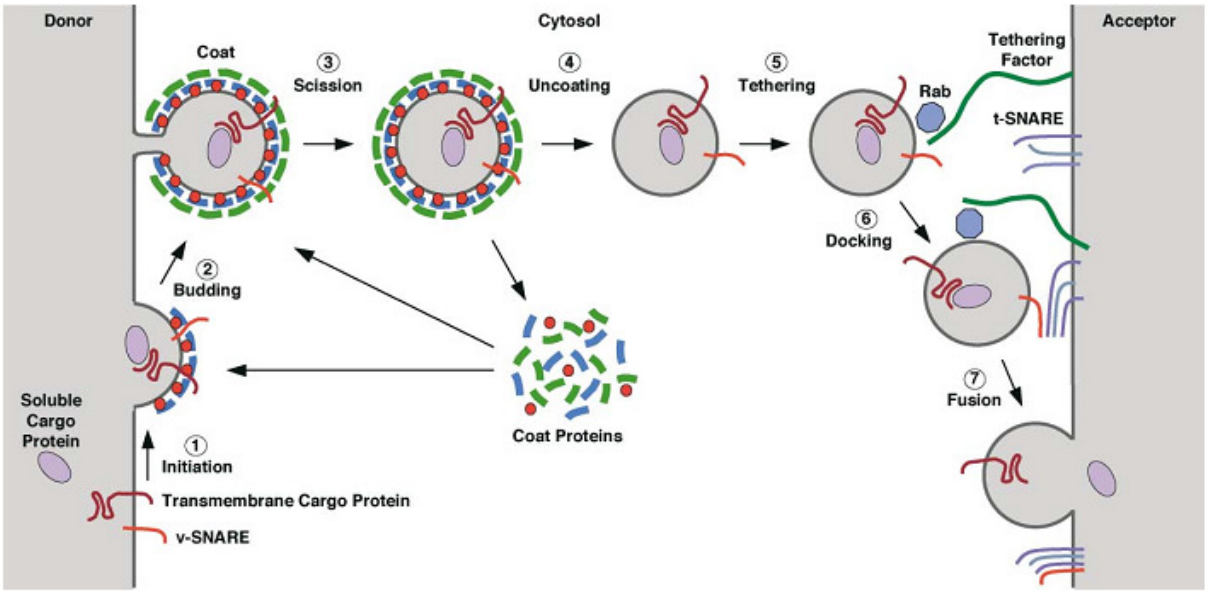



**Fig 11: Mechanisms of vesicle budding/fusion (taken from Bonifacino and Glick 2004)**
(1) Initiation: the coat proteins are recruited from the cytosol to the donor membrane by interaction with a specific small GTPase or a phosphoinositide. Transmembrane receptors and soluble cargo are gathered to the forming vesicle.
(2) Budding: structural proteins of the coat complex assemble at the bud surface and polymerise, thus bending the membrane and leading to the formation of a vesicle.
(3) Scission: the neck that links the newly formed vesicle to the donor membrane is disrupted by either direct action of coat proteins or with the help of accessory factors
(4) Vesicle uncoating: the components of the coat and the factors that initiated its formation are inactivated and disassembled.
(5) Tethering: the vesicle is brought to the vicinity of the acceptor membrane along the cytoskeleton and is tethered through the interaction of a specific Rab GTPase present at the vesicle surface and tethering factors specific to the acceptor compartment.
(6) Docking: the formation of the SNARE complex forces the two membranes to come closer.
(7) Fusion: the SNARE complex induces the fusion between the two membranes. The soluble cargo is released into the lumen of the acceptor compartment.

## *E. The sorting of vacuolar proteins depends on signal peptides*

Vacuolar proteins reach their target organelles *via* various specific ways. Membrane and soluble proteins seem to be directed differently: for example the soluble vacuolar protein phytohemagglutinin is not targeted any more to the plant vacuole upon treatment with the drugs BFA and monensin, whereas the same drugs have no effect on the vacuolar trafficking of the membrane protein α-TIP (Gomez and Chrispeels 1993; Park et al. 2004).

Several soluble vacuole proteins have a propeptide that is necessary and sufficient for their proper targeting and is cleaved once they reach the vacuole (it contains the Vacuolar Sorting Determinant). Vacuole proteins that have a faulty VSD are secreted, presumably via a default secretion pathway from the Golgi (Denecke et al. 1990; Neuhaus et al. 1994; Matsuoka and Nakamura 1999). Moreover, Matsuoka et al. (1995) have shown that if the propeptides of sweet potato sporamin and barley lectin are sufficient to drive the vacuolar sorting of the proteins, the drug wortmannin

inhibits only the sorting of the lectin. This result suggests that vacuolar proteins follow at least two different pathways depending on their propeptide.

Various VSD have been described so far, they differ by their sequences and position in the proteins. They have been sorted into three groups (Neuhaus and Paris 2005), depending on the existence of specific sequences and their position in the precursor protein: the sequence-specific VSDs (ssVSDs), the C-terminal VSDs (CtVSDs) and the condensation-dependent VSDs (conVSDs).

## 1. The sequence-specific Vacuolar Sorting Determinant

The best studied ssVSD are those found in the N-terminal propeptides (NTPP) of sporamin, a protein present in the storage vacuole of tuberous roots of sweet potato and of barley aleurain, a protease present in the lytic vacuole.

The NTPP of sporamin has been shown to contain a VSD through studies in tobacco suspension culture cells where sporamin was targeted to the vacuole, whereas a mutant form of the sporamin lacking its NTPP is secreted. Further studies have allowed identifying the peptide $^{23}$SRFNPIRL$^{30}$ as the minimum ssVSD of sporamin. Moreover, a mutational analysis by systematic replacements of N26, P27, I28 or L30 indicated the requirements at these positions. Indeed, more than a strict sequence specificity, the ssVSD of sporamin should preferably have a N26, non-acidic aa at position 27, (Ile or Leu) 28 and a large hydrophobic aa at position 30 (Matsuoka and Nakamura 1999). For sporamin and aleurain, the sequence of the VSD is more important than its location, since the propeptide of the sporamin is still functional when transferred to the C-terminus of the protein (Koide et al. 1997). In Brazil nut, an ssVSD is in fact located in a C-terminal propeptide (Kirsch et al. 1996), and in castor bean ricin the ssVSD is present in an internal propeptide (Frigerio et al. 2001).

## 2. The C-terminal Vacuolar Sorting Determinant

The proper sorting of some vacuolar proteins depend on a C-terminal propeptide, which was shown to be both necessary and sufficient. The best studied CtVSDs are those of barley lectin, tobacco chitinase A and bean phaseolin (Bednarek et al. 1990; Neuhaus et al. 1994; Frigerio et al. 1998). Contrary to ssVSDs, no consensus sequence could be determined for CtVSDs, but some studies suggest that they must be enriched in hydrophobic residues.

A mutational study of tobacco chitinase A showed that its C-terminal propeptide (GLLVDTM) is both necessary and sufficient to drive the protein to the vacuole. Interestingly, removal of the final Met or its replacement by a Phe or Lys had no effect, whereas its replacement by a Gly leads to a 50 % decrease of the sorting efficiency of the marker protein. No other single replacement had a similar effect (Neuhaus et al. 1994). A similar study of barley lectin's C-terminal propeptide also showed that there is no clear common sequence or structural organisation among CtVSDs. Moreover, the addition of two Gly at the C-terminus strongly lowered the sorting efficiency of the marker protein, confirming the idea that the sequence of the CtVSDs may be not crucial, but that the sorting relies more on the accessibility of the C-terminus of the proprotein (Dombrovski et al. 1993).

## 3. The condensation-dependent Vacuolar Sorting Determinant

These proteins do not present a clearly defined propeptide signal; instead their Vacuolar Sorting Determinant consists of different parts of the polypeptide or a very large region of the protein, which suggests a 3-dimensional structure is the sorting signal. These determinants have been described for the phytohemagglutinin of common bean and legumin-like proteins. The mechanism of sorting of such proteins is hypothesized to rely on condensation of the vacuolar proteins in the donor organelle. One determinant for condensation is thought to be enrichment in hydrophobic residues. For example, cereal prolamins aggregate in the ER where they form protein bodies. When isolated from the ER or the GA, prolamins are more hydrophobic and membrane–bound than when they are in the vacuole (Arcalis et al. 2004).

Some proteins may have more than one VSD. Glycinin is one of the major proteins of the seed storage vacuole of soybean, and is made of five major subunits. The C-terminal 10 amino acids of A1AB1b was sufficient to direct another protein to the PSV, but functional inactivation of this putative CtVSD did not block PSV sorting, indicating the existence of a second VSD. The three-dimensional structure of this subunit showed the existence of a disordered region, located at the surface of the molecule that could be a good candidate for a ssVSD. This sequence could be proven to be sufficient for vacuolar sorting. However, inhibition of the CtVSD combined with a mutation of the Ile297 to Gly present in the second identified VSD, still did not abolish the vacuolar sorting of A1AB1b, suggesting that there is a third sorting determinant in addition to CtVSD and ssVSD (Maruyama et al. 2006).

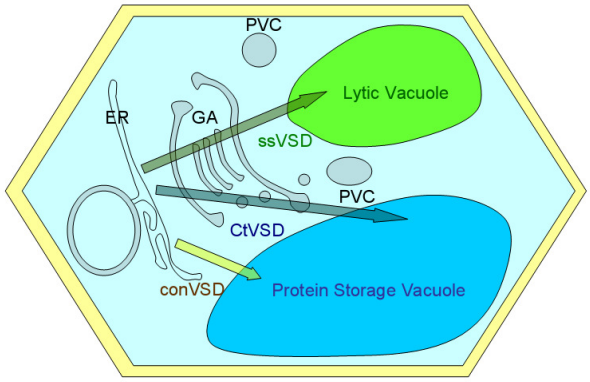



**Fig 12: the proposed pathways used by soluble proteins to reach the plant vacuoles.**
ssVSD proteins are sorted through the Golgi and prevacuolar compartments (PVC) to the LV.
CtVSD proteins follow a parallel and distinct route through Golgi and PVC to the Storage Vacuole (represented in this scheme by a PSV).
conVSD proteins form aggregates that are sorted from the ER or the Golgi to the PSV.

# III. The plant vacuole sorting receptors

## *A. The receptor-mediated sorting to lysosomes/vacuoles*

The implication of a receptor protein in targeting pathways has been postulated after the discovery of its sensitivity to saturation (Frigerio et al. 1998).

The postulated model is based on the sorting mechanism leading proteins to the lysosome in animals. Proteins destined to the lysosome are characterised by a specific oligosaccharidic motif: during their maturation in the ER/Golgi these proteins first acquire an N-linked oligosaccharide which is processed in such a way that a mannose-6-phosphate (M6P) residue is left attached to the initial oligosaccharide. A receptor present in the Golgi binds to the lysosome protein through its M6P; the complex is then incorporated in clathrin-coated vesicles that bud from the Golgi and are brought to intermediate compartments.

Two Mannose-6-phosphate receptors (M6PR) have been identified so far in vertebrates (Ghosh et al. 2003): the calcium-dependent (CD) M6PR and the calcium-independent (CI) M6PR. Both are type I integral membrane proteins, i.e with the N-terminal region in the lumen of the Golgi, followed by a single transmembrane segment and finally a C-terminal tail facing the cytosol. The CI-M6PR is significantly larger (300 kDa) than the CD-M6PR (46 kDa). The N-terminal end of CI-M6PR contains 15 repetitive domains, of which two are specific for M6P and one for Insulin-like Growth Factor, whereas its CD-M6PR counterpart has a single M6P-binding domain. Their cytosolic domains contain several sorting signals, some of which are phosphorylated or palmitoylated (Rosorius et al. 1993; Schweizer et al. 1996), and are involved in the receptor trafficking. In accordance with their structures, both proteins are involved in the sorting of M6P proteins, while the CI-M6PR also assumes the internalisation of the growth factor IGF-II for degradation in the lysosome and other physiological processes, such as cell growth and T-cell activation (Ikezu et al. 1995; Ikushima et al. 2000).

Additionally to the canonical M6P-dependent sorting mechanisms, which accounts for the majority of the protein sorting to the lysosomes, alternative mechanisms exist. For example, Shingolipid activator proteins (SAPs) use an alternative targeting mechanism to reach the lysosomes, as shown by analyses of human fibroblasts from patients with I-cell disease (ICD) that cannot add the mannose-6 phosphate to hydrolases that must be targeted to the lysosomes (Lefrancois et al. 2003). Alternative lysosomal sorting receptors are the five Vps10-D receptors, named after the homology of their N-terminal domain to the luminal domain of the yeast vacuolar receptor Vps10p (see below). Information at the molecular and cellular level confirms that the receptors of this protein family are multifunctional, bind several different ligands, and they engage in intracellular sorting as well as in endocytosis and signal transduction. Sortilin is involved in the endocytosis of lipoprotein lipase, neurotensin, and the proform of nerve growth factor (Nielsen et al. 1999; Navarro et al. 2001; Nykjaer et al. 2004), but it may also target proteins in Golgi for transport to late endosomes (Nielsen et al. 2001). Overall, there is only a modest sequence similarity between the Vps10p-Ds from Sortilin, SorLA, and the three SorCS molecules, but they all share two distinct structural features: (i) an N-terminal propeptide with a consensus sequence for cleavage by proteases of the subtilisin family of proprotein convertases and (ii) a ~120 amino acid, C-terminal segment (called 10CC) containing 10 conserved cysteins (Westergaard et al. 2004).

In contrast, in yeast cells, vacuolar proteins are recognised by a peptidic signal, e.g in the vacuolar Carboxypeptidase Y (CPY), shown to be the cargo of the vacuole sorting receptor Vps10p (Marcusson et al. 1994; Cooper and Stevens 1996). Additionally to vacuolar proteins, Vps10p has been shown to be involved in the delivery to the vacuole of misfolded proteins that exit from the ER (Hong et al. 1996). Vps10p is a 178 kDa type I integral membrane protein. As is the case of its mammalian counterparts M6PRs, the luminal part of Vps10p interacts with its vacuolar ligands. This region is made of two domains (1 and 2) that share some homology to each other. The domain 2 has been shown to contain two binding sites: one is specific to the vacuolar proteins CPY or preApeI, while the other has less specificity and could contain a binding site for malfolded proteins (Jorgensen et al. 1999).

Even if they share no similarities, which is not that surprising regarding the differences in the vacuolar determinants used in the different kingdoms, functional analogues for the M6PR and Vps10p receptors have been characterised in plants. Currently, two families of proteins are thought to assume this function, the VSR and the RMR proteins.

## *B. The VSRs (Vacuole Sorting Receptors)*

The first protein described as a plant vacuolar sorting receptor was the pea BP80. This 80 kDa integral membrane protein was isolated from the membrane of pea CCVs - vesicles that are known to be important in the trafficking from the Golgi to the vacuole – by its ability to bind to the N-terminal propeptide of barley aleurain in a pH-dependent manner (Kirsch et al. 1994). This protein was also able to bind *in vitro* to the ssVSD of sweet potato sporamin (Kirsch et al. 1994) and to the C-terminal ssVSD of Brazil nut 2S albumin (Kirsch et al. 1996).

Further studies have identified other related vacuolar sorting receptors, such as the seed-specific pumpkin PV72 or the Arabidopsis AtELP. These proteins form the

familiy named VSR (Vacuolar Sorting Receptor). Plant VSR are type I integral membrane proteins made of a first domain of approximatively 100 aa, a PA domain (Mahon and Bateman 2000), which is thought to bind the vacuolar cargo, followed by a large central region of approximatively 300 aa and finally by three EGF-like repeats, which may stabilise the interaction of the receptor and its target molecule (Cao et al. 2000). A single transmembrane domain is followed by a short cytosolic tail, containing a potential Tyrosine sorting motif which was shown to interact *in vitro* with the Arabidopsis µA-adaptin, a subunit of a putative AP complex located at the TGN (Happel et al. 2004). In a recent paper, the transmembrane and cytosolic parts of an *A. thaliana* VSR have been dissected to better identify the parts of the receptor involved in its trafficking (da Silva et al. 2006), confirming the importance of the Tyr motif.

From the available experimental data, the VSR proteins are thought to be involved in the sorting of ssVSD proteins to the LV. Nevertheless, it is now becoming clear that at least some of them are also able to drive proteins to the protein storage vacuole. In pumpkin cells, the transport of storage proteins from the ER directly to the PSV, bypassing the Golgi, is thought to be assumed by PAC vesicles (Precursor-ACcumulating). The pumpkin VSR protein PV72 was initially purified from these vesicles and is therefore presumed to traffic to the storage vacuole in these cells. In Arabidopsis, *atvsr1* KO seeds accumulate a part of their storage proteins in the cell wall (Shimada et al. 2002).

In *A. thaliana*, there are 7 variously named VSR proteins (Masclaux et al. 2005). These homologues have been named AtVSR 1 to 7 (Shimada et al.), AtBP80a to g (due to their homology to the pea BP80, see Hadlington and Denecke 2000) or AtELP1 to 7 (for EGF-Like receptor Protein, Ahmed et al. 2000). A phylogenetic analysis of VSR proteins from several plant species, both monocots (rice) and dicots (Arabidopsis, poplar, legumes, solanaceae) showed that all the available sequences can be grouped into three subfamilies (Neuhaus and Paris 2005).

| Neuhaus and Paris 2005, this study | AGI | Shimada et al. 2003 | Hadlington and Denecke 2000 | Ahmed et al. 2000 |
|---|---|---|---|---|
| AtVSR1;1 | At3g52850 | AtVSR1 | Atbp80b | AtELP1 |
| AtVSR1;2 | At2g30290 | AtVSR2 | Atbp80c | AtELP4 |
| AtVSR2;1 | At2g14720 | AtVSR4 | Atbp80a | AtELP2b |
| AtVSR2;2 | At2g14740 | AtVSR3 | Atbp80a' | AtELPa |
| AtVSR3;1 | At4g20110 | AtVSR7 | Atbp80f | AtELP3 |
| AtVSR3;2 | At2g34940 | AtVSR5 | Atbp80d | AtELP5 |
| AtVSR3;3 | At1g30900 | AtVSR6 | Atbp80e | AtELP6 |

**Table I: nomenclatures for the VSR proteins of *Arabidopsis thaliana*.**

## *C. The RMRs (Receptor Membrane RingH2)*

A new family of putative receptors was identified by its homology to the PA domain of the VSR proteins. They are composed of an N-terminal luminal domain consisting essentially of a single PA domain, but lacking 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).

It was hypothesised that the RMR protein could be a vacuolar receptor, mainly due to the homology of its luminal domain to the PA domain found to be important for the binding of VSR proteins to vacuole proteins (Cao et al. 2000).

A recent study on AtRMR2, one of the six *A. thaliana* RMR homologues, showed that it was highly expressed in protoplasts and that AtRMR2-HA (HA epitope-tagged AtRMR2) labelled a punctate structure and colocalised with ST-GFP (sialyl transferase fused to GFP a reporter of the GA) at the Golgi complex (Park et al. 2005). A mutated form of AtRMR2 used in this study provoked a mistargeting of the CtVSD vacuole protein phaseolin, but had no effect on the localisation of any of the two ssVSD reporters sweet potato sporamin or Arabidopsis aleurain-like protein. Moreover, the authors showed that AtRMR2 interacts *in vivo* with the CTPP of phaseolin at acidic pH. The complex dissociates at a neutral pH, corresponding to the environment found in the PSV. AtRMR2 functioned thus as a cargo receptor for PSV proteins by interacting with the CtVSD of phaseolin and was shown to colocalise with phaseolin on the way to the PSV.

| **Neuhaus and Paris 2005, this study** | **AGI** | **Jiang and Rogers 2000** | **Park et al. 2005** |
|---|---|---|---|
| AtRMR1 | At1G71980 | RMR, JR702 | AtRMR2 |
| AtRMR2 | At5G66160 | JR700 | AtRMR1 |
| AtRMR3 | At1G22670 | T22J18.16 | AtRMR3 |
| AtRMR4 | At4g09560 | AC006567 | AtRMR4 |
| AtRMR5 | At1G35630 | | AtRMR5 |
| AtRMR6 | At1G35625 | | AtRMR6 |

**Table II: nomenclatures for the RMR proteins in *Arabidopsis thaliana*.**

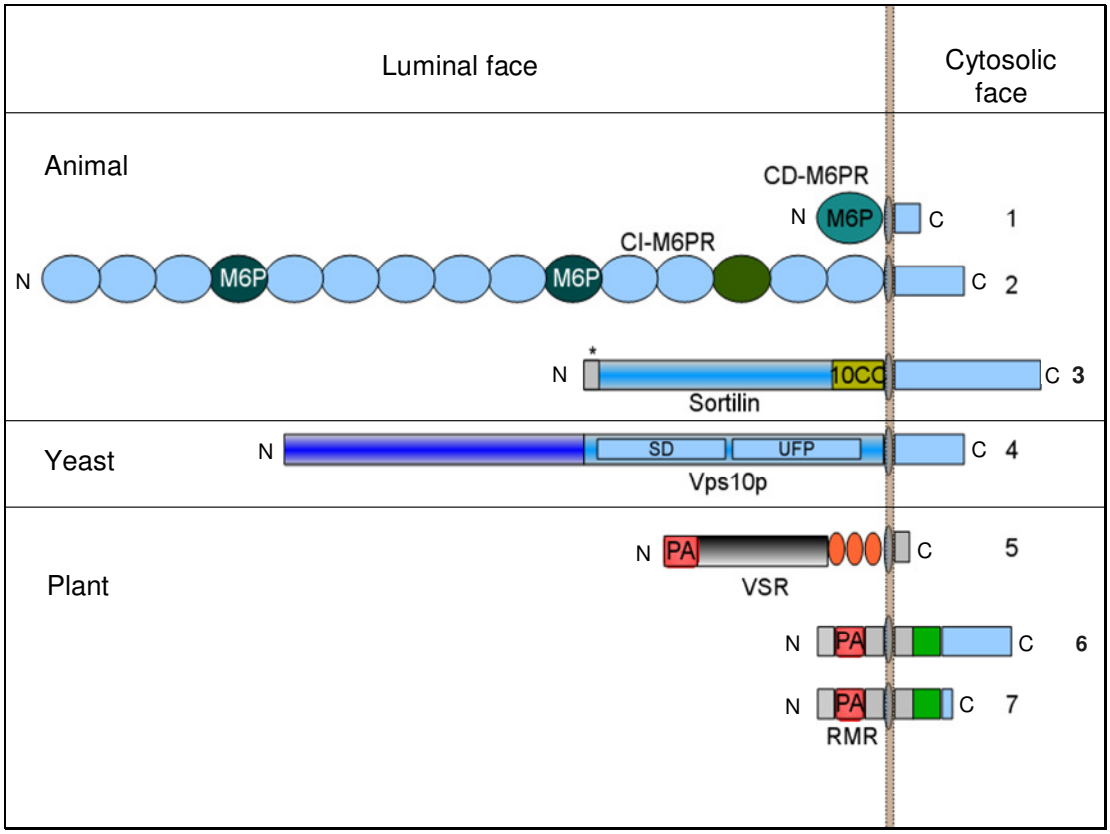



**Fig 13: Comparison of the structures of different families of vacuole/lysosome receptors. The lumen is on the left, the cytosol on the right.**
(1) CD-MPR.
(2) CI-MPR. The two MPR binding domains are represented in dark blue, whereas the IGF-II binding site is represented in green
(3) Sortilin. The luminal domain is characterised by the presence at its N-terminus a 44 aa propeptide (shown by an asterisk), a domain homologous to the domain 2 of Vps10p and a 10CC region important for the binding to the lysosomal targets.
(4) Vps10p. the two luminal Vps10 domains are represented in different shades of blue. The ligand-binding domains are represented by the boxes SD (specific for CPY) and UFP (for unfolded proteins).
(5) VSR. The luminal PA domain is represented in a red rectangle and the three EGF repeats are shown as orange ovals.
(6) and (7) AtRMR1. The PA domain is represented by a red rectangle, the cytosolic RingH2 region is shown as a green rectangle. The AtRMR1, AtRMR3 and AtRMR4 (6) have a longer cytosolic tail presenting a Ser-rich region.

# IV. Experimental aims

The data available on the VSR and the RMR protein families have inspired a model for vacuolar sorting of ssVSD and CtVSD proteins where VSR receptors would bind to ssVSD proteins and target them to the LV, whereas RMR proteins would act as receptors for CtVSD proteins and sort them to storage vacuoles.

The aim of this study was to test the involvement of AtRMR proteins in protein sorting to the vacuole and to gain further insights into their specificities.

More precisely, the questions addressed in this work are: (1) are RMR involved in vacuolar targeting, (2) are they specific to a certain class of VSD, (3) do the different AtRMR proteins have different functions.

Towards answering these questions, various experiments were carried out taking advantage of reverse genetic methods, which allowed us to study the effects of the depletion of AtRMR function on the sorting of vacuole reporters.

In a first strategy, I used protoplasts isolated from *A.thaliana* leaves. This model was helpful for these studies, as it allowed us to test the effect of dominant-negative versions of AtRMR1 on the retention of vacuolar proteins in the plant cell. I used fluorescent reporters to assess the intracellular effect of the dominant-negative constructs and enzymatic reporters, whose activity can be detected in the medium once they have been secreted and permit to calculate a secretion index that reflects the level of intracellular retention of the vacuolar reporters.

I also carried out tests on T-DNA insertion mutants of three of the AtRMR genes. I introduced fluorescent vacuolar reporters in these mutant genotypes and compared their fluorescence patterns to those observed in control wild type plants expressing the same fluorescent reporters.

In the following text, the results obtained are thus presented: (1) dominant-negative constructs of AtRMR1 influence the way CtVSD and ssVSD reporters reach the vacuole, (2) KO mutants of AtRMR1, AtRMR3 and AtRMR4 genes show similar defects in the sorting of CtVSD and ssVSD proteins and (3) I will present preliminary results obtained when studying the fitness of the mutants.

This thesis ends with a general discussion of our current models of receptor-mediated plant vacuolar sorting and of next experimental strategies to confirm and extend my results.

# Results

## I. AtRMR1 functions in the sorting of both ssVSD and CtVSD vacuolar proteins

The use of dominant-negative mutants has been shown to be a good alternative for genomic studies. In this method, overexpression of a modified version of the target protein (truncation, chimera) leads to competition between the endogenous protein and the dominant negative construct, so that the modified protein virtually replaces the endogenous protein (Herskowitz 1987). This technique has the advantage over antisense or silencing approaches that the dominant negative construct does not target the expression of the gene, which can be spread to other homologues. Secondly, such a chimeric protein can, in theory, be transiently expressed, whereas genetic approaches imply the production of stable mutants of the gene of interest, mutations that could be lethal if an early developmental function is impaired. Both naturally occurring and ectopically expressed dominant-negative genes have allowed, for example, to spot the involvement of oncogenes in cancer in animal cells (Ransone et al. 1990), to detail the machinery involved in gene expression in yeasts, prokaryotic and animal cells (Zhou et al. 1991), to study the molecular basis of some ligand-receptor interactions (Kashles et al. 1991). In plant cells, this strategy allowed to dissect events of plant development. In molecular transport mechanisms, the involvement of small GTPases and SNARE proteins is also better known thank to studies of dominant-negative proteins (Batoko et al. 2000; Geelen et al. 2002; Foresti et al. 2006). Dominant-negative mutants of AtVSR proteins have led to the conclusion that recycling of these plant vacuolar receptors is crucial for their function (da Silva et al. 2005). Park et al. (2005) have performed such experiments with a version of AtRMR2 (JR700) that lacks its luminal part. The deficient receptor is thought to be unable to bind to his vacuolar ligands, but still to be able to traffic as the endogenous receptors. Thus the truncated receptor should occupy limiting sites in the secretory pathway or exhaust limiting cytosolic factors that the endogenous receptor would have otherwise used. When overexpressed, this defective receptor led to the secretion of the CtVSD protein phaseolin but not of the ssVSD reporters sporamin-GFP or AALP-GFP (Park et al. 2005).

To study the function of the type I integral membrane protein AtRMR1 (clone JR702, in Jiang 2000), I exploited its structural organisation. It is hypothesised that RMR has a luminal domain, homologous to the PA domain found to be involved in the binding of the pea BP80 receptor to ssVSD (Cao et al. 2000), a transmembrane domain anchoring the receptor in the membrane and a cytosolic tail presumably involved in the regulation of receptor trafficking via interactions with cytosolic factors. Deletion of the N-terminal domain should disrupt its interactions with luminal ligands, whereas mutations in the C-terminal domain should interfere with its interaction with cytosolic factors and changing the transmembrane domain is also likely to change the way the construct is anchored to the membrane. Following this logic, four different constructs were studied. RMRΔlum, where the luminal domain is removed, should not be able to bind to luminal ligands. This construct should still be able to traffic similarly to the endogenous AtRMR1, so that RMRΔlum should compete with endogenous AtRMR1 for its trafficking machinery, thus leading to a saturation of the

sorting system. The RMRlumER construct, where the N-terminal domain alone is fused to the ER-retention motif HDEL, should lead to the accumulation in the ER of a soluble luminal domain of AtRMR1 and thus of its ligands. The RMRlumPM, where the N-t domain of AtRMR1 is fused to the 23 aa long TM of the LampI protein should lead to a missorting of the luminal domain of AtRMR1 and its ligands to the exterior of the cell (Brandizzi et al. 2002). The RMRcyt, consisting of the C-terminal cytosolic domain of AtRMR1, should deplete the cytosol of factors that would otherwise interact with the C-t tail of the endogenous receptor.

The experiments showed that overexpression of a truncated AtRMR1 lacking its luminal domain is sufficient to disturb the pattern of fluorescence of the ssVSD reporter Aleu$_{143}$GFP and to induce the secretion of ssVSD reporter Amy-Spo as well as CtVSD reporters Amy-bl and Amy-chi (Di Sansebastiano et al. 2001; da Silva et al. 2005). These three amylase reporters were retained in a microsomal fraction when the soluble luminal domain of AtRMR1 was overexpressed.

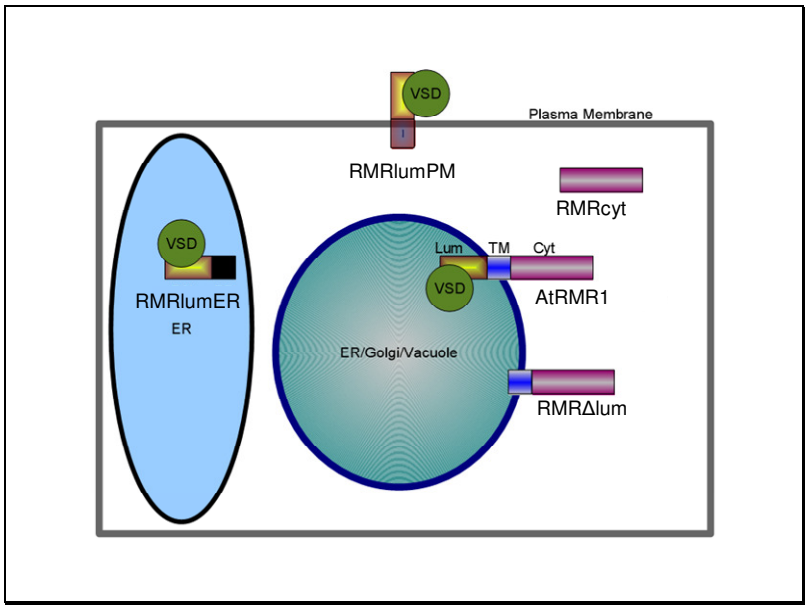



**Fig 14: Dominant negative AtRMR1 constructs used in this study.**
The complete AtRMR1 is schematised, with its luminal VSD-binding domain (Lum), its transmembrane domain (TM) and its cytosolic domain (Cyt).
The RMRΔlum construct consists of a deletion of the Lum domain of full length AtRMR1 and therefore should traffic like the receptor but should not be able to bind to VSD proteins.
The RMRlumER is made of the soluble Cyt domain fused to the ER-retention signal HDEL, so that it should be blocked in the ER together with its VSD ligands.
The RMRcyt construct consists of only the soluble Cyt domain of AtRMR1 and should compete with the endogenous protein for binding to the cytosolic regulators of its fonction.
The RMRlumPM is made of the soluble Lum domain fused to 23 aa of the LampI lysosomal protein that should send it to the PM together with its VSD ligands.

## *A. The overexpression of a truncated form of AtRMR1 causes a mislocation of a ssVSD-GFP reporter*

Previous work in the lab has shown that fusions between the green fluorescent protein and the VSDs of barley aleurain or tobacco chitinase revealed the ssVSD and CtVSD sorting pathways respectively (Di Sansebastiano et al. 2001; Fluckiger et al. 2003).

To study the importance of AtRMR1 in vacuolar sorting, we used as probes GFP fusions (see Annexes page 108) with either the whole 143 aa-long propeptide of barley aleurain N-terminal (called $Aleu_{143}GFP$), just the first 15 aa of this propeptide containing the ssVSD N-terminally fused to the GFP (called $Aleu_{15}GFP$), or the CtVSD of tobacco chitinase A C-terminaly fused to GFP (called GFPChi).

In control protoplasts transformed with $Aleu_{143}GFP$, the fluorescence was observed in the large central vacuole for 10 to 30 % of the fluorescent cells (fig. 15A). Upon the overexpression of the RMRΔlum construct, a significant proportion of the protoplasts harboured additional punctate fluorescence (fig. 15B). It is not clear which organelles are labeled, but the clear difference in fluorescence patterns reveals an effect of this construct on sorting of the $Aleu_{143}GFP$ reporter.

The $Aleu_{15}GFP$ and GFPChi reporters gave other results: in the control protoplasts transformed with the fluorescent reporters alone, a relatively small proportion of the protoplasts were labelled in the large central vacuole. Moreover, the fact that most of the protoplasts exhibiting a fluorescent vacuole were also strongly labelled in the ER makes the different fluorescent patterns hard to distinguish. Upon overexpression of the RMRΔlum construct, no noticeable difference in the fluorescence patterns could be reported. The protoplasts co-transfected with the RMRΔlum construct exhibited a weaker fluorescence that is hard to quantify by microscopy. But it is hard to conclude on the involvement of AtRMR1 in the sorting of $Aleu_{15}GFP$ and GFPChi from these results: the absence of a clear vacuolar fluorescence in the control protoplasts indicates that these two vacuolar reporters are not as efficiently sorted as $Aleu_{143}GFP$. One cannot expect the dominant negative construct of a putative receptor to have any influence on a reporter that hardly reaches the vacuole anyway.

Nevertheless, the western blot presented in Fig 16 tends to show that the GFPs are less retained in the cell when coexpressed with RMRΔlum. The GFP may thus be secreted in the medium, even if no GFP could be detected in the medium by western blots. This could be due to degradation of the fluorescent probe once in the medium, which we have no way to test.

The effect of the RMRΔlum construct repeatedly observed on the intracellular pattern of fluorescence – more precisely the decrease of fluorescence – of the GFP reporters does not allow us a clear conclusion. Even if it is unlikely, one cannot exclude the possibility that the construct affects the expression of the GFP constructs. Additionally, the observations must be interpreted very cautiously as even in the control protoplasts (i.e transfected with GFP reporters and empty vector) the pattern of fluorescence is hard to define. For example, a large proportion of protoplasts showed a faint fluorescence in the central vacuole as well as a clear labelling of reticulate/punctate structures that could correspond to anything from ER to Golgi and intermediate compartments.

These results show that the most obvious changes induced by the RMRΔlum dominant-negative construct can be seen only on single protoplasts. It is therefore difficult to firmly state an effect of the dominant-negative construct on the overall sorting of vacuolar reporters in a protoplast population, which would be more convincing. Even if the western blots tend to confirm the visual impression of a decreased fluorescence intensity when RMRΔlum was cotransfected. The non-detection of GFP in the medium does not allow us to quantify the effect.

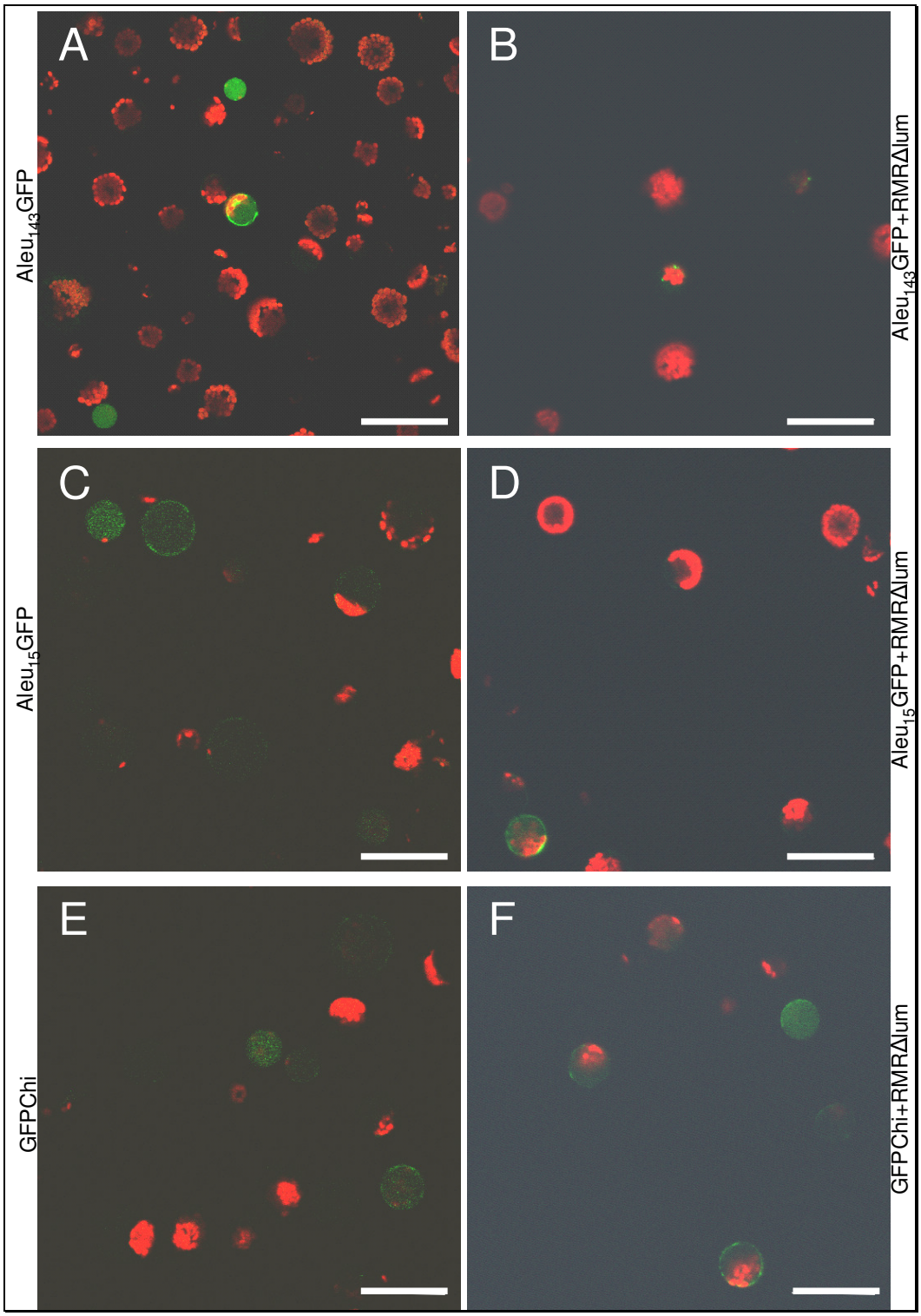



**Fig 15: pictures of transfected protoplasts isolated from A. thaliana leaves.**
Wild type protoplasts transfected with either an empty vector and Aleu$_{143}$GFP reporter, GFPchi and Aleu$_{15}$GFP (respectively pictures A, C and E) or the RMRΔlum construct and Aleu$_{143}$GFP, GFPchi and Aleu$_{15}$GFP (respectively pictures B, D and F).
Scale bars = 50 µm

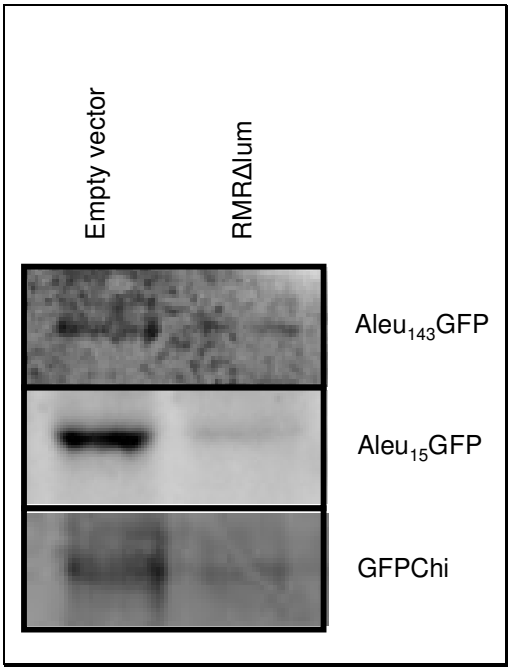



**Fig 16: western blots of the cell extracts of protoplasts.**
Immunodetection of GFP in the cell extracts of the transfected protoplasts shown in fig 15.

## *B. The truncated AtRMR1 leads to the secretion of CtVSD and ssVSD reporter proteins*

Da Silva et al. (2005 and 2006) used fusions between VSDs and barley α-amylase to monitor vacuolar sorting. They took advantage of the stability of the amylase in the culture medium to quantify the secreted vacuolar reporters. They thus showed that overexpression of a VSR receptor (AtBP80) lacking its ligand-binding domain increases secretion of the amylase fused to the ssVSD of the sweet potato sporamin. The advantage of this technique over the use of fluorescent reporters is the stability of the reporter in the medium, and the fact that instead of looking at single fluorescent reporter in single transfected cells, one can detect the abundant products of an enzymatic reaction involving each reporter enzyme molecule in the whole population of transfected protoplasts. With this assay it became possible to quantify the effect of an AtRMR1 dominant-negative constructs on the overall sorting of vacuole reporters in the whole population of protoplasts. More precisely, as the GFP reporters cotransfected with the RMRΔlum construct seemed to show less fluorescence than when cotransfected with the empty vector, I was interested in quantifying the overall secretion of the amylase reporters.

The dominant-negative construct was cotransfected with vacuolar amylase reporters in arabidopsis protoplasts and the culture and cell media were isolated after 24 hours of expression. The amylase activity was determined in these samples and a secretion index was calculated (da Silva et al. 2005). Figure 17A shows that the dominant-negative construct RMRΔlum induced secretion of the ssVSD reporter Amy-spo and the CtVSD reporters Amy-bl and amy-chi. The increase of secretion is approximately a third of the increase caused by the AtBP80 dominant-negative construct. Moreover, confocal pictures of protoplasts transfected with the RMRΔlum fused at its N-terminus with GFP (fig 17B) show that the construct is efficiently

expressed and localises to ER/dot-like structures, which could correspond to the expected ER/Golgi localisation of the endogenous receptor (Park et al. 2007).

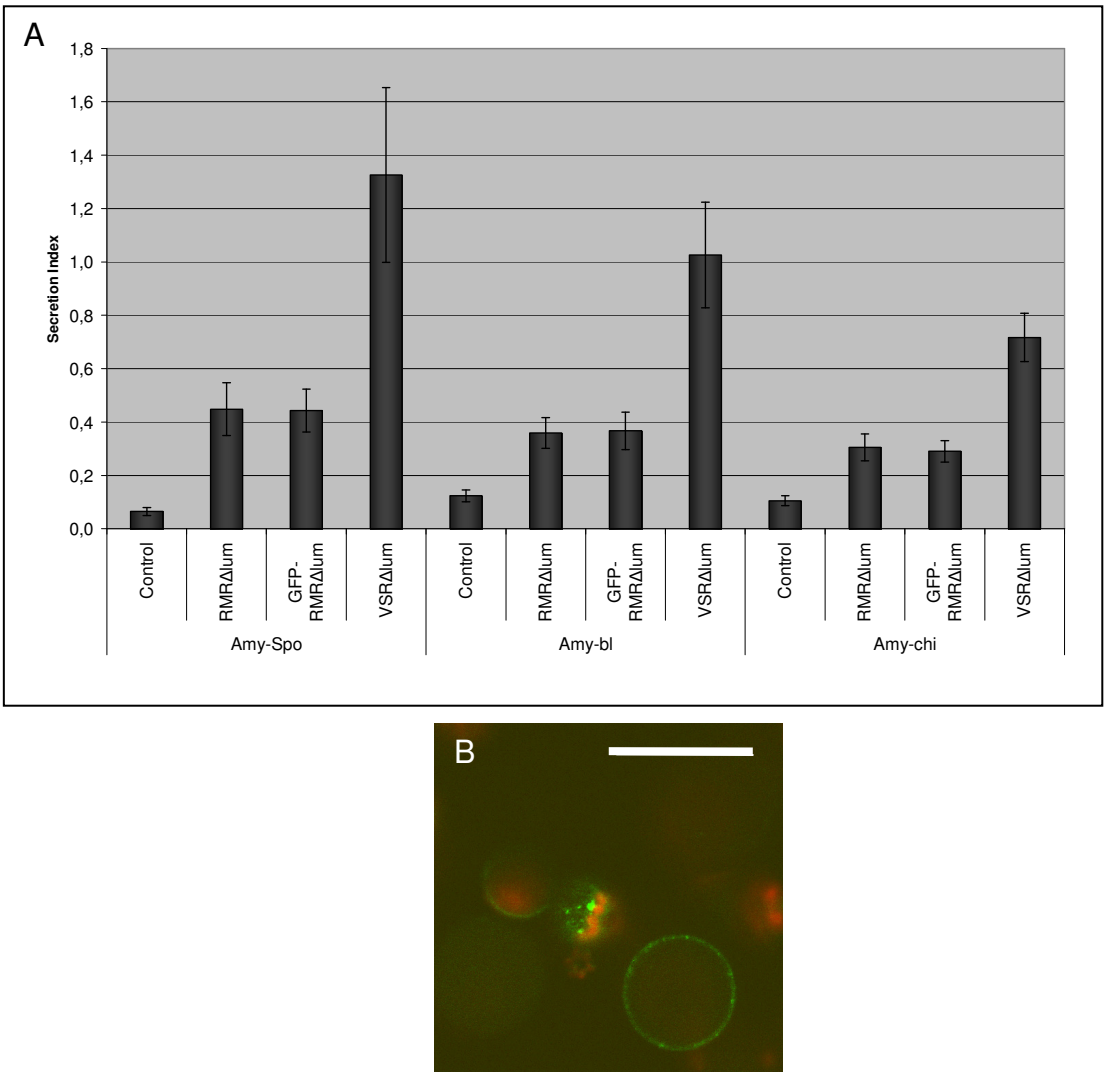



**Fig 17: secretion index of vacuole reporters.**
(A) A. thaliana protoplasts were transfected with one of the three reporters Amy-Spo (ssVSD), Amy-bl (CtVSD) or Amy-chi (CtVSD) and with either (Control) the empty vector, or the RMRΔlum construct, or the dominant-negative AtBP80a construct (VSRΔlum).
The α-amylase activity was measured in the medium and in cell extract and the secretion index was calculated.
(B) confocal picture of Arabidopsis leaf protoplasts transfected with the GFP- RMRΔlum construct. Scale bar 50 µm.

## *C. CtVSD and ssVSD proteins can bind to AtRMR1 in the ER*

The results described in the previous section denote the importance of the transmembrane and cytosolic domains of AtRMR1 in vacuolar trafficking. The dominant-negative construct was built to inhibit the movements of the AtRMR1 shuttle, without interfering with a putative binding of the endogenous protein to soluble vacuole proteins. To specifically test the *in vivo* binding of AtRMR1 to VSDs,

we constructed a gain-of-function receptor consisting of the fusion of the luminal domain of AtRMR1 with the ER-retention motif HDEL. This chimeric protein should be constitutively retained in or retrieved to the ER and thereby retain its luminal ligands. Similar truncated receptors have been used in other studies, based on VSR (PV72 or AtBP80) and have been shown to inhibit efficiently the sorting of vacuolar proteins and to cause their retention in the ER (Watanabe et al. 2004; da Silva et al. 2005).

I analysed the effect of RMRlumER on the sorting of the same amylase reporters. The RMRlumER didn't cause any change in the secretion index in protoplasts, compared with the empty vector. However, a simple fractionation, allowing to separate the vacuolar and cytosolic contents released upon an osmotic shock and the microsomal fraction (da Silva et al. 2005), indicated that the RMRlumER construct caused a cellular redistribution of the enzyme reporters: the proportion of reporter present in the microsomal fraction increased in the presence of the RMRlumER construct to the detriment of the cytosolic/vacuolar fraction.

In conclusion, the AtRMR1 gene possesses the characteristics of a vacuolar sorting receptor: (1) a dominant negative mutant of AtRMR1 induces the secretion of ssVSD and CtVSD reporters and (2) The luminal domain of AtRMR1 is able to retain the VSDs of barley lectin (CtVSD) and of sporamin (ssVSD), probably through specific interactions.

This is not in accordance to the previous model that postulates AtRMR1 proteins as vacuolar receptors specific for CtVSD proteins only, but not for ssVSD proteins that are sorted by VSR receptors.

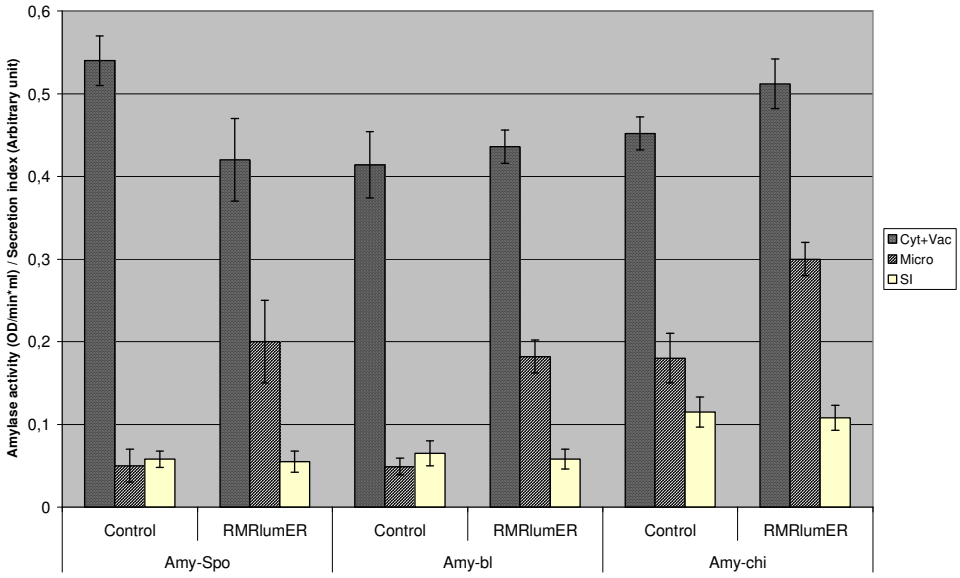



**Fig 18: intracellular distribution of vacuolar reporters.**
Protoplasts isolated from *A. thaliana* leaves were transfected with either the Amy-Spo, Amy-bl or Amy-chi reporters and (Control) an empty vector or (RMRlumER) the RMRlumER construct. A simple fractionation was performed, allowing the separation of cytosolic and vacuolar contents (Cyt+Vac fraction) and the microsomal content (Micro fraction). The amylase activity was recorded for medium and total cell extract (to calculate the secretion index) and for both fractions.

## *D. The overexpression of the soluble cytosolic domain of AtRMR1 has no incidence on the sorting of vacuolar reporters*

By comparison of the AtRMR1 sequences with VSR receptors it appears that AtRMR1 proteins have a relatively long cytosolic tail. Moreover, this tail includes a RingH2 domain, which is well documented to be implicated in protein-protein interactions (Kim et al. 2002). The cytosolic tail of VSR has been shown to contain a domain necessary for its binding to the µA subunit of the clathrin coat and the trafficking of the receptor (Happel et al. 2004). Thus it was tempting to test the effect of the overexpression of a soluble form of the cytosolic domain of AtRMR1 on the vacuole sorting. If this domain alone is involved in the recruitment of cytosolic factors involved in the formation of the AtRMR1 cargo vesicle, overexpression of the cytosolic tail should interfere with the recruitment of the endogenous AtRMR1 receptor, ultimately leading to a saturation of the vacuolar sorting machinery.

As shown in the figure 19, the overexpression of the soluble cytosolic domain of AtRMR1 had no effect on the secretion of amylase reporters.

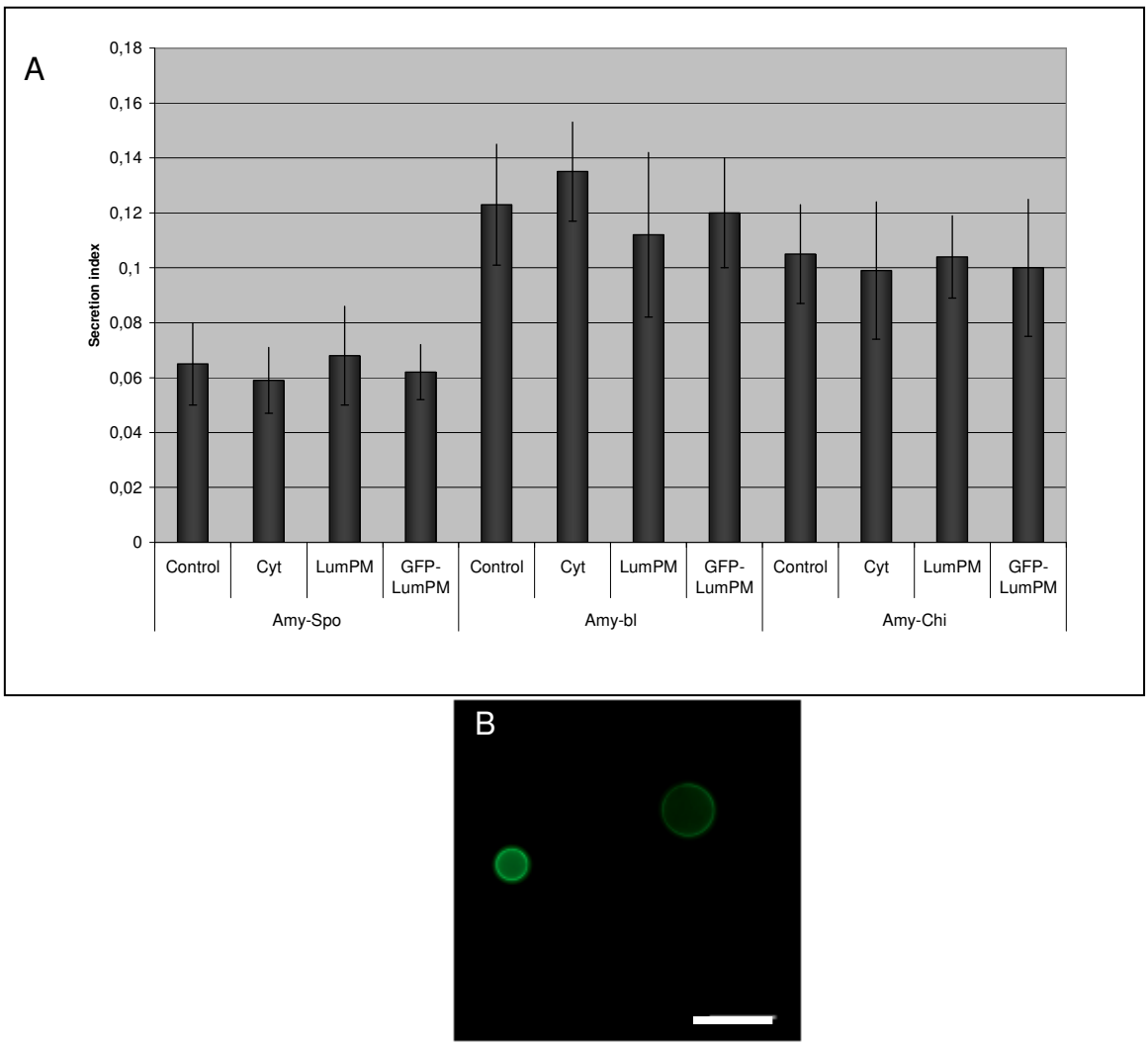



**Fig 19: secretion index of vacuolar reporters.**
(A) Protoplasts isolated from *A. thaliana* leaves were transfected with either the Amy-Spo, the Amy-bl or the Amy-chi reporters and an empty vector (Control) or the RMRcyt construct (Cyt) or the RMRlumPM construct (lumPM) or the GFP-RMRlumPM construct(GFP-LumPM). The amylase activities present in the medium and retained in the cells were measured to calculate the secretion index.
(B) Fluorescence microscope observation of protoplasts transfected with the GFP-RMRlumPM construct. Scale bar 50 µm.

## *E. The targeting of the luminal domain of AtRMR1 to the plasma membrane does not lead to secretion of the vacuolar reporters*

In our previous results (page 49), we saw that the RMRΔlum construct is able to saturate the vacuolar sorting machinery, probably by displacing the endogenous receptor from the cargo vesicle. But it does not necessarily mean that AtRMR1 is a vacuolar receptor, AtRMR1 could just be a part of the molecular machinery involved in this cellular process. We therefore attempted to force AtRMR1 to secrete actively and specifically its vacuolar targets, without interfering too much with the saturation of

the system. For this purpose we fused the luminal domain of AtRMR1 to the transmembrane domain of the LampI protein. This transmembrane domain, called TM23, was shown previously to be sufficient to drive a reporter protein to the plasma membrane in an oriented fashion (Brandizzi et al. 2002). We built the construct so that the luminal domain of AtRMR1 would be accessible to the ER/Golgi lumen and finally point to the extracellular medium, thus releasing its targets. I also used a version where the GFP protein was added to the N-terminus of the RMRlumPM.

Fig 19A shows that none of the RMRlumPM or GFP-RMRlumPM did work as expected as the secretion index was unaffected, whereas the construct seems to be expressed and sent to the PM as seen by fluorescence imaging of the GFP-RMRlumPM (fig 19B).

## *F. Preliminary conclusion: AtRMR1 is a receptor for both CtVSD and ssVSD proteins*

In plants, all tested soluble vacuolar proteins reach the vacuole through the secretory pathway. Depending on specific peptides present in their sequences, called VSD, these proteins are thought to follow two or even three different pathways. These last years several laboratories have worked on the identification of putative vacuole sorting receptors. Proteins of the VSR family have been shown to interact *in vitro* and *in vivo* with various ssVSD reporter proteins and to be involved in their sorting. Recently a new class of vacuole receptors, the RMRs, has emerged. One member of this family in *A.thaliana*, AtRMR2 was shown to bind to the CtVSD of phaseolin and a dominant negative mutant induced a change in the intracellular location of a CtVSD fluorescent reporter (Park et al. 2005). More recently AtRMR1 has been shown to bind *in vitro* and *in vivo* to the CtVSD of phaseolin. The specificity of the binding was confirmed by the fact that it was blocked when the VSD was C-terminally coupled to the resin or when two glycins were added to the C-terminal VSD (Park et al. 2007). Until now no involvement of a AtRMR1 protein in the sorting of ssVSD proteins had been reported. My results, in contrary, show that the presence of AtRMR1 is important for the sorting of both CtVSD and ssVSD reporters and that the same reporters are ligands of a soluble ER-resident AtRMR1.

Such an apparent contradiction could arise from some key differences in the methodologies and experimental strategies underlying the different publications. Clearly the results reported by Park in 2005 are the one closest to the experiments depicted on page 47. Nevertheless it may be of importance to note that the authors used different GFP reporters than those presented here: they used the CtVSD from bean phaseolin instead of tobacco chitinase or barley lectin as used in the present study; they studied the sorting of sporamin-GFP and *Arabidopsis* aleurain-like protease AALP-GFP ssVSD reporters. Park (2005) used AtRMR2, whereas Park (2007) and I worked on AtRMR1. An other important remark is that the authors looked *in vitro* at the ability of AtRMR2 to bind to VSDs, whereas I tested it *in vivo* with the RMRlumER construct. The *in vivo* and *in vitro* behaviour of RMR proteins may differ in a way that no experiment has yet identified.

Park et al. (2007) report that they did not see an effect of a similar construct as the RMRΔlum mentioned here. It is consistent with the critics raised at page 47 concerning the relevance of using GFP reporters to monitor the effect of inhibitors on protein sorting to the plant vacuole. They gave good evidence for the *in vitro* binding of the chitinase A VSD to the luminal domain of AtRMR1. The results they showed

with the ssVSD of barley aleurain however are less convincing as they seem sensitive to the experimental conditions. Indeed they correlate the observed retention of the luminal domain of AtRMR1 to the fact that the resin used to test the binding to the sporamin ssVSD may harbour more bound VSD peptides than the resin coupled to the CtVSD of tobacco chitinase A or phaseolin. In this respect, the *in vivo* affinity for the sporamin ssVSD shown by the RMRlumER construct may reflect a weak but physiologically relevant binding of the receptor to ssVSD ligands. This weak binding may explain the *in vitro* observations made by Park et al. (2007).

The fact that the cytosolic domain alone was unable to interfere with the vacuolar sorting indicates that the transmembrane domain, or at least a membrane attachment, is important for that function. Such a construct has been shown to have a similar effect when a VSR backbone is used (da Silva et al. 2006), so it ultimately shows that to efficiently interact with – and consequently trap - the endogenous partners of AtRMR1, the cytosolic tail needs to be attached to the transmembrane domain. It is however not clear why the plasma membrane construct did not induce the secretion of vacuolar reporters. The TM23 segment could be implicated in the segregation of the construct in a part of the secretory pathway where vacuolar proteins are not accessible to the luminal domain of AtRMR1 (the way proteins enter the ER may influence their sorting). A possible explanation could also be that the TM domain is important in the binding to the VSD, and hence replacing it by TM23 could interfere with the binding.

The fact that our dominant-negative constructs lacking the TM domain (i.e RMRcyt and R2PM) do not influence the vacuolar sorting of the reporter used show that this domain may have a more active role than anchoring the receptor in the membrane. More precisely, it is possible that the RMRlumPM traffics via a different route than the endogenous AtRMR1, where it has poor chances to interact with VSD, due to unfavourable conditions of pH, calcium concentration, etc. The RMRcyt inefficiency may be explained if we imagine these AtRMR1 docking sites constituting tightly regulated regions in the endomembrane system where the receptors are concentrated together with their soluble cytosolic partners, whereas the soluble cytosolic domain of AtRMR1 may be too diluted to efficiently compete with these concentrated endogenous receptors.

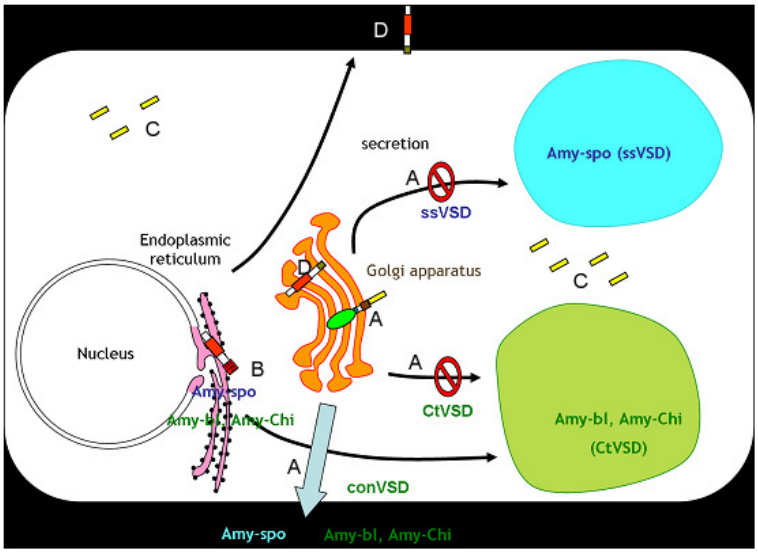



**Fig 20: summary of the results of the dominant negative approach.**
(A) The RMRΔlum construct blocks the vacuolar sorting of Amy-Spo (ssVSD), Amy-bl (CtVSD) and Amy-chi (CtVSD), leading to a saturation of the traffic machinery and the secretion of the amylase reporters.
(B) The RMRlumER constructs retains the amylase reporters in microsomal fractions.
(C)The RMRcyt does not interfere with the vacuolar sorting of the reporters.
(D) The RMRlumPM construct reaches the PM but does not lead to secretion of the vacuolar reporters.

In conclusion, the AtRMR1 protein has all the requirements for a vacuole sorting receptor, involved in both CtVSD and ssVSD protein sorting.

In *A. thaliana*, AtRMR1 is one of the six expressed AtRMR proteins. Contrary to the relative uniformity of the AtRMR1 homologues present in other plant genomes (see annexes page 115), the six AtRMR genes present important differences, mainly in the cytosolic tail, that could reflect a functional divergence of the AtRMR. For instance, the various AtRMR could have different VSD ligands. It is therefore of great interest to test the involvement of this various proteins in vacuolar sorting and especially to compare their ligand spectra. This thesis will continue with the study of KO mutants of three *AtRMR* genes.

# II. Single KO mutants for AtRMR1, AtRMR3 and AtRMR4 genes are impaired in vacuolar sorting

Since genomes of living beings started to be sequenced, reverse genetic techniques have become widely used to study the functions of genes of interest. This powerful technique can be applied to a large variety of organisms covering all three kingdoms. Depending on the organism, various mutagenesis techniques can be used to create banks of mutants that can be genetically characterised.

In the plant field, the most common approaches use mutants created by physical methods (radiations), chemical point mutation (using EthylMethyl Sulfonate) (Sega 1984) or the insertion in the gene of interest of a large DNA element (transposon or T-DNA tagging; (Topping et al. 1995; Sundaresan 1996; Azpiroz-Leehan and Feldmann 1997; Bouche and Bouchez 2001).

All these techniques have the big disadvantage to be random, which implies that to obtain a mutation in a particular gene, one has to screen a large number of mutants. Among these techniques, the T-DNA insertion mutation presents the advantage of being easily screened by PCR and southern blot, as the sequence of the transposed element is known.

Nowadays, reverse genetic work has been simplified by the existence of banks of mutated seeds. Using the sequence of a gene of interest, its accession number or its locus number as a query, one can browse these banks online (http://www.arabidopsis.org/; http://signal.salk.edu/) to identify seed pools with a mutation in or close to the query sequence and finely obtain a portion of the seeds for further testing.

## *A. Identification of AtRMR KO mutants in the library of the SALK Institute*

The genome of *A. thaliana* contains six AtRMR genes (see the alignement of the six genes in the Annexes). I screened *in silico* the library of the SALK institute (http://signal.salk.edu/cgi-bin/tdnaexpress) using either the cDNA sequences of the 6 AtRMR or their accession numbers.

I first identified three lines and obtained homozygous plants for each. The results reported in the following sections have been obtained with these plants; three other lines (SALK_052180, SALK_009129, SALK_039663) have later completed our collection but haven't been tested so far.

Fig 21 shows the structure of the different AtRMR genes and the location of the inserts. In the three lines we have tested, the insert is located at the 3' end of the gene (AtRMR3 and AtRMR4) or in the 3' untranslated region (AtRMR1).

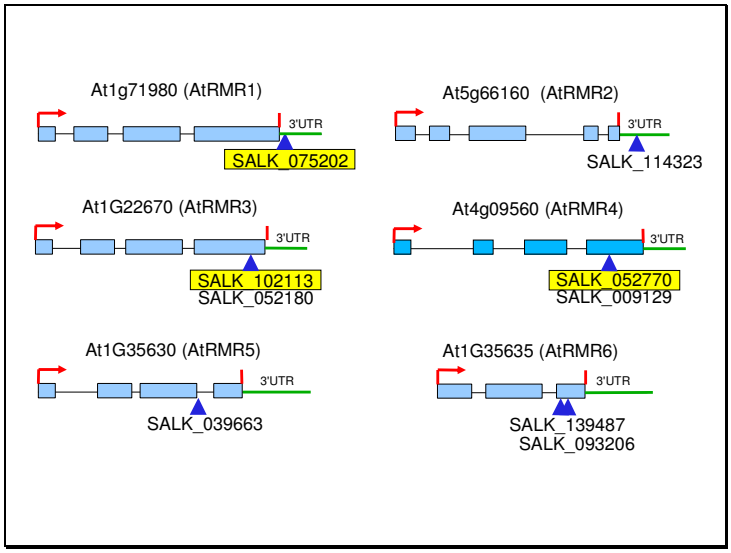



**Fig 22: Structure of the T-DNA insertions in the *atrmr1*, *atrmr3* and *atrmr4* mutants. T-DNA insertions are marked by yellow arrows.**
The lines used in this study are marked by yellow rectangles.
The lines presenting an insertion in the AtRMR2 and AtRMR6 loci were not yet available during this study.

Such insertions could have different impacts on the expression of the genes: they could lead to a truncated protein or impair the stability of the mRNA and thus the production of the encoded protein.

I screened these plants for their homozygosity both by looking at the segregation in their progeny of the resistance to kanamycin and by PCR. The screen by PCR is based on the use of two primers that target the plant genomic sequence upstream and downstream of the T-DNA insert and a third primer that is specific for the T-DNA (fig 22A). When the reaction is carried out using the three primers on wt plants, the approximately 1000 bp genomic region is amplified. If the plant is homozygous for the insert, the PCR product corresponds to the 500 bp sequence that separates one genomic-specific primer and the T-DNA-specific primer. A heterozygote produces both PCR fragments.

RT-PCR proved that in the three KO only one AtRMR is deleted (fig 22B).

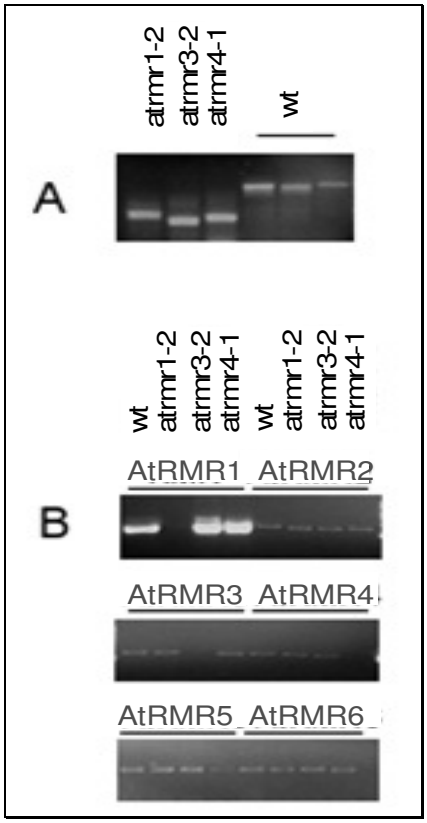



**Fig 22: molecular characterisation of the mutants.**
(A) screen for homozygotes by PCR.
(B) RT-PCR made on homozygous plants show they are single KO.

## *B. Crosses between transgenic GFPChi plants and either atrmr1 or atrmr4 show a different pattern of fluorescence compared to control GFPChi plants*

To test an impairment in the sorting of vacuolar proteins in the KO mutants, I used markers already available in the lab, i.e reporter plants expressing the GFPChi or $Aleu_{143}$GFP reporters or agrobacteria bearing the GFPChi and $Aleu_{143}$GFP reporter genes under the 35S promoter.

My first intent was to test *in planta* the effect of the depletion of AtRMR proteins on the GFPChi reporter, as they were supposed to be the receptors for CtVSD proteins (cf our first model in the introduction p. 48).

I first crossed the *atrmr1* and *atrmr4* and reporter plant expressing the GFPChi and compared them to wt col0 crossed the same way to GFPChi reporter plants.

I tested crosses for the kanamycin resistance, fluorescence (luckily, the cotyledons show fluorescence, Fig 23, pictures A, D and G) and by PCR to detect plants being both homozygous for the KO insertion in the *AtRMR* gene and having the GFPChi transgene. These plants are termed hereafter *atrmr1*(-/-)XGFPChi and *atrmr4*(-/-)XGFPChi.

For each cross I made confocal observations. Fig 23-A to C show a typical fluorescence pattern observed in reporter plants expressing the GFP fused to the CtVSD of tobacco chitinase A: in cotyledons, fluorescence is restricted to the ER and

spindle-shaped compartments that look like ER-bodies (Matsushima et al. 2003) in epidermal cells and no fluorescence is observed in mesophyll cells. The roots show a strong fluorescence in small punctate structures and ER bodies. The crosses between *atrmr1* and *atrmr4* and the GFPChi reporter plants exhibit strikingly different patterns: in cotyledons, approximately 50 to 70 % of the epidermal cells showed a clear fluorescence in the large central vacuole, these cells were also characterised by strongly fluorescent punctate structures accumulated at the edge of the cell (fig. 23 arrow). In fully developed leaves the more striking phenotype is an almost complete absence of fluorescence. Indeed fluorescence is only occasionally present in patches at the periphery of the cells.

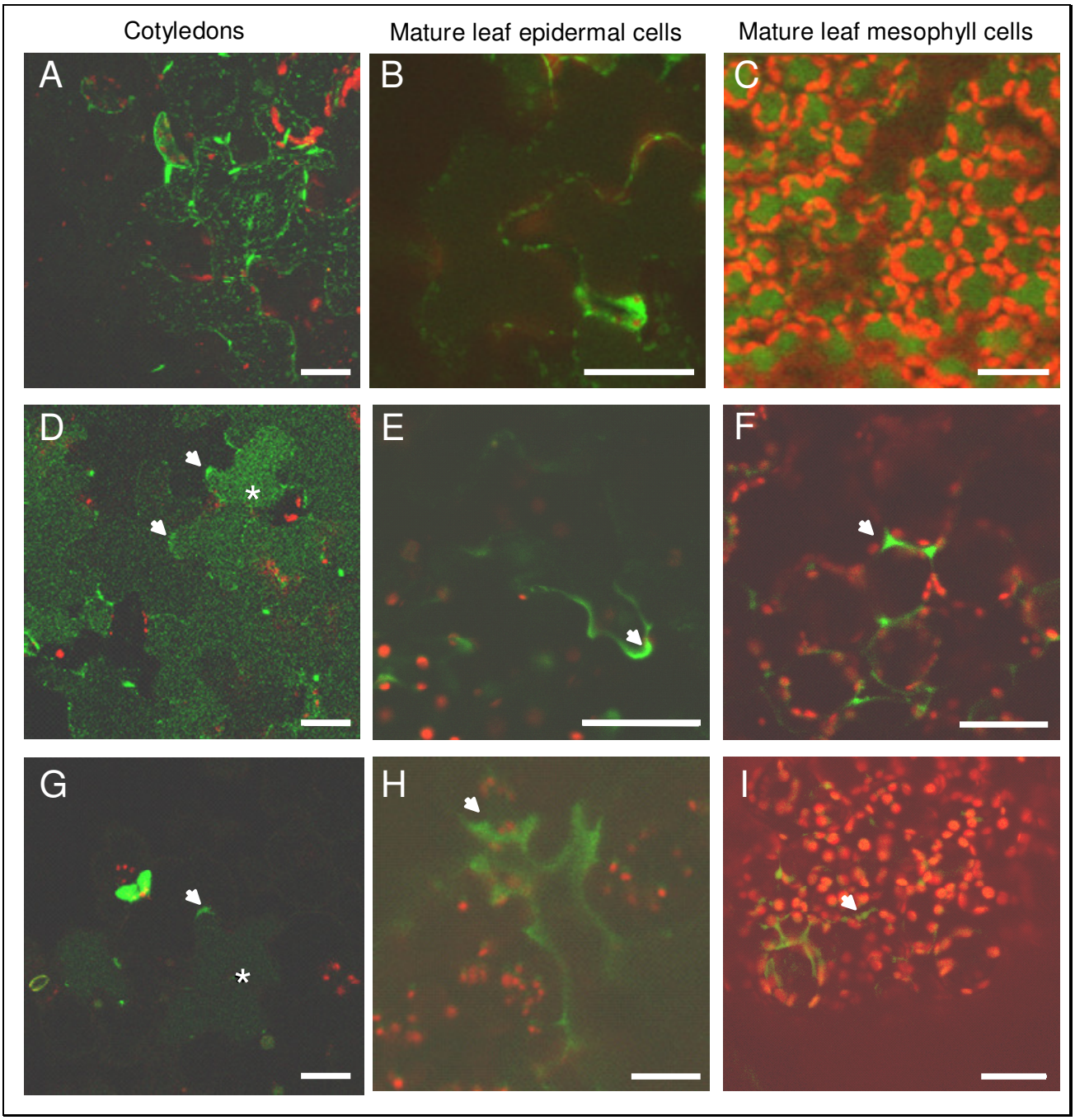



**Fig 23: localisation of the GFPChi vacuolar reporter in *A. thaliana* WT plants and in two KO lines.**
Confocal pictures of cotyledons, mature leaf epidermal cells and mature leaf mesophyll cells in GFPChi plant (respectively pictures A, B and C), *atrmr1(*-/-)XGFPChi (pictures D, E and F) and *atrmr4*(-/-)XGFPChi (pictures G, H and I).
Arrows point to fluorescence accumulated at the periphery of the cell. Asterisks indicate fluorescent central vacuoles in epidermal cells. Scale bars = 100 µm.

The observed almost complete absence of fluorescence could be due to the fact that during the maturation of the leaves, the *de novo* synthesised fluorescent reporter

is totally secreted and possibly degraded in the apoplast. But it is not possible to exclude the possibility that the fluorescent reporter is not synthesised. To test the effect of the KO on the sorting of vacuolar reporters in mature leaves cells, the protoplasts system is a model of choice. In these cells, the need to rebuild a cell wall stimulates the secretory apparatus which could amplify the possible effects of the mutations on the sorting of the vacuolar reporter. Protoplasts can also be easily separated from the medium, allowing the separate analysis of the proteins present in the cells or secreted into the medium.

Fig. 24 presents the patterns of fluorescence observed in protoplasts isolated from a GFPChi reporter plant, an *atrmr1*(-/-)XGFPChi plant and an *atrmr4*(-/-)XGFPChi plant. In the control GFPChi protoplasts we see that the vast majority of the cells show an ER-like/punctate pattern, a small proportion of small, chloroplast-rich protoplasts – presumably originating from photosynthetic mesophyll – have a fluorescent central vacuole. In the two KO backgrounds, the fluorescence is drastically reduced and mainly restricted to the ER. In these protoplasts, the transient overexpression of full length *AtRMR1* gene enhances greatly the fluorescence and some protoplasts exhibit fluorescence in the central vacuole. This last observation indicates that the full length AtRMR1 is able to complement both the *atrmr1* and *atrmr4* KO.

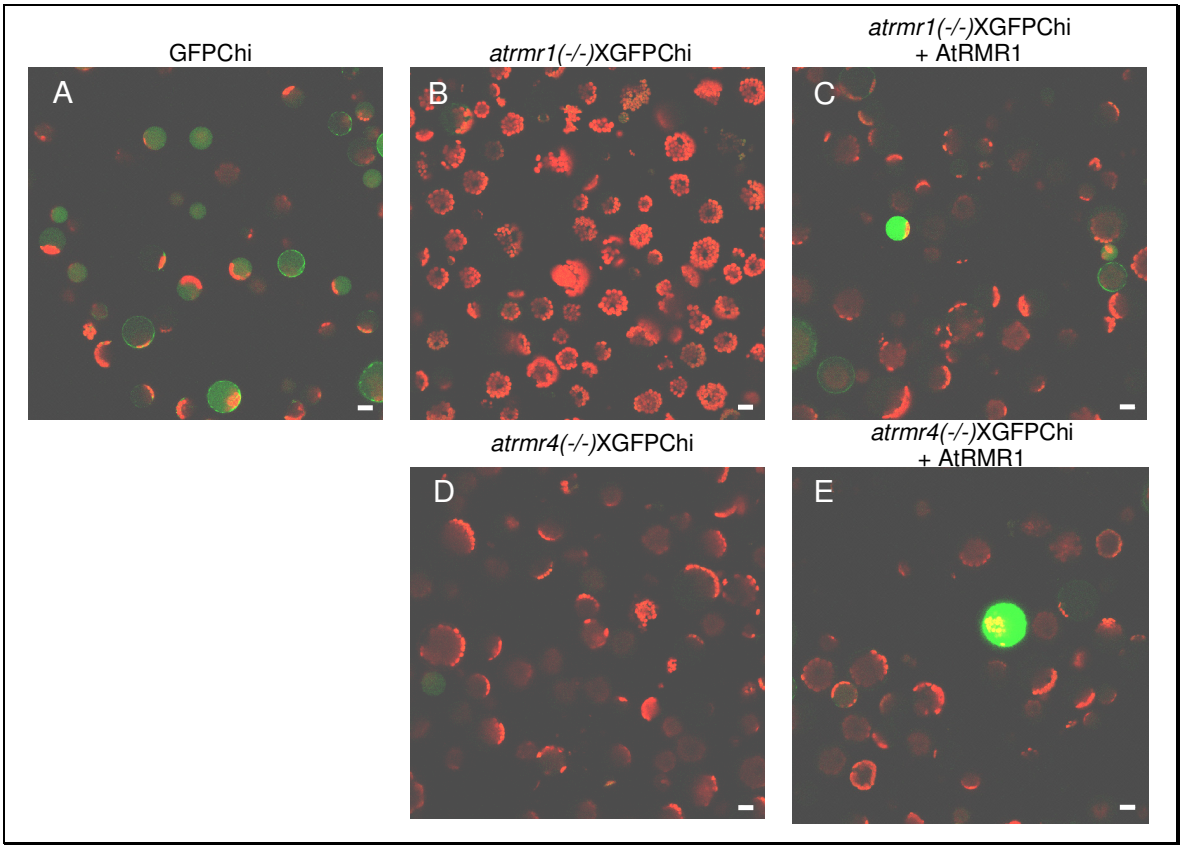



**Fig 24: The AtRMR1 cDNA restores the vacuolar sorting of GFPChi in leaf-derived protoplasts from *atrmr1(-/-)*XGFPChi and *atrmr4(-/-)*XGFPChi.**
Control GFPChi transgenic plant transfected with an empty vector (A); *atrmr1*(-/-)XGFPChi transfected with an empty vector (B) or the cDNA of AtRMR1 under the control of the 35S promoter (C): *atrmr4*(-/-)XGFPChi transfected with either an empty vector (D) or the full length cDNA of AtRMR1 under the control of the 35S promoter (E). Scale bars = 10 µm

The western blot analysis of these protoplasts confirmed the influence of the overexpressed full length AtRMR1 in the two crosses. While the control GFPChi plant showed the strongest GFP signal, in the protoplasts isolated from the crosses, the signal was barely detectable. Upon transfection with the AtRMR1 full length cDNA, the GFP signal in the crossings is strongly increased, even if it did not reach the levels observed in control plants. Moreover, in the control plants as well as in the mutants complemented with the AtRMR1 cDNA we clearly saw two bands, corresponding most likely to a partial maturation of the GFPChi fusion once it reached the vacuole. This means that the AtRMR1 construct can restore at least partially the sorting of the GFPChi reporter in these protoplasts.

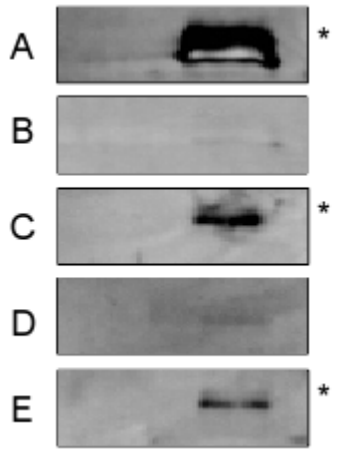



**Fig 25: The AtRMR1 cDNA induces a intracellular accumulation of GFPChi in leaf-derived protoplasts from *atrmr1(-/-)*XGFPChi and *atrmr4(-/-)*XGFPChi.**
Immunodetection of GFP in extracts of leaf-derived. The left part of each line corresponds to the medium and the right part to the cell fraction; the asterisk corresponds to higher molecular weight GFPChi (unprocessed).
(A) Transgenic GFPChi protoplasts transfected with an empty vector.
(B) *atrmr1*(-/-)XGFPChi transfected with the empty vector.
(C) *atrmr1*(-/-)XGFPChi transfected with the cDNA of AtRMR1 under the control of the 35S promoter.
(D) *atrmr4*(-/-)XGFPChi transfected with the empty vector.
(E) *atrmr4*(-/-)XGFPChi transfected with the cDNA of AtRMR1 under the control of the 35S promoter.

As the crossing of mutants and reporter plants appeared particularly time consuming, especially due to the lack of distinct screening methods for the KO and the GFPChi, we decided to continue our investigations by introducing the reporter proteins into the KO background by agrotransformation.

### *C. atrmr1, atrmr3 and atrmr4 seem to accumulate Aleu$_{143}$GFP and GFPChi in the apoplast in agroinfiltrated leaves*

The stable transformation of most higher plants is a tedious and time-consuming technique. An interesting alternative is the use of transient assays mediated by agroinfection. Transient expression provides a rapid method for assaying the function

of some types of gene: transgenes can often be assayed within a few days of infiltration (Wroblewski et al. 2005).

I transformed leaves of Columbia wt, *atrmr1*, *atrmr3* and *atrmr4* plants with either Aleu$_{143}$GFP or GFPChi plasmids by agrobacterium infiltration. Three days after infiltration, a clear fluorescence could be observed in control wt plants (fig. 26-A and B). The fluorescence patterns were in accordance to what was seen in stably transformed wt plants: the GFPChi fluorescence was seen in the large central vacuole of the mesophyll cells, while it appeared in small dots/ER in the epidermal cells. The Aleu$_{143}$GFP appeared in the large central vacuole of the epidermal cells, and was not detected in the mesophyll.

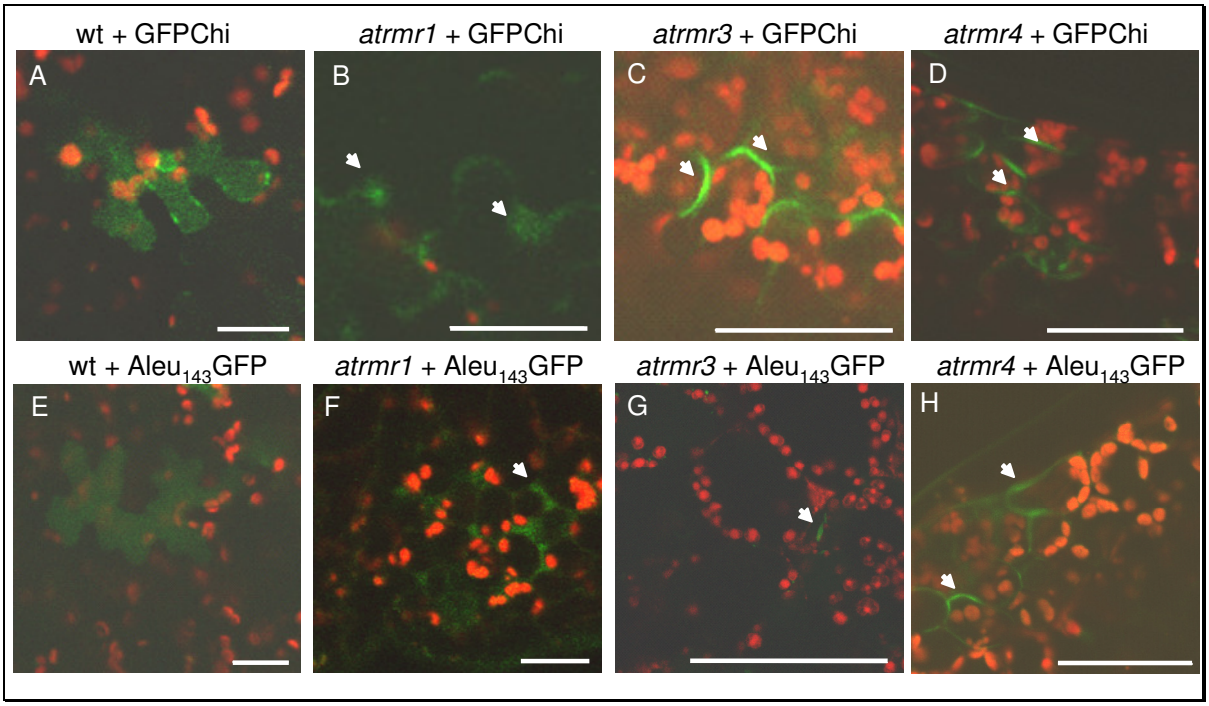



**Fig 26: localisation of GFP reporters in agroinfiltrated leaves of *A. thaliana*.**
Wild type, *atrmr1*, *atrmr3* and *atrmr4* KO leaves transiently expressing either the GFPChi reporter –respectively pictures A, B, C and D- or the Aleu$_{143}$GFP reporter –respectively pictures E, F, G and H.
Scale bars = 100 µm

Even if agroinfiltration allows to test the various genetic backgrounds without the use of time-consuming techniques such as crossing and stable transformation, it has a big disadvantage. In our case, this technique is not very efficient as it can be performed only on mature leaves. As shown in the crosses (page 60), we could expect to observe only a few fluorescent cells, so we had to screen many leaves.

Nevertheless, the fluorescence patterns give clues to what happens in the cells.

## *D. Leaf-derived protoplasts from mutant plants are less fluorescent than those from wt plants when transfected with Aleu$_{143}$GFP or GFPChi*

To confirm the intracellular retention defect of GFP-VSD in agroinfiltrated leaves, the fluorescent reporters were used to transfect protoplasts isolated from leaves of

the three mutant backgrounds and compared to leaf protoplasts from the wild type Columbia genetic background.

Fig 27 shows the typical fluorescence patterns. Control protoplasts exhibited the usual patterns. In the three different mutant backgrounds, the fluorescence was clearly lower than was seen in wild type protoplasts.

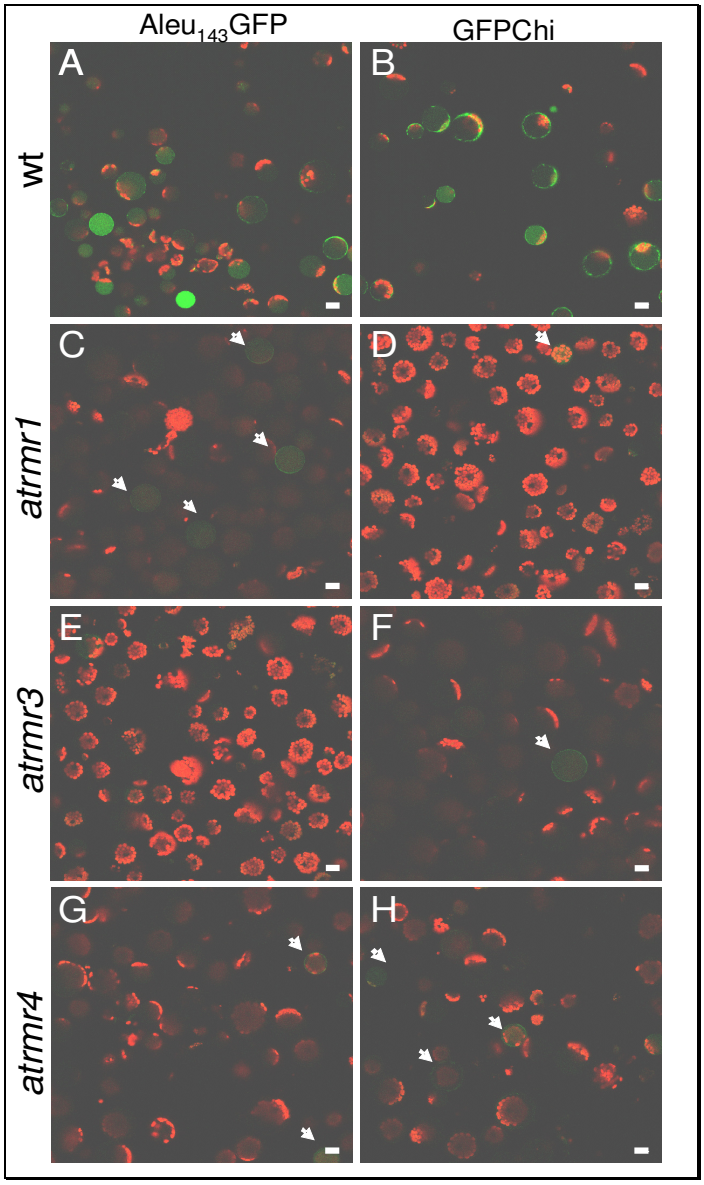



**Fig 27: localisation of GFP reporters in protoplasts isolated from *A. thaliana* leaves.**
Wild type, *atrmr1*, *atrmr3* and *atrmr4* protoplasts transiently expressing either the $Aleu_{143}$GFP reporter –respectively pictures A, C, E and G- or the GFPChi reporter – respectively pictures B, D, F and H. Arrows point faintly fluorescent protoplasts.
Scale bars = 10 µm

## *E. Conclusion*

The genome of *A. thaliana* presents five genes homologous to *AtRMR1*. As convincing evidence supports the idea of AtRMR1 being a vacuolar sorting receptor (Jiang et al. 2000; Park et al. 2005; Park et al. 2007), it is tempting to think that the six RMR genes constitute a family of vacuolar sorting receptors. Together with the seven VSR genes found in *A. thaliana*, thirteen proteins could possibly have this function. This raises the question of the specificity of these molecules. To address this question I decided to study KO mutants available from the SALK institute.

The aim of this study was to introduce vacuolar reporters in the different mutant background obtained so far in the lab. Whole plant experiments performed on crossings between KO for *AtRMR1* and *AtRMR4* and the CtVSD reporter GFPChi showed that in the mutant, the intracellular targeting of vacuolar proteins is disturbed. Interestingly the observed phenotype was the same for the two different backgrounds which was not expected if they act on different pathways. The CtVSD GFPChi and the ssVSD reporter $Aleu_{143}$GFP were transiently introduced in KO mutants for *AtRMR1*, *AtRMR3* and *AtRMR4* by agroinfiltration of leaves. In the mutant backgrounds, the fluorescence was restricted to discrete peripheral parts of the cells, whereas in the wild type genetic background GFPChi was mainly observed in ER or ER-associated organelles whereas $Aleu_{143}$GFP was seen in the large central vacuole.

Taken together, these results are in favour of a theory where the three genes are involved in the sorting of both CtVSD and ssVSD proteins.

# III. Preliminary observations on KO mutants: searching for a straightforward phenotype

In the previous chapter I reported results obtained by introducing reporter proteins into AtRMR mutants via various methods.

These plants lacking one of these putative vacuolar sorting receptors appeared to be impaired in the vacuolar trafficking of the vacuolar reporters.

Since each line lacks a single AtRMR, it is possible to test the importance of each putative receptor and whether various versions of the receptors are able to complement the vacuolar sorting defect. To further study the involvement of AtRMR proteins in vacuolar sorting, it would be helpful to be able to discriminate RMR-deficient from lines where the sorting machinery is restored, without having to introduce reporter proteins. This strategy requires a clear phenotype for the mutants.

The first hint of a mutant phenotype came from early observations of mutant plants sown on MS plates: when grown in parallel with wt plants some of the KO plants showed a strong purple coloration of the cotyledons. According to literature, this colour is due to an accumulation of anthocyanins in the vacuole, probably in response to a stress (Piao et al. 2001; Giacomelli et al. 2006). Plant vacuoles are involved in the plant response to various stresses: they store defence compounds against pathogens, participate in plant cell osmoregulation, cell turgor and plant fitness. Therefore mutants impaired in vacuolar sorting are likely to be impaired in the resistance to these stresses.

I report here preliminary results of a study of the effects of salt on *atrmr* KO plants.

## *A. atrmr1, atrmr3 and atrmr4 show an increased sensitivity to salt stress*

Wt, *atrmr1*, *atrmr3* and *atrmr4* plants were grown on soil for three weeks. I then injected into the soil a 3 M NaCl solution, so that the final concentration of salt in the soil was approximately 300 mM (Hamiduzzaman et al. 2005). The plants that survived the salt treatment for three days were allowed to recover after watering. A week later the surviving plants were counted (fig 28, upper graph). The *atrmr3* and *atrmr4* plants seemed less susceptible (or more resistant) to the salt stress under these conditions. But after the salt treatment, some pots exhibited salt crystals at the surface, putting suspicion on the homogeneity of the salt treatment.

To avoid this crystallisation, I proceeded similarly with 150 mM salt. In this case, the salt treatment did not cause plant death, but leaves became wilted, and therefore I counted rosettes showing this response. Under these conditions no difference between the different lines could be detected. Other attempts failed to reproduce the previously observed effect.

As mentioned before, the main difficulty was to apply on soil a uniform concentration of NaCl. I decided to switch to *in vitro* conditions. Firstly I tested the susceptibility of the different genetic backgrounds to increasing concentrations of NaCl (fig 28, lower graph). Mutants appeared slightly more susceptible to increasing concentrations of salt up to 150 mM NaCl, where the mutant seeds didn't germinate anymore, whereas wt seeds still did.

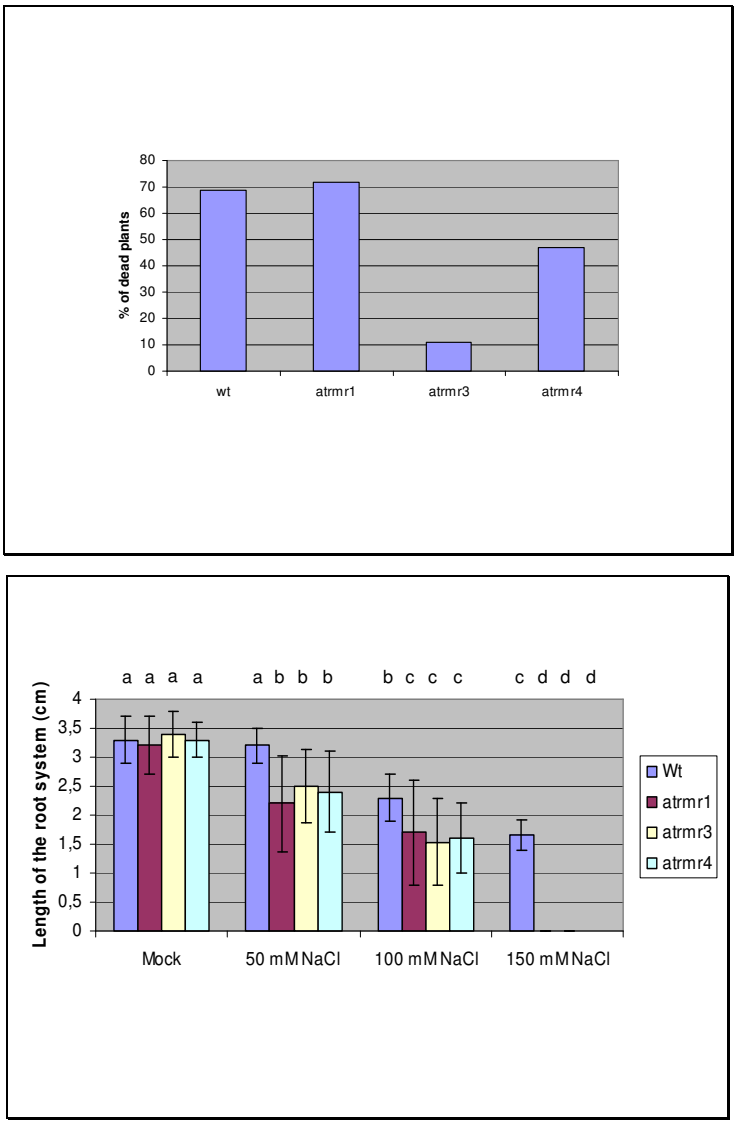



**Fig 28: Effect of NaCl on wt and KO plants.**
Upper graph: 3 weeks old plants treated during 3 days with 300 mM NaCl. The graph represents the proportion of plants that do not recover the salt stress.
Lower graph: Effect of increasing concentrations of NaCl on the growth of the root system of plants sawn on MS plates during 2 weeks. The graph represents the length of the root system of the plants.
Values presented are means +/- standard error of the mean (Tamhane's test, α=0.05).

## *B. Effect of wortmannin on plant grown in vitro*

The drug wortmannin has been shown to be an inhibitor of vacuolar sorting (Matsuoka et al. 1995; da Silva et al. 2005). Our previous data show that the KO mutants are impaired in the vacuolar sorting. Thus I hypothesised that these plants would be more sensitive to the drug than wt.

I looked at the fitness of wt and KO plants sawn on 7 µM wortmannin (fig 29). No difference was seen. The fact that KO plants had the same sensitivity to wortmannin as wt would be in accordance to the fact that no recycling of the AtRMR proteins has been reported, as wortmannin inhibits the retrograde trafficking from the PVC to the

Golgi apparatus (da Silva et al. 2005). In this respect it would be interesting to compare the susceptibility to wortmannin of *atrmr* and *atvsr1* KO, known to be impaired in the sorting of vacuolar proteins in seeds (Shimada et al. 2003).

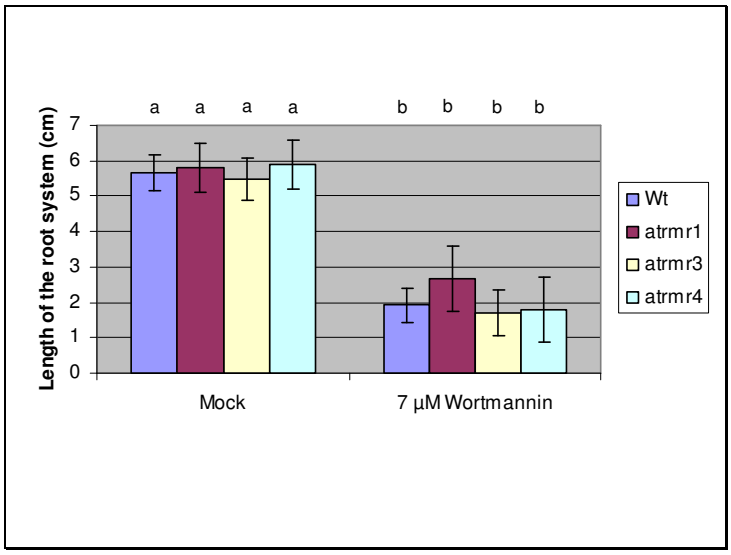



**Fig 29: Effect of wortmannin on wt and KO mutants.**
Seeds were sawn on MS medium supplemented with 7 µM wortmannin and the length of the root system was measured 20 days after germination.
Values presented are means +/- standard error of the mean (Tamhane's test, α=0.05).

## *C. Conclusion*

The purpose of these experiments was to find a convenient and straightforward way to test the ability of genes to complement the effect of the KO. There seems to be a slight increase in the susceptibility to 100 mM NaCl on MS plates in the mutants. The most obvious phenotype observed was a strong inhibition of the germination of the KO seeds sawn on 150 mM NaCl MS medium, whereas wt plants could still germinate. It would be thus of great interest to test the ability of atrmr plants complemented with AtRMR cDNA to germinate on 150 mM NaCl.

It is striking to note that the only phenotypes observed for plant vacuolar receptor KO plants are linked to seed germination (Laval et al. 2003; Shimada et al. 2003), whereas their *in vitro* binding to various VSDs have been widely documented (Kirsch et al. 1994; Kirsch et al. 1996; Cao et al. 2000; Park et al. 2005; Park et al. 2007). Recently, Fuji et al. (2007) showed that in the seeds of an AtVSR1 KO, the fusion between the GFP and the CtVSD of the seed storage protein β-conglycinin is secreted. The seeds of these KO plants transformed with the conglycinin reporter, seen under a fluorescence microscope, appear more fluorescent than wild type plants expressing the same reporter. The authors then used this criterium to screen a bank of EMS-mutagenised wt GFP-conglycinin plants based on the fluorescence of their seeds. They could thus identify several mutants, including namely *atvsr1*, *gfs2/kam2* (an homologue of rme8 involved in the endocytosis and regulation of endosomal structures in *Caenorhabditis. elegans*) and an unknown gene, encoding a membrane protein that seems to be specific to higher plants and may thus participate in a novel plant-specific type of vacuolar sorting system.

I have initiated a physiological study to find clear phenotypes of atrmr mutants. This could help reverse genetic studies of our receptors, if a functional AtRMR gene can restore the ability of atrmr seeds to germinate on 150 mM NaCl.

A more interesting phenotype would be one that could be rescued easily. For example, the size of the root system observed in complemented mutants grown on 100 mM NaCl could be an indicator for the ability of a transgene to restore AtRMR function and would still allow recovering the transgenic plants with failed complementation of the mutant. This way we could further test the vacuolar sorting efficiency of both efficient and deficient AtRMR constructs. For this purpose more conditions and other stresses must be tested.

# Discussion

## I. General conclusion

The vacuole is a major organelle of the plant cell, both by its size (vacuoles usually take up 30 to 90 % of the volume of the cell) and by its physiological roles. It is a very plastic organelle that can fulfill simultaneously lytic and storage functions within a single cell. Experimental data led to the emergence of a model where vacuoles have the ability to acquire specialised functions by changing dynamically their contents to fit the needs of the cell. It is now commonly accepted that two types of vacuoles exist that can be distinguished biochemically by soluble or membrane specific markers and by their internal pH. Such a well-defined endomembrane organisation supposes the existence of specific mechanisms of import of proteins as vacuoles do not have their own genome. Most of the soluble vacuole proteins are first synthesised as precursors that exhibit propeptides containing a peptidic sequence (VSD) necessary and sufficient to drive the protein to the vacuole. Once the protein reaches the corresponding vacuole, the propeptide is cleaved and the active protein is released in the correct organelle.

Different mechanisms may lead the VSD proteins to the vacuoles. First the commonly observed physical aggregation of some of these proteins (mostly in seeds) may trigger their packaging into cargo vesicles. A second mechanism implies the specific interaction of the VSD sequence with vacuolar sorting receptors, as is the case in the animal model of mannose-6-phosphate receptors involved in the sorting of specific hydrolases to the lysosomes – mammalian equivalents to the plant vacuoles. Some hybrid mechanisms, where receptors act together with the aggregation of vacuolar proteins, could also be invoked (Park et al. 2007).

Therefore these last years, great efforts have been made to identify plant vacuolar receptors. From the first studies that showed the involvement of pea BP80 to recent papers which further characterised VSR as well as RMR proteins in *A. thaliana* - and a growing number of monocots and dicots - we have more insights on the existence and function of Vacuole Sorting Receptors. Two families of proteins have been shown to be such receptors: the VSR proteins (such as BP80, AtVSR, PV72) have been shown to be involved in the sorting of ssVSD proteins, whereas RMR proteins (AtRMR1, AtRMR2) seem to specifically bring CtVSD reporters to Protein Storage Vacuoles.

The aim of this study was to test the involvement of AtRMR proteins in the sorting of vacuolar proteins. To answer key questions on the function of a AtRMR protein and the synergy between the various AtRMR genes, a dominant-negative approach based on the dissection of AtRMR1 according to its functional domain and the study of the sorting of various vacuole reporters in *A. thaliana* KO mutants were carried out.

Our results give unprecedented insights into the function of AtRMR1 in vacuolar sorting: a dominant negative AtRMR1 influences both ssVSD and CtVSD sorting pathways and KO of *AtRMR1*, *AtRMR3* and *AtRMR4* have similar defects in the sorting of ssVSD and CtVSD reporters.

## *A. AtRMR1 functions as a vacuolar receptor for both ssVSD and CtVSD proteins*

Our studies using both fluorescent and enzymatic reporters show that: firstly the overexpression of a dominant-negative mutant of AtRMR1 whose luminal domain is depleted leads to secretion of vacuolar reporters (page 50). Secondly, when the luminal domain of AtRMR1 is retained in a soluble form in the lumen of the ER, it delays the sorting of the same reporters to the vacuolar fraction (page 51).

These results strongly argue in favour of the hypothesis of AtRMR1 protein being a vacuolar sorting receptor for both CtVSD and ssVSD proteins.

As is the case for a dominant-negative mutant of AtBP80 lacking its luminal domain, the RMRΔlum construct is thought to traffic like the endogenous AtRMR. Therefore when overexpressed, this dominant-negative construct should compete with the endogenous receptors for critical trafficking regulators and in consequence inhibit their traffic. This is confirmed by confocal images showing the localisation of the GFP-RMRΔlum construct in ER / punctate structures that could correspond to ER and Golgi structures, expected locations of the endogenous receptor (Park et al. 2007). This construct induced an increase of the secretion of vacuole reporters (page 50). Da Silva et al (2005) reported a similar phenomenon when they blocked the vacuolar trafficking with the drug wortmannin. This effect is thought to be due to a saturation of the biosynthetic pathway that ultimately leads to the secretion of the proteins that the cell cannot sort properly any more. Therefore, a trafficking competitor of AtRMR1 provokes a saturation of the vacuolar sorting machinery, which indicates that the integrity of AtRMR1 compartments is necessary for the proper sorting of the ssVSD and CtVSD reporters.

When the luminal domain of AtRMR1 is fused to the HDEL ER-retrieval signal, the vacuole reporters are retained in microsomal / ER compartments (cf page 51). A similar construct, where the luminal domain of the pea PV72 is fused to the ER retrieval signal, retains the ssVSD of sweet potato sporamin (Watanabe et al. 2004). When the same region of AtBP80 is used in tobacco protoplasts, it leads to the retention of CtVSD and ssVSD proteins. Here the hypothesis is that the luminal domain of AtRMR1 efficiently binds to the VSD sequences of the reporter proteins and as it is retrieved to the ER, it brings back the reporter to the ER. This binding could occur in the ER itself or in the Golgi.

Two other constructs used in the present study didn't have the expected effects on vacuolar sorting but still give valuable indications on the function of AtRMR1. I used a construct where the luminal domain of AtRMR1 is fused to a 23 amino acid long portion of the LampI transmembrane domain – shown to be sufficient to direct a protein to the plasma membrane (Brandizzi et al. 2002). This chimeric protein should bind the target VSD protein and transport it to the plasma membrane, presenting it on the exterior surface of the cell and ultimately leading to its secretion. The results (page 53) show that this construct does not lead to secretion of any of the studied CtVSD and ssVSD proteins. This indicates a loss of binding of the luminal domain to the same reporters that were efficiently retained by the similar RMRlumER. The fact that the same peptide sequence is able to bind *in vivo* to a protein when it is fused to the HDEL peptide and not when it is fused to the 23 amino acid of the LampI protein suggests that the environment of the luminal domain of AtRMR1 is important for its activity or that the replacement of the transmembrane and cytosolic domains of

AtRMR1 by the TM23 leads to an inactivation of the luminal domain of AtRMR1. Hence the ability of AtRMR1 to bind to its VSD targets may be strongly dependent on its trafficking and it would be interesting to study whether this is due to a conformation change induced by the transmembrane and cytosolic domains or to unfavourable pH or salt concentrations in the compartments where the TM23 traffics.

The cytosolic domains of AtRMR proteins share regions that are known in other proteins to be involved in protein-protein interactions – the Ring-H2 domain, a Ser-rich region – and other regulatory elements – putative palmitoylation and phophorylation sites. For this reason, the cytosolic tail of AtRMR1 is thought to interact with various proteins involved in its trafficking, as it has been reported for VSR proteins (Happel et al. 2004). Similarly to the RMRΔlum construct, overexpression of the cytosolic domain of AtRMR1 should interact with endogenous partners and thus render them unavailable for the endogenous receptor and ultimately block the AtRMR1 cargo. Our experiments show that the cytosolic tail of AtRMR1 alone does not lead to secretion of vacuole reporters, whereas the transmembrane and cytosolic parts of the same protein induce secretion. Da silva et al (2006) reported that the soluble cytosolic tail of AtBP80 does not induce secretion of vacuolar reporters. This can be explained if the transmembrane domain of AtRMR1 plays an active role in the traffic of the protein so that the ectopically expressed cytosolic tail is not enough to compete with the endogenous protein; the endogenous partners of the cytosolic tail may also be mainly concentrated in membranes enriched in endogenous AtRMR1, and hence unavailable to the ectopically expressed soluble cytosolic tail.

### *B. At least AtRMR1, AtRMR3 and AtRMR4 are important in this system*

In our working hypothesis, the 7 AtVSR genes were thought to assume the sorting of ssVSD proteins to the LV, whereas the 6 AtRMRs would specifically direct the CtVSD proteins to the storage vacuole.

The silencing of all six AtRMRs in reporter plants performed in our lab (Okmeni, PhD thesis) showed the implication in whole plants of these proteins in the sorting of both CtVSD and ssVSD vacuolar reporters.

More specifically, the study of crosses between *atrmr1* and *atrmr4* KO plants on one hand and GFPChi plants on the other hand, and also transient expression of vacuolar reporters in *atrmr1*, *atrmr3* and *atrmr4* showed that when one of these three proteins is missing, the traffic to the vacuoles of both ssVSD and CtVSD proteins is strongly disturbed.

This result is neither in accordance to a model where the different AtRMR proteins would have different functions (in this case the three KO lines should have different phenotypes) nor to a model where their function overlap (the other non-affected AtRMRs should complement the single KO). The AtRMR1 transcript is by far the most present in rosette leaves (see Annexes table XXII). If the level of transcript is correlated to the amount of proteins present in the rosettes, the absence of AtRMR1 proteins could not be compensated in the rosette by AtRMR3 or AtRMR4, which are less expressed than AtRMR1. This could explain the phenotype of *atrmr1* plants. But on the other hand the strong presence of AtRMR1 protein in the *atrmr3* and *atrmr4*

plants should be enough to complement the defects in these less abundant proteins, which is not the case.

Nevertheless, the full length AtRMR1 was shown to complement the vacuolar sorting defect of atrmr4 KO protoplasts, an effect that could however not be quantified with our system and especially couldn't be quantitatively compared to the complementation of the *atrmr1* KO with the AtRMR1 cDNA.

# II. New models for the sorting mechanism involving RMRs

## *A. Sequential rearrangement of the receptors*

As said in the introduction (page 43) our starting model included two different receptor-mediated pathways, assumed by the VSR and RMR protein families that were thought to specifically interact and sort respectively the ssVSD and the CtVSD types of vacuolar proteins.

EM studies in developing seeds localised AtRMR1 in the *cis*- and median-Golgi cisternae, together with the PSV protein cruciferin, whereas AtVSR1 antibodies labelled the *trans*-Golgi (Hinz et al. 2007). So AtRMR1 could act prior to AtVSR1 in the secretory pathway to separate as early as in the median-cisternae the vacuolar cargo mostly destined to the PSV, from the rest of the Golgi lumen. AtVSR1 would then relay the action of AtRMR1 in prevacuolar compartments/TGN to more finely sort the vacuolar proteins destined to the lytic vacuole.

Park et al. (2007) also hypothesised a sequential arrangement: the CtVSD proteins would be segregated from the rest of the protein bulk flow at the median Golgi, where they would follow an AtRMR-mediated route to the PSV and subsequently would be internalised into storage vacuoles together with the receptors, while the AtVSRs would take charge of the rest of the sorting at a later Golgi compartment.

Our results add to this discussion the *in vivo* ability of AtRMR proteins to drive the sorting of ssVSD. Previous works in the lab (Okmeni, 2006, PhD thesis) showed that when the family 3 of AtVSR proteins is silenced in a $Aleu_{143}$GFP reporter plant, the sorting of the fluorescent vacuolar protein is disturbed. This is not the case in GFPChi plants and the silencing of the families 1 and 2 of AtVSR proteins has no apparent effect on the sorting of either of the two different fluorescent reporters. Therefore this work demonstrates a clear ssVSD-specificity of AtVSR proteins *in vivo*. In contrast, in plants where all the AtRMR genes were silenced, she observed that both the ssVSD reporter $Aleu_{143}$GFP and the CtVSD reporter GFPChi were missorted.

This could be explained by a sequential model (Fig. 30, model 1A) where RMR, present in the *cis*- and median- Golgi, would sort both CtVSD and ssVSD proteins from the rest of Golgi resident proteins and lead them first to prevacuolar compartments. In this model, VSR proteins, with a more stringent VSD-specificity, would further sort the ssVSD from the rest of the RMR cargo. VSRs would then lead their cargo to the LV, whereas RMR would follow its route together with its cargo to the PSV. In this model, the deletion of AtVSR proteins would lead to the secretion of only the VSR cargo, whereas the absence of RMR proteins would have an effect on all the receptor-dependant cargo. Nevertheless, in this model, the silencing of VSR

receptors would lead to the rerouting of ssVSD to the default prevacuolar and/or to PSV compartments, not to their secretion, which contradicts the results of Okmeni.

In an alternative sequential model (Fig. 30, model 1B) the VSR proteins act prior to the RMR, directing the ssVSD proteins to a prevacuolar compartment, the rest being sorted later in the secretory pathway by RMR. In this model, the depletion of RMR proteins would result only in the secretion of CtVSD cargo, not to the secretion of ssVSD.

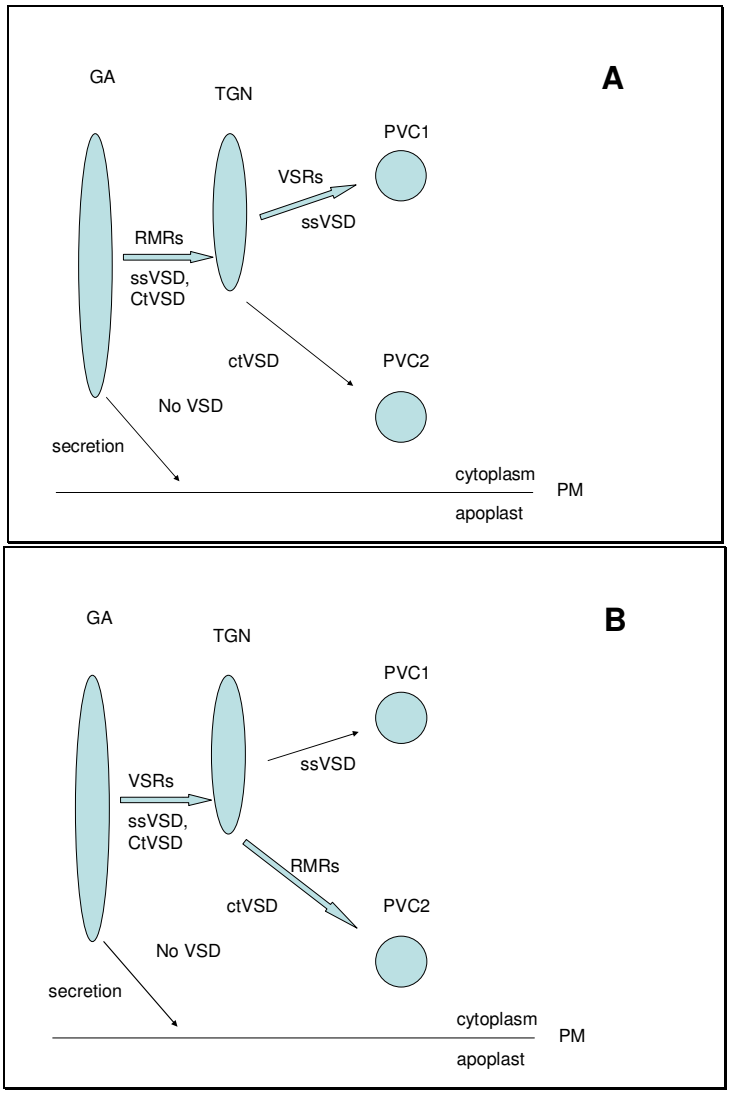



**Fig 30: sequential model of vacuolar proteins sorting (model 1A and B).**
Soluble vacuole proteins exit indiscriminately the GA via RMR vesicles, whereas proteins that lack VSD are secreted. There the ssVSD proteins are sorted to a specific class of prevacuole (PVC1), whereas ctVSD proteins reach a second type of prevacuole. Large arrows represent sorting steps where receptors are involved, simple arrows represent default bulk flow steps.

One can also imagine a cooperative model 2 (Fig. 31) where ssVSD are sorted via a RMR and VSR-dependent pathway, whereas CtVSD proteins would follow a RMR-dependent route. This model fits with the results obtained by Okmeni and the ones presented in this thesis.

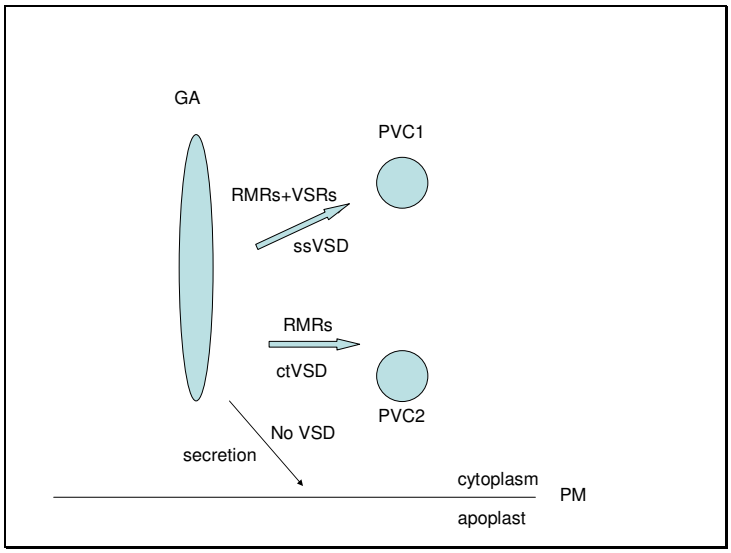



**Fig 31: the cooperative model (model 2).**
ssVSD proteins are sorted to the PVC1 via a RMRs and VSRs-dependent pathway, whereas the sorting of CtVSD proteins to the PVC2 is only RMRs-dependent and proteins that carry no VSD are secreted.

An AtRMR2 dominant-negative construct (Park et al. 2005) has been shown to induce a mislocation of the ctVSD protein phaseolin, but not of the two ssVSD proteins phaseolin or Arabidopsis aleurain-like protein. This does not fit to our previous model, where RMR proteins are general-purpose receptors and suggests a further specialisation of AtRMR2, at least in *A. thaliana*. This could reflect a divergence and specialisation of the RMR receptors in *A. thaliana* that is dicussed later in this thesis (cf p 89). If we go back to the first model and replace the default transport step that follows the first RMRs-dependent traffic by a AtRMR2-dependent CtVSD-specific sorting step, we come to a third model (Fig. 32). This model fits with the effects of the silencing of the VSR receptors performed by Okmeni, the effects of the AtRMR1 dominant-negative construct presented in this study and the results of Park et al. (2005) on the function of AtRMR2.

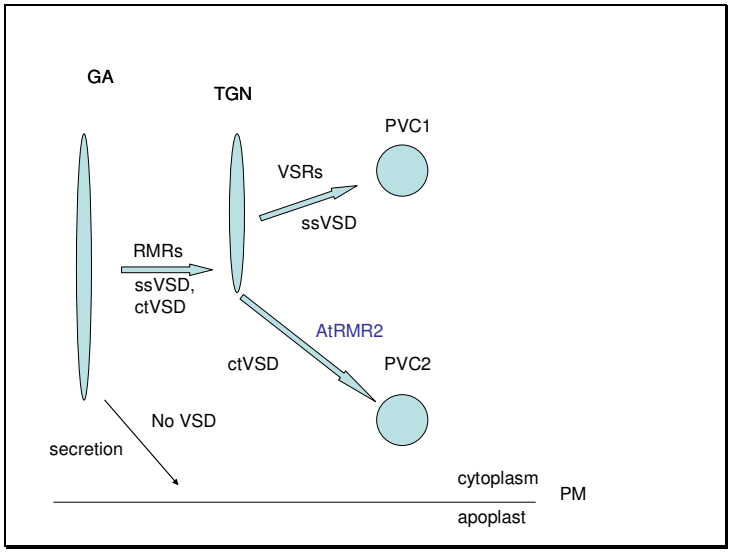



**Fig 32: Model 3.**
Vacuolar proteins are sorted from the GA to the TGN by RMRs independently of their type of VSD. VSRs subsequently sort ssVSD proteins to the PVC1, whereas AtRMR2 sorts CtVSD proteins to the PVC2.

However, none of these three models could explain the effect of the three different KO on the vacuolar sorting. Our results give good indications that AtRMR1, AtRMR3 and AtRMR4 could act on both ssVSD and CtVSD sorting. The analysis of 2455 experiments with Affimetrix 22 K arrays using Genevestigator (https://www.genevestigator.ethz.ch/at/) show that these three genes are expressed in the rosette leaves where our observations were made. If the RMRs act independently, the lack of AtRMR1, which is expressed approximately ten times more than AtRMR3 or AtRMR4, would mean the depletion of most of the RMRs present in the leaves and therefore fits with the observed impairment of the AtRMR1 mutant in vacuolar sorting. But then how could we explain that *atrmr3* and *atrmr4*, where only 5% of the RMRs are expected to be absent, have a similar phenotype than AtRMR1? These results suggest that at least these three receptors are inter-dependent.

## *B. Possible interactions between the different receptors*

The AtRMR1-AtRMR3-AtRMR4-dependency of the vacuolar sorting could be explained by interactions between these three proteins that would build up a functional receptor complex. This is compatible with both models 2 and 3, but as EM data suggest that RMRs act prior to the VSR, we will base the model 4 on a sequential arrangement of RMRs and VSRs. In this new model (Fig 33), AtRMR1, AtRMR3 and AtRMR4 form receptor complexes, the exact compositions of which cannot be discussed here, that are necessary for the sorting of both ssVSD and CtVSD proteins to an intermediate compartment where they are further sorted by VSRs or AtRMR2.

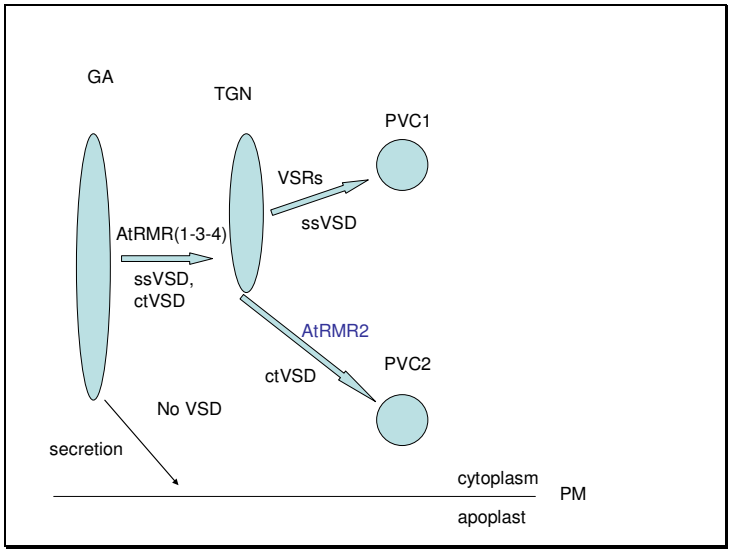



**Fig 33 Involvement of AtRMR complexes (model 4).**
AtRMR1, AtRMR3 and AtRMR4 form homo- or heterocomplexes that sort the ssVSD and CtVSD proteins from the GA to the TGN. These proteins are later sorted by VSRS to the PVC1 (in the case of ssVSD proteins) or AtRMR2 to the PVC2.

Such interactions have been already described in other kingdoms. In the mammalian system, CD-MPR and CI-MPR have been shown to form homodimers and it is thought that these interactions regulate the recognition of M6P targets: (Roberts et al. 1998; Byrd et al. 1999; Byrd and MacDonald 2000). In *Saccharomyces cerevisiae*, a putative homologue of CD-MPR has been shown to have a synergistic effect with Vps10p on the sorting of vacuolar hydrolases (Whyte and Munro 2001). Therefore, the plant vacuolar receptors could, as their animal and yeast couterparts, form such receptors oligomers.

The alignement of the peptidic sequences of the various plant RMRs show that the RMR proteins in arabidopsis are not as conserved as the ones of other plants (see Annexes page 115). Especially, in rice, poplar and moss, RMR proteins share the same C-terminal Cys residue that is present only in AtRMR1 in Arabidopsis. This could reflect a late evolution of RMR proteins in crucifers. From an ancestral RMR that forms a functional oligomer the family could have diverged to several RMRs that would have accumulated mutations (fig. 34). These mutations would have diminished the functionality of homooligomers, thus favouring the formation of heterooligomers involving receptors with complementary mutations. We then would have two situations: in plants such as rice, the RMR proteins are highly similar and form functional homocomplexes; in contrary, in A. thaliana, the AtRMRs have accumulated diverse mutations that lead to a at least partial loss of function of homooligomers, but fully functional heterocoomplexes. This model could explain why the specificity of a single receptor tested *in vitro* (Park et al. 2005; Park et al. 2007) could not fully reflect the effect of its deletion *in vivo* (this study). Moreover, in this model, the homooligomers are still present but less efficient than heterooligomers. This could explain why the leaf protoplasts isolated from the *atrmr4*(-/-)XGFPChi plants could be complemented by ectopically expressed AtRMR1. The overexpression of AtRMR1 could have lead to the formation of a large number of AtRMR1 homooligomers, less efficient than heterooligomers. In such a case, the quantity of receptors could have overcome their lack of quality (cf fig. 35).

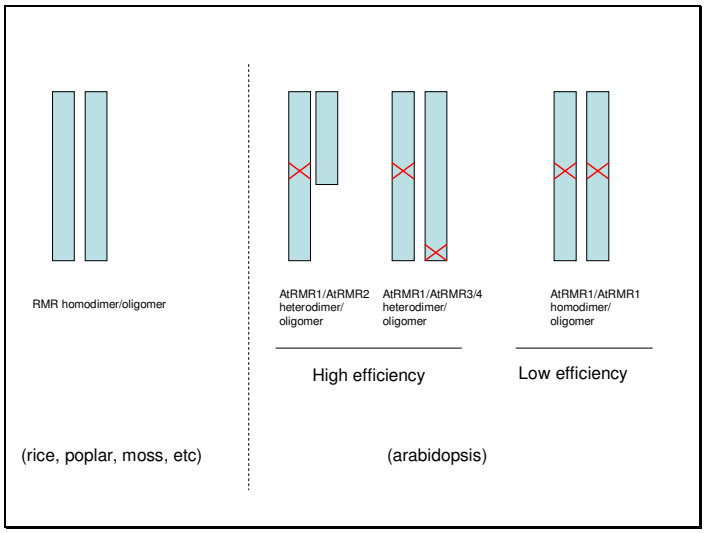



**Fig 34: possible evolution of RMR complexes.**
An ancestral RMR protein forms homodimer/oligomer that constitutes the functional form of the receptor.
In arabidopsis, the various AtRMR proteins have accumulated distinct mutations that diminish the fuctionality of the homodimers/oligomers, but the formation of heterocomplexes that involve proteins with complementary mutations can reconstitute a functional complex.

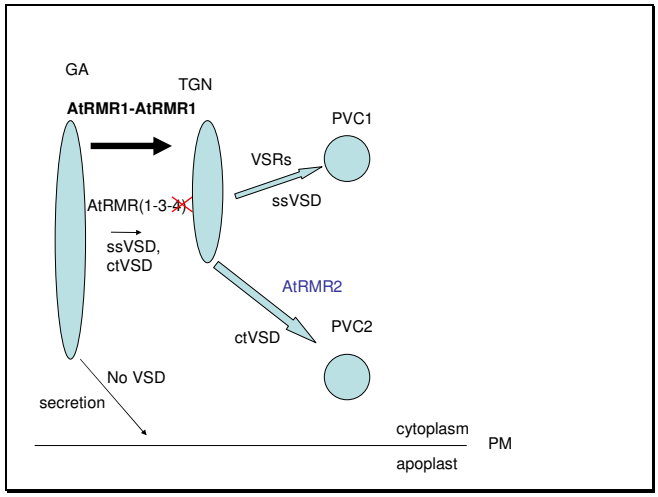



**Fig. 35: Effect of the ectopic expression of AtRMR1 in *atrmr4*(-/-)XGFPchi.**
The deletion of AtRMR4 leads to a loss of efficient AtRMR heterocomplexes. The overexpression of AtRMR1 and the consequent increase of AtRMR1 homocomplexes, though less efficient, can compensate lack of heterocomplexes.

In the previous models, the discussion on how vacuolar proteins reach the different vacuoles was not treated. Three different mechanisms (fig. 36) could explain the formation and maturation of the different plant vacuoles.

Firstly, most commonly accepted model postulates that the formation and maintenance of vacuolar compartments is achieved by vesicular shuttles. In this

model, the PVC and the vacuoles are fixed organelles that receive their contents via vesicules that traffic between them. In favor of this model, EM studies have shown that during their transport, vacuolar proteins are present in vesicles (Hillmer et al. 2001; Wenzel et al. 2005). Recently, a dominant-negative syntaxin Syp21 has been shown to inhibit the PVC-to-vacuole traffic *in vivo* (Foresti et al. 2006). Other publications have demonstrated the implication of components of the vesicle trafficking machinery in the PVC-to-vacuole pathways (Bassham and Raikhel 1998; Jin et al. 2001; Rojo et al. 2003; Sohn et al. 2003).

Secondly, vacuoles could be formed by maturation of the prevacuolar compartments followed by their fusion or aggregation. In mammalian cells, early endosomes are thought to mature after the endocytosis to become late endosomes that are intermediate organelles connected to the biosynthetic pathway and ultimately fuse to the lysosome. For example, Futter et al. (1996) have observed by electron microscopy close interactions and fusion of late multi-vesicular endosomes to lysosomes in mammalian cells. This is in agreement with a direct maturation and fusion of PVCs to the vacuole. In plants, such a mechanism has not been clearly identified yet. Yamada et al. (2005) suggest that endosomes fuse with a vacuole under sucrose starvation by an autophagic mechanism that involves vacuolar proteases, without direct experimental evidence. For instance, no EM observations have confirmed this theory in plant so far. Nevertheless, the formation of the complex PSV in seeds nevertheless supports such a mechanism: in these cells, the MVB is considered as PVC and has been shown to be internalised in the PSV in such a way that can be explained only if the whole intermediate compartment has been engulfed in the vacuole. Moreover, in developing Arabidopsis embryos, the vacuolar receptors AtRMR1 and AtVSR1 are both present in MVBs, but in the PSV, only AtRMR1 can be found (Hinz et al. 2007). This could reflect maturation of the MVBs (through the loss of AtVSR1) and their later fusion with the PSV which releases AtRMR1 into the vacuole lumen.

Thirdly, pre-existing specialised vacuoles (Epimashko et al. 2004) can fuse during the development of the plant, leading to the formation of a hybrid vacuole (Paris et al. 1996; Jauh et al. 1999). Small vacuoles were shown to fuse during stomatal aperture in *V. faba* (Gao et al. 2005).

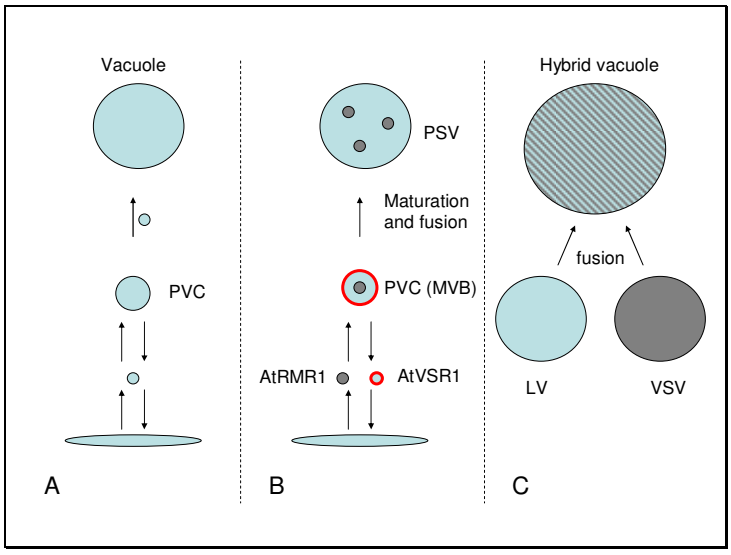



**Fig. 36: post-PVC events leading to the formation of vacuoles.**
(A) Vesicle shuttle from the PVC to the vacuole.
(B) Maturation of PVC to a vacuolar compartment characterised by the exclusion of AtVSR1 from the vacuole and internalisation of AtRMR1.
(C) Fusion of specialised LV and VSV in a large hybrid vacuole.

# III. Outlooks

The results presented here challenge the commonly assumed model of receptor-mediated vacuole sorting.

They show that at least three AtRMR proteins are vacuole sorting receptors involved in both the CtVSD and ssVSD pathways.

Moreover they redirect the study of this mechanism. They shed some light on the importance of dissecting the spatial distribution of the different receptors, their putative interactions, instead of concentrating on their specificities to VSD.

So, further efforts have to be invested in this direction. New projects are currently underway in the lab. If successful they should lead to some interesting conclusions.

In the first place, our results have to be confirmed and quantified. For this purpose we need to purify the proteins secreted by the KO plants, compare them to those secreted upon overexpression of the RMRΔlum construct and the GFP-BP80 construct. If the receptors do have a narrow spectrum of vacuolar ligands, this should have consequences on the secretome. The RMRlumER construct, which has been shown to retain vacuolar proteins in a microsomal fraction, has a Myc tag that should allow its purification. We can use it as a bait to immunoprecipitate the *in vivo* ligands of AtRMR1. Now that KO for *AtRMR2, AtRMR5* and *AtRMR6* genes are also available in the lab, we will use them to complete our views on the VSD specificity of the AtRMR proteins.

We also have to address the question of the putative interactions between the various receptors. This idea came from our studies of KO plants, and it has especially

been motivated by a very recent observation (Egidio Stigliano, PhD student). Using an antibody raised against the luminal domain of AtRMR1 he detected two bands on a western blot in extracts from wild type plants. The lower size band corresponds to the expected molecular weight of AtRMR1, so that the upper band could correspond to a post-translational modification of the receptor. In extracts from *AtRMR3* or *AtRMR4* KO however, the same antibody detected predominantly the lower band.

To obtain better insights in this question, we have chosen a Bi-Molecular Fluorescence Complementation (BiFC) strategy. In this technique, two interacting proteins are fused to the Nt half of the GFP for one and to the Ct half of the GFP. This way, once the two partners interact, the two halves get close enough to reconstitute the structure of the full length GFP and hence its fluorescence. BiFC therefore allows to detect the interaction of two proteins of interest and to localise this event in the cell. Moreover, Hu et al. (2003), using fragments of fluorescent proteins with different spectral characteristics, showed that it is possible to detect simultaneously in a single cell the formation of more than one protein complex. For example, with this technique, it would be possible to localise simultaneously the formation of AtRMR1ntYFP-AtRMR2ctCFP and AtRMR1ntYFP-AtRMR4ctYFP complexes, the two complexes leading to the formation of fluorescent chimera having an excitation spectrum close to the one of YFP and emission spectra close to, respectively, CFP and YFP.

Finally, we plan to use the mutants for complementation studies which should allow us to find key elements in AtRMR proteins that are involved in the vacuolar sorting. For this purpose, the determination of a clear phenotype (initiated in the present study) would be of great help. It could be used to screen among a population of complemented mutants plants where the phenotype is recovered by the AtRMR transgene. The use of NaCl reported in this study could be a possible screen, but we should first find conditions where the mutant phenotypes are more evident and still viable. Unfortunately, the microarray data collected by Genevestigator do not show any evident condition where the AtRMR genes are regulated, conditions which could have been good candidates for a screening method (see Annexes page 117).

# Material and methods

# I. Bacteria culture and transformation

## *A. Bacterial strains and plasmids*

### 1. Bacterial strains

*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 plasmids.

Agrobacterium tumefaciens strain GV3101

### 2. Plasmids and vectors

Plasmids and vectors are listed in the table with their relevant characteristics and references.

| Plasmids/vectors | Relevant characteristics | references |
|---|---|---|
| pGEM-T-Easy | $Amp^r$ , Sp6 and T7 promoter | Promega |
| pGY1 | $Amp^r$, 35S promoter and terminator | Neuhaus et *al.*, *1991* |
| pAmy-spo | $Amp^r$, 35S promoter and terminator | (Pimpl et al. 2003) |
| pAmy-bl | $Amp^r$, 35S promoter and terminator | (Pimpl et al. 2003) |
| pGREEN | $Amp^r$, 35S promoter and terminator. Contain gene for the magnesium chelatase | Turnage et *al.,*2002 |
| RMRΔlum | RMRΔlum PCR product inserted in pGY1 multiple cloning site (MCS) with BamHI and SalI | This study |
| RMRlumER | RMRΔlum PCR product inserted in pGY1 multiple cloning site (MCS) with BamHI and SalI | This study |
| RMRlumPM | RMRΔlum PCR product inserted in pGY1 multiple cloning site (MCS) with BamHI and SalI | This study |
| RMRcyt | RMRΔlum PCR product inserted in pGY1 multiple cloning site (MCS) with BamHI and SalI | This study |

**Table III: plasmids and vectors.**
r: resistant.

## *B. Antibiotics used in selective media*

Antibiotics used in this study are listed in the table below

| 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 |

**Table IV: antibiotics working and stock concentrations.**

## *C. Bacterial culture and transformation*

### 1. Freezing and storage of E.coli strains

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

### *2.* Standard growth conditions for *E.coli*

E.coli bacteria were grown in LB-medium at 37°C. Liquid cultures were shaken at 280 rpm. According to the resistance marker carried by the plasmids antibiotics were added to the media.

### 3. Preparation of heat-shock competent *E.coli* XL1-Blue cells

5 ml LB medium containing tetracycline (50 mg/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. The culture was then chilled on ice for 15 min, and the bacteria were pelleted for 15 min at 4°C and 5000 rpm in the Sorvall GSA rotor. The pellet was resuspended in 32 ml RF1 buffer and was left for 20 min on ice. Following a centrifugation step of 15 min at 4°C and 5000 rpm in the Sorvall GSA rotor, the pellet was taken up in 8 ml RF2 and incubated for 20 min on ice. Eppendorf tubes were precooled in cold room. The competent bacteria suspension was portioned in aliquots of 100 µl and was frozen in liquid nitrogen. Tubes were stored in a -80°C freezer.

### 4. 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 1 µl of purified DNA sample or 5 µl 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℃. The mixture was then placed on ice for 10 min. Finally, all of the bacterial suspension was plated on LB-plates with the corresponding antibiotics and was grown overnight at 37ºC.

| | |
|---|---|
| LB-medium | 0.5% NaCl; 0.5% (w/v) yeast extract, 1% (w/v) bacto tryptone in H2O |
| LB-Amp-medium | LB-medium with 50 µg/ml Ampicillin |
| 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, 2 mM $MgSO_4$ |
| YEB-medium plates | 1,6% Agar was added to the liquid medium |
| YEB-Kan-Gent-Rif-medium | YEB-medium with 50 µg/ml kanamycin, 25 µg/ml gentamycin, 50 µg/ml rifampicin |
| YEB-Rif-Gent-medium | YEB-medium with 25 µg/ml gentamycin, 50 µg/ml rifampicin |
| RF-1 Buffer | 100 mM KCl;30 mM $MnCL_2$; 30 mM KOAC pH=7.5; 10 mM $CaCl_2$; 15% glycerol; adjust the pH to 5.8 with 200 mM acetic acid and filter sterilised |
| RF-2 Buffer | 10 mM MOPS pH=6.8; 10 mM KCl; 50 mM CaCl2; 15% glycerol, was adjusted to pH=6.8 with 1 M NaOH and filter sterilised |

**Table V: growth media for bacteria.**

# II. Plant handling and plants transformation techniques

## *A. Arabidopsis thaliana lines*

During my thesis, two lines of A. thaliana ecotype Wassilevskija were used: (1) the wild type and (2) the transgenic line expressing the GFP-Chi in the storage vacuoles (Flückiger et al., 2003).

Four lines of *A. thaliana plants* ecotype col0 were also used: (1) the wild type, (2) an insertional Knock-out (KO) of *AtRMR1* (SALK_075202), (3) an insertional KO of *AtRMR3* (SALK_102113) and (4) an insertional KO of *AtRMR4* (SALK_052770). The mutant lines were kindly provided by the SALK institute.

## *B. Seed sterilisation*

Seeds were poured into Eppendorf tubes. 1 ml of a solution containing 2% bleach in ethanol, 0.005% Triton X-100 was added and seeds were shaken vigorously for 10 min using a bench top shaker. The solution was removed with a micropipette, and then seeds were washed three times with ethanol 100%. 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 (1962), modified to contain half the amount of $NH_3$ and $NO_2$. Plates contained kanamycin (50 µg/ml) 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 cabinet with a 16h light / 8h dark photocycle at 22°C.

## *C. Germination conditions*

Plant germination was carried out in a 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 µE $s^{-1}$ $m^{-2}$.

After 3 to 4 weeks in Petri dishes plants were transferred individually to soil and were incubated in a growth chamber with 8 h of light.

## *D. Protoplast techniques*

### 1. *A. thaliana* leaf protoplasts isolation and PEG-mediated transfection

This method is adapted (from Jin et al. 2001).

We used 3–4 weeks old *Arabidopsis* plants (*A. thaliana* var Columbia 2) grown on agarose plates (0.5x MS, 1% w/v sucrose) under long day conditions. The rosettes of these plants were cut using a sterile razor blade. The leaves from each plate were placed in 12 ml of enzyme solution in a new plate (ø 15cm) and lacerated with a sterile razor blade. They were incubated in the Enzyme solution without shaking over night in darkness at room temperature.

Protoplasts were released by very carefully shaking the plates. The macerate was filtered to separate the released protoplasts from the undigested leaf debris through a 100 µm mesh. During the whole transformation procedure 3 ml disposable plastic Pasteur pipettes with an opening of about 2 mm were used for pipetting the protoplasts. Once filtered, protoplasts were transferred to 15 ml plastic tubes using a Pasteur pipette (for large numbers of plates filtrates were pooled in 50 ml plastic tubes). 2 volumes of W5 solutions were added and the whole suspension was centrifuged 5' at 50 g in a swing-out rotor (Beckman) at room temperature. The supernatant was discarded and the pellet resuspended in W5 and pooled to a final volume of 20 ml.

3 ml of protoplasts were carefully overlaid on top of 4 ml 21% w/v sucrose in 15 ml plastic tubes with a Pasteur pipette (for large numbers of plates, 10 ml of protoplasts were overlaid on 15 ml of sucrose 21 % in 50 ml plastic tubes). Tubes were centrifuged 10' at 50 g in a swing out rotor at room temperature. At this step, intact protoplasts accumulated at the sucrose surface and were collected carefully with a Pasteur pipette and pooled in a plastic tube (3 ml of overlaid protoplasts give 1.5 ml in a 15 ml tube). 10 ml of W5 solution were added. The tubes were centrifuged 5' at 50 g in a swing out rotor at room temperature, the supernatant were discarded.

Protoplasts were resuspended and pooled in 5 ml of W5 solution and incubated for 30' on ice. Intact round-shaped protoplasts were counted using a hemocytometer.

Protoplasts were centrifuged 5' at 50 g in a swing out rotor at room temperature and the supernatant removed. The protoplasts were finally resuspended to a final concentration of $2.10^6$ protoplats per ml in MaMg solution.

2 µl of plasmid DNA (5 µg/µl; total 10 µg) and 5 µl sheared carrier DNA (10 µg/µl; total 50 µg) were added to 300 µl protoplasts and gently mixed. Immediately 325 µl of 40 % PEG solution were added and mixed by shaking gently. To pipette protoplasts and PEG solution, I used 1 ml micropipette with tips cut at their extremities. The mixes were incubated for 30' at room temperature.

10 ml W5 solution were added over about 10' (300 µl at a time) and gently mixed.

Protoplasts were centrifuged 5' as previously. The supernatant was removed. Finally the protoplasts were resuspended in 4 ml of W5 solution, transferred to a 15 ml plastic tube and incubated for 24 h in darkness at room temperature.

## 2. Amylase assay

The assay was adapted from da Silva (2005).

For each sample, ten transfections where done as described (p x) and pooled in a 50 ml plastic tube. Protoplasts were gently separated from the medium by centrifugation at 50 g for 10 min. 200 µL of the supernatant were transferred in an Eppendorf tube, 200 µL of Amy 1X solution were added and the tube was put on ice (Medium sample M). The rest of the supernatant was discarded.

For the fractionation, 800 µL of Amy 1X solution was added to the pellet (approximatively 200 µL). The samples were placed on ice for 5 min and centrifuged at 25000 g for 15 min. This causes an osmotic shock, leading to the release of the soluble, mainly cytosolic and vacuolar, proteins in the medium, whereas the proteins present in microsomes were pelleted. After the centrifugation, 200 µL of the supernatant were transferred to an Eppendorf tube (fraction S1), the rest was carefully removed.

The pellet was resupended in 1 ml of Amy 1X solution and sonicated to release the soluble proteins presents in the microsomal fraction. The samples were centrifuged at 25000 g for 15 min and finally 200 µL of the supernatant transferred into an eppendorf tube (fraction S2).

To 30 µL of each sample (M, S1, S2), 30 µL of Amylase Substrate solution were added and the reaction carried out at 37°C for various times. The reaction was stopped by the addition of 200 µL of Tris 1% solution. The absorbance at 395 nm of the product was recorded with a microplate reader and enzymatic activity calculated as published (Denecke et al. 1990).

| | |
|---|---|
| Enzyme solution | 400 mM mannitol, 5 mM MES, 8 mM $CaCl_2$, 1 % w/v Cellulase Onozuka R-10 (Serva), 0.25 % w/v Macerozyme R-10 (Serva). pH=5.6 with KOH 0.1 N. Filter sterilised. |
| W5 | 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, 1.5 mM MES. pH=5.6 with KOH 0.1 N. |
| MaMg | 0.4 M mannitol, 15 mM $MgCl_2$, 5 mM MES. pH=5.6 with KOH 0.1 N. |
| PEG 40% | 40 % w/v PEG 4000 (Fluka), 0.4 M mannitol, 0.1 M $Ca(NO_3)_2$. pH=7 – 8 with KOH 0.1 N. Filter sterilised. |
| 21 % sucrose | 21 % w/v sucrose (Fluka) in water. Filter sterilised. |
| Amylase buffer 20X | 25.0 mM Tris/HCl pH=8.0; 50 mM glucose; 10 mM EDTA pH=8.0 |
| Amylase substrate | 10 mL of sterile bidistilled water added to the content of a bottle of amylase HR reagent (Megazyme) |
| Tris 1% pH=11 | 1 g of tris-base dissolved in 100 mL of water. |

**Table VI: solutions used for protoplast handling.**

## *E. Transient transfection via agroinfiltration of A. thaliana leaves*

Plants were grown for three weeks under short day conditions (8h light, 16 hrs dark). Rosettes were then cut and leaves further cut in squares of approximately 1 $cm^2$. Leaf pieces were placed five in a row in the tank of a 20 ml syringe, 10 ml of agrobacterial culture (see following section for culture details) were added and the piston of the syringe was put back in place. The excess air present in the tank was chased, then the syringe was blocked and a vacuum was applied in the tank by pulling slowly the piston. The air bubles formed at the surface of the leaves were removed by gently shaking the syringe. The piston was then carefully released, thus stopping the vacuum. The infiltrated leaves, appearing dark, were rinced with water to remove excess infiltration medium and bacteria and plated on a ½ MS plate.

### 1. 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 400 ml) and was grown at 28ºC until $OD_{600}$ reached 0.5-0.8. 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. 100 µl aliquots of the cell suspension were stored in -80ºC freezer.

### 2. Cold shock transformation of *A. tumefaciens*

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 a 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 28ºC with shaking. Then they were centrifuged for 5 seconds and resuspended in

200 µl of YEB, and cells suspension were spread onto selective YEB plates and incubated at 28°C until visible colonies appear.

### 3. Preparation of Agrobacteria for agroinfiltration

Agrobacteria are grown in 10 ml of YEB medium without antibiotics in a 50 ml Erlenmeyer. The bacteria are grown until an $OD_{600}$ of 1. The cells are collected by centrifugation at 3000 g for 20' and resuspended in a volume of infiltration medium such as their $OD_{600}$ was approximately 0.6-1.

## *F. Characterisation of mutant plants*

Screen for homozygous plants for the KO

AtRMR KO plants were first screened thank to the kanamycin resistance carried by the T-DNA inserted. Sterilised seeds were sawn on MS+kan plates. Plates were grown on plates 10 days, when the kanamycin-resistant phenotype could clearly be observed and resistant plants transferred to soil. The homozygoty was confirmed by PCR and the KO by RT-PCR.

| | |
|---|---|
| MS- medium | 0.22 % MS salt, 0.05 % MES, 1%sucrose, the pH was adjusted to 5.8 using a solution of 1 N KOH |
| MS-medium plates | 0.8% agar was added to the liquid medium |
| MS-Kan-medium | MS-medium with 50 µg/ml Kanamycin added after autoclaving |
| Infiltration Medium | Glucose 0.5 %, 50 mM MES pH=5.6, 2 mM $Na_3PO_4$ |

**Table VII: growth media for plant culture.**

## *G. 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 CS2.0, and green was attributed to GFP and red to chloroplasts. Images with transmitted light were also collected using the same confocal microscope.

# III. Molecular biology

## *A. Isolation and purification of DNA*

### 1. Isolation of plasmid DNA from *E. coli*

#### a) Small-scale or mini-preparation

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

After centrifugation at 12 000 rpm at 4°C for 10 min, 1 volume of chloroform was added to the supernatant and mixed by vortexing. The mixture was centrifuged for 10 min at RT and at 12000 rpm. 400 µl of the upper phase was transferred to a fresh tube.

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

#### b) Maxi-preparation of plasmid DNA

400 ml 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 400 ml culture at 15000 rpm for 15 min at 4°C. The pellet was resuspended in 8 ml 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 kept on ice for 10 minutes. 8 ml of Solution III was added and the mixture was vortexed, chilled on ice for 10 min, then centrifuged at RT, at 15000 rpm for 10 min (Beckman centrifuge). The supernatant was transferred into a new tube and was treated with 2 Vol of chloroform. DNA in the aqueous phase was precipitated by adding 16 ml of isopropanol and the mixture was incubated at RT for 10 min. The pellet was recovered by centrifugation at 15000 rpm for 15 minutes at RT and washed with 70% Ethanol, then resuspended in 2 ml of TE, and 2 ml of 5M LiCl. The mixture was centrifuged at 3700 rpm for 10 min and 4 ml of isopropanol was added to the supernatant. After centrifugation at 3700 rpm for 10 min, the pellet was washed with 3 ml of 70% Ethanol, dried, dissolved in 500 µl of TE containing the RNAse A (20 µg/ml), then kept at 37°C for 2 hours. DNA was precipitated by adding 1ml of 99% ethanol. After 5 minutes, DNA was collected by

centrifugation at 12000 rpm for 5 min and was rinsed with 70% EtOH. It was then was dried 15 minutes at RT and resuspended in 400 µl of TE for 5 minutes at 65°C.

## *B. Characterisation of DNA molecules*

### 1. Enzymatic digestion of plasmid DNA

#### a) 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 30 µl. 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 30 µl, 1 µl of concentrated mini-preparation or 8 µl of mini-preparation DNA was used.

#### b) Preparative digestion of plasmid DNA

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

#### c) 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 weighed in a flask and was suspended in 0.5 x TBE and dissolved by heating in a microwave oven for 3 min. 2 µl of ethidium bromide was added to 50 ml of agarose, the gel was poured into 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.

#### d) Estimation of DNA concentration

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

#### e) DNA precipitation

The DNA solution obtained after the inactivation of restriction enzymes may have to be concentrated for further cloning. 0.1 volume of 3 M sodium acetate pH 6.8 was added to increase the salt concentration. Then 2.5 vol 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 20 min. The ethanol was sucked away, 70% ethanol was added and after another 5 min centrifugation step the supernatant was removed completely and the DNA was dried

at 65°C for 5 min. The dry DNA was taken up in sterile water (10 to 20 µl in case a 100 µl digestion had been performed)

## 2. DNA sequencing

The sequencing reaction was performed according to the dideoxynucleotide method described by (Sanger et al. 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.

First step: Reaction beginning

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

1 µl of 500-700 ng template (ds-DNA)

1 µl of IRD700 forward primer (1.0 pmol/µl)

1 µl of IRD800 reverse primer (1.0 pmol/µl)

3 µl of distilled water

1 drop of mineral oil

The components were mixed well by pipetting 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 1ul of the appropriate template/primer combination was added to the A, T, G, and C tubes and mixed well.

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

Second step: PCR-program:

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

Table VIII: PCR reaction for DNA sequencing.

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

Depending on the comb, 1.0 to 1.5 µl of samples were loaded onto a 33 cm 7% polyacrylamide gel. The gel was run using 1X TBE buffer. The sequencer apparatus was a LI-COR 4000L sequencer (LI-COR Biosciences).

The data were automatically collected and simultaneously recorded during electrophoresis.

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

## *C. Cloning*

### 1. Strategies used for AtRMR1-derived dominant-negative constructs

#### a) General strategy

I amplified the adequate part of the AtRMR1 cDNA clone (JR702, Jiang et al. 2000) which had been cloned into the pGal vector by PCR and subcloned them in the pGEM-T vector. The sequences of the PCR products were checked. I used the restriction enzymes whose restriction sites had been introduced in the constructs by PCR to further clone them into the final plasmid for expression in plants.

#### b) RMRΔlum

A first PCR reaction was done using the primers TMcyt and ASRMR and the AtRMR1 cDNA as matrix. The checked PCR product was introduced into the pGY1 vector using the restriction enzymes BamHI and SalI.

#### c) RMRlumHDEL

The construct was obtained by a PCR reaction using the primers SRMR and ASRMRlumMycHDEL and the AtRMR1 cDNA as matrix. The checked PCR product was introduced into the pGY1 vector using the restriction enzymes BamHI and SalI.

#### d) RMRlumPM

A first PCR reaction was done with the primers SRMRFL and ASFLT and the TM23 construct in pGY1 (Brandizzi et al. 2002). The amplified product was used as a megaprimer for a second PCR reaction together with the primer SRMR and the AtRMR1 as matrix. The checked PCR product was introduced into the pGY1 vector using the restriction enzymes BamHI and SalI.

#### e) RMRcyt

The construct was obtained by a PCR reaction using the primers Scyt and ASRMR and the AtRMR1 cDNA as matrix. The checked PCR product was introduced into the plasmid pGY1 using the restriction enzymes BamHI and SalI.

## 2. Isolation of DNA from agarose gel

### a) Direct DNA extraction from the gel

This technique did not need special material. The gel was placed under a UV lamp and a 20 µl pipette was used to isolate the DNA. The tip was stuck into the gel where the desired DNA band was seen and the DNA was sucked out.

### b) DNA isolation using Wizard SV gel and PCR clean-up system

For the purification of DNA fragments which could not be extracted with the 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 (Promega, protocol: "Wizard® and Gel and PCR clean-Up System protocol". Handbook, 1/05, p.6-7).

The gel slice was weighed and put into an Eppendorf tube. 10 µl of Membrane binding solution per 100 mg 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 16000 rpm for 1 min, and the column was washed twice by adding 700 µl of Membrane Wash Solution. Between each washing step, the column was centrifuged for 5 min at 16000 rpm. The resulting DNA was collected in a new tube by adding 20 µl of nuclease free water followed by a centrifugation for 1 min at 16000 rpm. 5 µl of the DNA was analyzed on a gel and DNA was pure enough for sequencing and further cloning techniques.

## 3. Ligation of DNA fragments

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

Ligation mixture:

4.5 µl of DNA fragment

1 µl of linearised vector

7.5 µl of 2x rapid ligation buffer / 1.5 µl of 10x T4 Ligase buffer

1 µl of T4 ligase (1U/µl)

Water to complete to 15 µl if 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 page 88).

## 4. Polymerase Chain Reaction (PCR)

### a) Primers

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

| | |
|---|---|
| M13 | GTAAAACGACGGCCAGTG |
| Rev | CAGGAACAGCTATGACCATG |
| 120 | TGACGCACAATCCCACTATCCTTCGCA |
| 121 | TGTAGAGAGCTGGTGATTTC |
| SAT1N | TGGATGCGGAAATTAGAGGG |
| AAT1N | GGTACACTTATTTCTGTTTGTGGC |
| SAT2Nb | GATCAGAGCCATAATGGCAC |
| AAT2N | TTCTCCGAAGCTACATCGAAG |
| SAT3N | GTCTCTGGTTTGTGATTGAGC |
| AAT3Nb | TGCATTTGTATCCACCCCATG |
| SAT4N | AGATGTGAATGAGTGTGAGGAGAA |
| AAT4N | TTATGCAAATGTCGTGTTCTCTTATG |
| SAT5N | GCTTTGAAGATATGGAACGG |
| AAT5N | GTATAGACTCACTCCAATCCATC |
| SAT6N | GCATTAAAGGTATGGAACGGTC |
| AAT6N | GACTCACTCCAGTCTATCTTCAGG |

**Table IX: primers for sequencing and molecular characterisation of the KO plants.**

| | |
|---|---|
| TMcyt | ATGAATCGTTTGGTCCTACTTTTATATGTTTGTACTGTTTCTTGTTT AGCTTCAAGATCTGATACGAAAGTTTGGTTGATCCCA |
| ASRMR | GTCGACACAGTCTGGAAGCGA |
| SRMR | TATGGATCCATGAATCGTGCTTTGGTT |
| SRMRFL | GTCGACACAGTCTGGAAGCGA |
| ASFLT | GTCGACTTAGGTCCGTATGAGGGG |
| Scyt | GTCGACACAGTCTGGAAGCGA |
| ASRMRlum MycHDEL | ATGAATCGTTTGGTCCTACTTTTATATGTTTGTACTGTTTCTTGTTT AGCTTCAAGATCTGATACGAAAGTTTGGTTGATCCCA |

**Table X: primers for dominant negative constructs**

PCR was performed using a Biometra Tgradient thermocycler (Biometra, Goettingen) with standard programs.

Most PCR reactions were made in a final volume of 50 µl, or 20 µl for PCR on colonies.

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

**Table XI: typical PCR reaction mixture**

## b) General program

| | Reaction | Temperature | Time | Cycle |
|---|---|---|---|---|
| Denaturation | | 95°C | 3min | 1 |
| Synthesis | Denaturation<br>Annealing<br>Polymerization | 94°C<br>54°C<br>72°C | 30sec<br>45 sec<br>1' 30 sec | 35 |
| Complete synthesis<br>Cool down | | 72°C<br>10°C | 10'<br>∞ | 1 |

Table XII: typical PCR program

## c) 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 $MgCl_2$, forward and reverse primers, Taq polymerase enzyme and water was prepared. This master mix was aliquoted into 25 µl 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 $H_2O$

12,5 µl Taq buffer containing $MgCl_2$

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

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

Table XIII: program for PCR on colony.

PCR products were run in an agarose gel as in page 95.

## *D. Reverse Transcriptase-PCR (RT-PCR)*

### 1. 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 and Sacchi 1987). It is a solution containing phenol and guanidine thiocyanate. RNA isolation is complete in less than one hour.

Leaves were frozen in liquid nitrogen and ground in a precooled mortar. They were transferred into an Eppendorf tube and 500 µl 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, at 14000 rpm for 10 min, the resulting supernatant was transferred into a new Eppendorf tube and 200 µl 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 20 minutes. The aqueous phase was transferred into a new tube and 500 µl of isopropanol was added. The tube was kept at room temperature for 25 min and then centrifuged at 4°C at 14000 rpm for 15 min. Finally the pellet was washed with 1ml of 75% ethanol (made with DEPC water) and was resuspended in 15 µl of nuclease-free water.

#### a) 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 genomic DNA. 1 µl of DNAse was added to the RNA extract and the reaction mixture was completed to a final volume of 20 µl 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.

#### b) 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 1 µl of bromophenol blue. For the

electrophoresis 1x TAE buffer was used. With good RNA preparations the 16S rRNA and 23 rRNA must be visible.

### c) 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 40 µg/ml RNA.

### d) Reverse Transcriptase Reaction

The Reverse transcriptase reaction copies mRNA into cDNA. The ImProm-II Reverse Transcription System (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.

**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:

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

Table XIV: composition of a PCR mixture.

1 µl RNA (2 µg/µl)

1 µl oligo$(dT)_{15}$ (0.5 µg/µl)

3 µl nuclease-free water

Final volume: 5 µl

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

• The tube was then centrifuged at 4°C, 800 rpm for 5 sec.

• The reaction mix 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:

4 µl nuclease-free water

4 µl ImProm-II 5x Reaction buffer

4 µl $MgCl_2$ (25 mM)

1 µl dNTP mix (10 mM)

1 µl RNase inhibitor (20 U)

1 µl ImProm-II reverse transcriptase

Final volume: 15 µl

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

| Reaction | Temperature | Time |
|---|---|---|
| Annealing | 25°C | 5 min |
| Extension | 42°C | 2 hours |
| Reverse transcriptase inactivation | 70°C | 15 min |

Table XV: Reverse transcription temperature cycle.

1 µl of the first strand cDNA was directly used in a PCR reaction in a final volume of 50 µl. The reaction condition was the same as in typical PCR reaction (see page 96).

### 2. Solutions used for molecular biology

| | |
|---|---|
| Solution I | 25 mM Tris-HCl pH=8, 50 mM glucose (Fluka), 10 mM EDTA pH=8. |
| Solution II | 0.2 N NaOH, 1 % SDS. Prepared freshly. |
| Solution III | 3 M potassium acetate, 11.5 ml of glacial acetic acid for 100 ml final solution. |
| TE-buffer | 10 mM Tris/HCl, 1 mM EDTA,pH=8.0 |
| Bromophenol blue-mix | 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll |
| Ethidium Bromide | 0.5 µg/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 |
| RNA 2X Loadding buffer | 3% Ficoll; 0.05% Xylene cyanol; 0.05% bromophenol blue |
| RNA extraction Buffer | 1 Vol of 2 M Tris pH=8.0; 2 Vol of EDTA pH=8.0; 1 Vol 20% SDS |
| 6M Lithium Chloride | |
| 70% EtOH | 70 ml of 100% ethanol diluted in 30 ml of DEPC water |
| DEPC water | 500 µl of DEPC in 1 l of water stir very well and autoclaved. |

Table XVI: solutions for molecular biology.

## *E. Enzymes, chemicals and kits*

### 1. Enzymes

| **Enzymes** | **Buffer used** | **Manufacturer** |
|---|---|---|
| BamHI | D | promega |
| SalI | D | promega |
| T4 DNA Ligase | 10X ligase buffer | Promega |
| Taq polymerase | 10X Taq buffer | Promega |
| Pfu polymerase | 10X Pfu buffer | Promega |

### 2. Chemicals

| **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 |

### 3. 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

For protoplast transfection, the plasmids have been isolated with the JETSTAR 2.0 Plasmid Midiprep Kit (Genomed), as indicated by the kit manual.

# IV. Protein techniques

## *A. Protein extraction*

### 1. Whole plant extracts

We extracted proteins from small quantity material in the simplest way. 0.1g of leaves was cut and put into an Eppendorf tube and 30 µl of 50 mM 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 10 min. 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.

### 2. Proteins extracted from protoplasts

After transfection, A. thaliana leaf protoplasts were gently pelleted by centrifugation at 90 g for 15'. 2 mL of the supernatant was carefully transferred to a fresh 12 ml plastic tube and placed on ice (secreted fraction). The pellet was resupended with 2 ml of W5 and gently centrifuged. The supernatant was discarded and the pellet (approximately 200 µl), transferred to a fresh 1.5 ml plastic tube, sonicated and placed on ice (cell fraction). In the different fractions, proteins were precipitated by addition of 2 volumes of acetone and placed overnight at 4 °C. Samples were centrifuged at max speed, the pellet was rinced with EtOH 70%, allowed to dry and resuspended with 20 µl of protein extraction buffer and kept at -20 °C. Thus the cell fractions were concentrated approximately 10 times, the secreted fractions 100 times.

## *B. Proteins analysis by 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. 20 µl 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 |
| 1.5 M Tris pH 8.8 | 2.00 ml | |
| Protogel | 4.00 ml | 0.650 ml |
| 0.5 M Tris pH 6.5 | 1.25 ml | |
| 10% SDS | 0.08 ml | 0.05 ml |
| APS 10% | 0.04 ml | 0.025 ml |
| TEMED | 0.005 ml | 0.015 ml |
| **Final volume** | **8 ml** | **5 ml** |

Table XVII: composition of a SDS-PAGE gel.

Protogel stock: 30% Acrylamide/Bisacrylamide.

Electrophoretic transfert of proteins

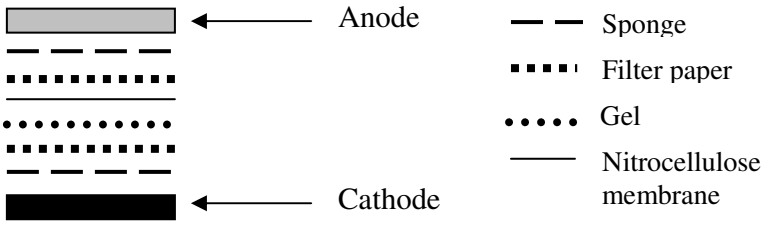
After SDS-PAGE, the proteins were transferred from the gel onto a Millipore membrane (Immobilon™-PSQ 0.2µm Membrane).

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 122 mA for 1 to 2 hours. After this time the blot could be used for probing.

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, the blot was washed again 4 times in 1x PBS containing 0. 2 % of Tween and 4 times in 1x PBS.

The secondary antibodies immunoblotted were revealed with the ECL plus detection kit (GE Healthcare) as indicated in the manual of the kit. The chemiluminescent product of the HRP-coupled secondary antibody was detected by a Chemidoc XRS System (Biorad).

## *C. 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 30 min with shaking every 5 min. Then, the blot was rinsed 10 times for 10 min each with 1x PBS. After the washes, it was blocked and probed**.**

## *D. Solutions used for protein techniques*

| | |
|---|---|
| Protein Electrophoresis buffer | 196 mM glycine / 0.1% SDS / 50 mM Tris-HCl pH=8.3 |
| 0,5M Tris-HCl pH6,5 | 60.57 g was dissolved in water then the pH was brought to 6.5 using a solution 2 N HCl. Autoclaved for sterilisation |
| 1.5M Tris-HCl pH 8,8 | 181.71 g was dissolved in water then the pH was brought to 8.8 using a solution 2 N HCl. Autoclaved. |
| 10% SDS | 10 g of SDS in 100 ml of water |
| 10% APS | 10 g of APS in 100 ml filter sterilized |
| Protein Transfer buffer | 3.03 g Tris –base; 14.4 g Glycine; 200 ml Methanol |
| 2X protein loading buffer | 125 mM Tris-HCl pH=6.8, 10% β-mercaptoethanol, 10% SDS, 10% glycerol |
| PBS buffer | NaCl : 8 g, KCl: 0.2 g, $Na_2HPO_4$: 1.15 g, $KH_2PO_4$: 0.21 g, 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, 50 mM Tris pH=6.8, 100 mM, β-mercaptoethanol |
| Protein extraction buffer | 50 mM Tris-HCL buffer pH=7.6 supplemented with 150 mM NaCl, 5 mM EDTA, 0.1% SDS, and 0.1% β-mercaptoethanol |

# Annexes

## I. DNA sequences

### Sequence of AtRMR1 cds (JR702), AGI: 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
```

### Sequence of AtRMR2 cds (JR700), AGI: 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
```

## Sequence of AtRMR3 cds (T22J18), AGI: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
```

## Sequence of AtRMR4 cds (CAB 78079), AGI: 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
```

## Sequence of AtRMR5 cds (F1504.19/1), AGI: 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
```

## Sequence of AtRMR6 cds (F1504.19/2), AGI: 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
```

Sequences fused to GFP for the Aleu143, aleu15 and Chi constructions.

```
ATGGCCCACGCCCGCGTCCTCCTCCTGGCGCTCGCCGTCCTGGCCACGGCCGCCGTCGCCGTC
M  A  H  A  R  V  L  L  L  A  L  A  V  L  A  T  A  A  V  A  V
GCCTCCTCCTCCTCCTTCGCCGACTCCAACCCGATCCGGCCCGTCACCGACCGCGCCGCCTCG
A  S  S  S  S  F  A  D  S  N  P  I  R  P  V  T  D  R  A  A  S
ACGCTCGAGTCCGCCGTCCTCGGCGCGCTCGGCCGCACCCGCCACGCCCTCCGCTTCGCGCGC
T  L  E  S  A  V  L  G  A  L  G  R  T  R  H  A  L  R  F  A  R
TTCGCCGTCAGGTACGGCAAGAGCTACGAGAGCGCGGCGGAGGTGCGGAGGCGGTTCCGGATC
F  A  V  R  Y  G  K  S  Y  E  S  A  A  E  V  R  R  R  F  R  I
TTCTCCGAGAGCCTCGAGGAGGTGCGCTCCACCAACCGGAAGGGCCTCCCCTACCGCCTCGGC
F  S  E  S  L  E  E  V  R  S  T  N  R  K  G  L  P  Y  R  L  G
ATCAACCGCTTCTCGGACATGAGCTGGGAGGAGTTCCAGGCGACCCGTCTCGGCGCGGCGCAG
I  N  R  F  S  D  M  S  W  E  E  F  Q  A  T  R  L  G  A  A  Q
ACCTGCTCGGCGACGCTCGCCGGCAACCACCTCATGCGGGACGCCGCC
T  C  S  A  T  L  A  G  N  H  L  M  R  D  A  A
```

**Sequence of Aleu143 including the signal sequence and the whole propeptide of barley aleurain, including the ssVSD.**

```
ATGGCCCACGCCCGCGTCCTCCTCCTGGCGCTCGCCGTCCTGGCCACGGCCGCCGTCGCCGTC
M  A  H  A  R  V  L  L  L  A  L  A  V  L  A  T  A  A  V  A  V
GCCTCCTCCTCCTCCTTCGCCGACTCCAACCCGATCCGGCCCGTCACCGACCGCGCCGCCTCG
A  S  S  S  S  F  A  D  S  N  P  I  R  P  V  T  D  R  A  A  S
ACGCTCGAGTCC
T  L  E  S
```

**Sequence of aleu15 including the signal sequence and the first 15 amino acids of the propeptide, including the ssVSD.**

```
GATCTTTTAGTCGATACTATG
D  L  L  V  D  T  M
```

**Sequence of the C-terminal propeptide of tobacco chitinase, including the CtVSD.**

Sequences of the dominant negative constructs

```
GGATCCATGAATCGTGCTTTGGTCCTACTTTTATATGTTTGTACTGTTTCTTGTTTAGCTTCAGAT
      M   N   R   A   L …                                   … A   S* D
ACGAAAGTTTGGTTGATCCCAAGTTTTGAGAACTCGGCGTGGTCTATTATGGCGGTTTCGTTTATC
T   K   V   W …
TCGCTGCTTGCAATGTCGGCTGTTCTCGCTACTTGTTTCTTTGTGCGTAGGCATCGAATAAGAAGG

CGGACATCTCGGTCCTCTCGAGTGCGTGAGTTTCACGGTATGAGCCGCCGCTTGGTGAAAGCAATG

CCGAGTCTTATATTCAGTTCGTTTCATGAAGATAACACTACTGCATTCACTTGTGCTATTTGCCTT

GAAGACTACACTGTTGGAGACAAGCTCAGGCTCTTACCTTGCTGTCACAAGTTTCATGCTGCGTGT

GTTGACTCATGGTTAACCTCTTGGAGAACTTTCTGTCCGGTGTGCAAACGAGATGCAAGAACGAGC

ACGGGAGAGCCTCCAGCTTCAGAGAGCACGCCATTGCTCTCATCTGCTGCATCGTCTTTCACTTCT

TCCTCTCTGCACTCTTCAGTCAGATCATCTGCACTATTGATTGGTCCTTCCTTGGGCTCATTACCA

ACTTCAATCTCTTTCTCTCCCGCATACGCAAGCTCATCCTATATTAGACAATCATTCCAGTCTTCC

TCTAACCGTCGATCACCTCCAATAAGCGTAAGTCGAAGCTCAGTGGATCTCAGACAACAAGCAGCT

TCTCCATCTCCATCACCATCACAGAGATCATACATTTCCCATATGGCTTCTCCACAGTCACTAGGT

TACCCAACTATCTCCCCTTTCAACACGAGGTACATGTCACCGTATAGACCTAGCCCGAGCAATGCA

TCACCTGCAATGGCTGGATCATCGAATTATCCGTTGAATCCACTGCGTTACAGTGAATCAGCTGGA

ACTTTCTCTCCATACGCCTCTGCAAACTCGCTTCCAGACTGTTAGGTCGAC
                          … L   P   D   C   Stop
```

**RMRΔlum Sequence from BamHI to SalI.**
The sequence from AtRMR1 are represented in blue.
The asterisk is placed where the signal peptide of AtRMR1 is fused to the TM and cytosolic domain of AtRMR1.

```
GGATCCATGAATCGTGCTTTGGTCCTACTTTTATATGTTTGTACTGTTTCTTGTTTAGCTTCAAGC
      M   N   R   A   L …
AAAGTTATTTTGATGAGGAATAACATCACTCTCTCTTTTGATGACATCGAAGCTAACATCGCTCCG

TCAGTGAAGGGTACAGGTGAAATTGGAGTGGTTTATGTGGCTGAGCCTCTTGACGCTTGTCAAAAT

CTTATGAATAAACCAGAACAGAGCTCCAATGAAACTTCTCCTTTTGTGTTGATTGTTAGAGGAGGC

TGTAGTTTTGAAGAGAAAGTTAGAAAAGCTCAGAGAGCTGGTTTCAAAGCTGCTATTATCTATGAC

AATGAAGACCGTGGAACATTGATAGCAATGGCAGGTAACTCTGGAGGTATAAGGATTCATGCGGTC

TTTGTTACGAAAGAAACGGGAGAAGTTTTAAAGGAGTATGCGGGTTTCCCCGATACGAAAGTTTGG

TTGATCCCAAGTTTTGAGAACTCGGCGTGGTCTGAGCTCTATAAGGAGCAGAAGCTGATCTCCGAG
                    … S   A   W   S   E   L   Y   K   E   Q   K   L   I   S   E
GAGGACCTGGCTAGCTCTGAGCACGACGAGCTATAAGTCGAC
E   D   L   A   S   S   E   H   D   E   L   Stop
```

**RMRlumER construct from BamHI to SalI.**
The luminal domain of AtRMR1 is represented in blue.
The Myc tag is represented in green.
The HDEL ER-retention signal is in red.

```
GGATCCATGAATCGTGCTTTGGTCCTACTTTTATATGTTTGTACTGTTTCTTGTTTAGCTTCAAGC
      M   N   R   A   L …
AAAGTTATTTTGATGAGGAATAACATCACTCTCTCTTTTGATGACATCGAAGCTAACATCGCTCCG

TCAGTGAAGGGTACAGGTGAAATTGGAGTGGTTTATGTGGCTGAGCCTCTTGACGCTTGTCAAAAT

CTTATGAATAAACCAGAACAGAGCTCCAATGAAACTTCTCCTTTTGTGTTGATTGTTAGAGGAGGC

TGTAGTTTTGAAGAGAAAGTTAGAAAAGCTCAGAGAGCTGGTTTCAAAGCTGCTATTATCTATGAC

AATGAAGACCGTGGAACATTGATAGCAATGGCAGGTAACTCTGGAGGTATAAGGATTCATGCGGTC

TTTGTTACGAAAGAAACGGGAGAAGTTTTAAAGGAGTATGCGGGTTTCCCCGATACGAAAGTTTGG

TTGATCCCAAGTTTTGAGAACTCGGCGTGGTCTGAACTATACAAGTCGACTATCGAAGGTAGAGAA
                    … S   A   W   S   E   L   Y   K   S   T …
GCTGAAGCTCTGATCCCCATCGCTGTGGGTGGCGCGCTAGCGGGGCTGGTCCTCATCGTCCTCATC

GCCTACCTCGTCGGCAGGAAGAGATCTTAAGTCGAC
            G   R   K   R   S   Stop
```

**RMRlumPM construct from BamHI to SalI.**
The luminal domain of AtRMR1 is represented in blue.
The TM23 sequence is in brown.

```
GGATCCATGAGAAGGCGGACATCTCGGTCCTCTCGAGTGCGTGAGTTTCACGGTATGAGCCGCCGCT
      M   R   R   R   S …
TGGTGAAAGCAATGCCGAGTCTTATATTCAGTTCGTTTCATGAAGATAACACTACTGCATTCACTTG

TGCTATTTGCCTTGAAGACTACACTGTTGGAGACAAGCTCAGGCTCTTACCTTGCTGTCACAAGTTT

CATGCTGCGTGTGTTGACTCATGGTTAACCTCTTGGAGAACTTTCTGTCCGGTGTGCAAACGAGATG

CAAGAACGAGCACGGGAGAGCCTCCAGCTTCAGAGAGCACGCCATTGCTCTCATCTGCTGCATCGTC

TTTCACTTCTTCCTCTCTGCACTCTTCAGTCAGATCATCTGCACTATTGATTGGTCCTTCCTTGGGC

TCATTACCAACTTCAATCTCTTTCTCTCCCGCATACGCAAGCTCATCCTATATTAGACAATCATTCC

AGTCTTCCTCTAACCGTCGATCACCTCCAATAAGCGTAAGTCGAAGCTCAGTGGATCTCAGACAACA

AGCAGCTTCTCCATCTCCATCACCATCACAGAGATCATACATTTCCCATATGGCTTCTCCACAGTCA

CTAGGTTACCCAACTATCTCCCCTTTCAACACGAGGTACATGTCACCGTATAGACCTAGCCCGAGCA

ATGCATCACCTGCAATGGCTGGATCATCGAATTATCCGTTGAATCCACTGCGTTACAGTGAATCAGC

TGGAACTTTCTCTCCATACGCCTCTGCAAACTCGCTTCCAGACTGTTAGGTCGAC
                                … S   L   P   D   C   Stop
```

**RMRcyt sequence from BamHI to SalI.**
The AtRMR1 cytosolic sequence is represented in blue.
An additional M has been inserted at the N-terminus of the construct (in red) to ensure its expression.

Other constucts not tested yet

The following constructs have been designed to test the *in vitro* binding properties of the luminal domains of AtRMR proteins with GFP-VSDs.

To cope with the fact that AtRMR1 is glycosylated, at least when expressed in Drosophila cells (Park et al. 2007), I chose to use Saccharomyces cerevisiae cells to

express the soluble luminal domains of AtRMR proteins. None of the GFP construct being glycosylated in plants, I chose to express it in bacteria. For these purposes, I used the pGal yeast expression vector and the pET22b(+) vector for E. coli, both already available in the lab (the pET22b vector being kindly provided by Dr. Nicolas Humbert).

pGalRMRlumHis construct

To clone the luminal domains of AtRMR proteins, I planed to use the restriction sites of EcoRI and BamHI present in the multiple cloning site of pGal (Humair et al. 2001), I also added to the sequence six His residues for pull down assays and detection of the constructs by western blot. Up to know, the luminal binding domains of AtRMR1, AtRMR2 and AtRMR4 have been subcloned in pGEM-T.

Sequence of RMRlumHis :

```
GAATTCATGAATCGTGCTTTGGTCCTACTTTTATATGTTTGTACTGTTTCTTGTTTAGCTTCAAGCA
      M  N  R  A  L …
AAGTTATTTTGATGAGGAATAACATCACTCTCTCTTTTGATGACATCGAAGCTAACATCGCTCCGTC

AGTGAAGGGTACAGGTGAAATTGGAGTGGTTTATGTGGCTGAGCCTCTTGACGCTTGTCAAAATCTT

ATGAATAAACCAGAACAGAGCTCCAATGAAACTTCTCCTTTTGTGTTGATTGTTAGAGGAGGCTGTA

GTTTTGAAGAGAAAGTTAGAAAAGCTCAGAGAGCTGGTTTCAAAGCTGCTATTATCTATGACAATGA

AGACCGTGGAACATTGATAGCAATGGCAGGTAACTCTGGAGGTATAAGGATTCATGCGGTCTTTGTT

ACGAAAGAAACGGGAGAAGTTTTAAAGGAGTATGCGGGTTTCCCCGATACGAAAGTTTGGTTGATCC

CAAGTTTTGAGAACGTCGACCACCACCACCATCATCATTAACTCGAGAATGGATCC
… S  F  E  N       H  H  H  H  H  H  Stop
```

Sequence of theRMRlumHis construct.
The sequence from the AtRMR1protein sequence is represented in blue, the six His repeat in green.

Sequence of AtRMR2lumHis :

```
GAATTCATGAGACTCGTCGTCTCAAGCTGTCTACTAGTTGCAGCTCCTTTTCTCTCCTCTCTGTT
      M   R   L   V   V …
ACGAGTCTCACTCGCCACTGTTGTCCTCAATTCCATCTCCGCCTCTTTTGCCGATCTCCCAGCCA

AATTTGACGGCTCCGTGACCAAAAACGGAATCTGTGGAGCTCTATACGTCGCAGATCCTCTCGAC

GGTTGCTCACCGCTTCTCCACGCCGCCGCATCCAACTGGACGCAACACAGAACTACTAAGTTCGC

TTTGATAATCAGAGGCGAATGTTCTTTTGAGGATAAGCTGCTCAATGCCCAGAACTCAGGTTTTC

AAGCTGTGATTGTCTATGACAACATTGACAACGAAGATCTCATCGTCATGAAGGTGAACCCTCAG

GACATTACAGTTGATGCAGTCTTCGTTTCAAATGTCGCCGGTGAGATTTTGAGAAAGTACGCGAG

AGGCCGAGATGGTGAATGCTGCCTTAATCCGCCAGACAGAGGGGTCGACCACCACCACCATCATC
                                … P   P   D   R   G         H   H   H   H   H   H
ATTAACTCGAGAATGGATCC
  Stop
```

Sequence of theAtRMR2lumHis construct.
The sequence from the AtRMR2protein sequence is represented in red, the six His repeat in green.

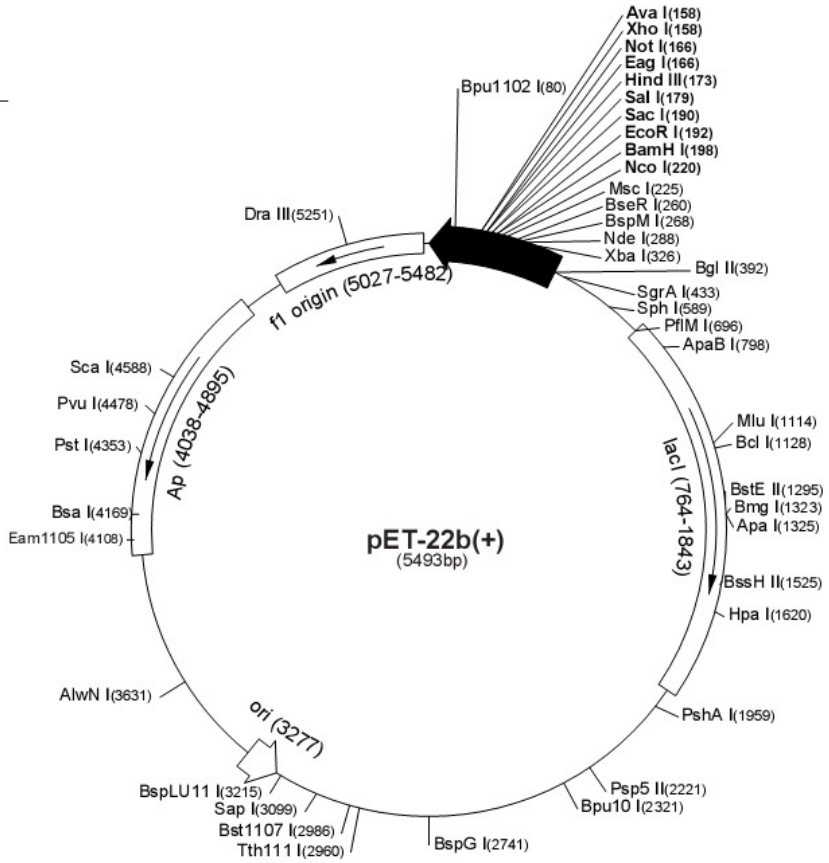
Sequence of AtRMR4lum His :

```
GAATTCATGATCCGTTCTTCGATTGTAATTTTATCTCTGTTACTAATTTCACACTTGGTTTCTGCA
      M   I   R   S   S …
AAAGTTCTGTTGATCGGTAACAGCACATCTCTCTCCTTCGACGACGTCGAAGCCACTTTCACTCCG

ATGATTAAGAGATCGGATCAAGGCGGTGTGTTGTATGTAGCAGAGCCACTCGATGCTTGTTCGGAT

TTGGTGAATACGGTGAATGTGAAAAATGGAACTACTGTGTCTCCTCCGTATGTGTTGATTATCCGC

GGTGGTTGTAGTTTCGAAGATAAGATTAGGAATGCTCAAAAGGCTGGTTATAAAGCTGCTATTGTT

TATGACTATGAAGATTTTGGGTTCTTAGTATCAATGGAGCGAAACCCCTCTGGTGTACTTATTTAT

GGTACGTTTGTCTCCAAAGCAACTGGGGAAGTACTTAAAGAGTATGCGGGTCGTACCGATTTTGAA

GTGTGGCTCATGCCAAGTTTCGAGACTGTCGACCACCACCACCATCATCATTAACTCGAGAATGGA
            … P   S   F   E   T         H   H   H   H   H   H   Stop
TCC
```

Sequence of theAtRMR4lumHis construct.
The sequence from the AtRMR4 protein sequence is represented in Bordeaux, the six His repeat in green.

Map of the pET22b(+) (Novagen)

| pET-22b(+) sequence landmarks | |
|---|---|
| T7 promoter | 361-377 |
| T7 transcription start | 360 |
| pelB coding sequence | 224-289 |
| Multiple cloning sites | |
| (Nco I - Xho I) | 158-225 |
| His• Tag coding sequence | 140-157 |
| T7 terminator | 26-72 |
| lacI coding sequence | 764-1843 |
| pBR322 origin | 3277 |
| bla coding sequence | 4038-4895 |
| f1 origin | 5027-5482 |




**Taken from Novagen TB038 (http://www.merckbiosciences.co.uk/)**

Up to know, Aleu$_{143}$GFP, GFPchi and GFPKDEL have been cloned in the BamHI restriction site of pET22b(+) using two BamHI sites added by PCR to the 5' and 3' ends of GFP constructs. In the pET22b(+), the translation start is upstream the BamHI site and not in the same coding frame, therefore, to add my constructs in the right coding frame, I added an adenosine between the 5'-end BamHI site and the coding sequences of the GFP constructs by PCR.

GGATCCAATGGCCCACGCCCGCGTCCTCCTCCTGGCGCTCGCCGTCCTGGCCACGGCCGCCGTCGCC
M A H A R V L L L A L A V L A T A A V A
GTCGCCTCCTCCTCCTCCTTCGCCGACTCCAACCCGATCCGGCCCGTCACCGACCGCGCCGCCTCGA
V A S S S S F A D S N P I R P V T D R A A S
CGCTCGAGTCCGCCGTCCTCGGCGCGCTCGGCCGCACCCGCCACGCCCTCCGCTTCGCGCGCTTCGC
T L E S
CGTCAGGTACGGCAAGAGCTACGAGAGCGCGGCGGAGGTGCGGAGGCGGTTCCGGATCTTCTCCGAG

AGCCTCGAGGAGGTGCGCTCCACCAACCGGAAGGGCCTCCCCTACCGCCTCGGCATCAACCGCTTCT

CGGACATGAGCTGGGAGGAGTTCCAGGCGACCCGTCTCGGCGCGGCGCAGACCTGCTCGGCGACGCT

CGCCGGCAACCACCTCATGCGGGACGCCGCCGCTAGCGCAATGAGTAAAGGAGAAGAACTTTTCACT

GGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAG

AGGGTGAAGGTGATGCAACATACGGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAACTACC

TGTTCCATGGCCAACACTTGTCACTACTCTCACTTATGGTGTTCAATGCTTTTCAAGATACCCAGAT

CATATGAAGCGGCACGACTTCTTCAAGAGCGCCATGCCTGAGGGATACGTGCAGGAGAGGACCATCT

TCTTCAAGGACGACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAGGGAGACACCCTCGTCAA

CAGGATCGAGCTTAAGGGAATCGATTTCAAGGAGGACGGAAACATCCTCGGCCACAAGTTGGAATAC

AACTACAACTCCCACAACGTATACATCATGGCCGACAAGCAAAAGAACGGCATCAAAGCCAACTTCA

AGACCCGCCACAACATCGAAGACGGCGGCGTGCAACTCGCTGATCATTATCAACAAAATACTCCAAT

TGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGAT

CCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCA

TGGATGAACTATACAAATAAGGATCC

**Sequence of the Aleu$_{143}$GFP fragment inserted in pET22b(+) from BamHI to BamHI.**
The aleu peptide sequence is represented in red.
The adenosine inserted to respect the coding frame of the pET22b(+) is represented in green.

GGATCCAATGAAGACTAATCTTTTTCTCTTTCTCATCTTTTCACTTCTCCTATCATTATCCTCGG

CCGAATTCAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGT

GATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCAACATACGGAAAACT

TACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTT

TCTCTTATGGTGTTCAATGCTTTTCAAGATACCCAGATCATATGAAGCGGCACGACTTCTTCAAG

AGCGCCATGCCTGAGGGATACGTGCAGGAGAGGACCATCTTCTTCAAGGACGACGGGAACTACAA

GACACGTGCTGAAGTCAAGTTTGAGGGAGACACCCTCGTCAACAGGATCGAGCTTAAGGGAATCG

ATTTCAAGGAGGACGGAAACATCCTCGGCCACAAGTTGGAATACAACTACAACTCCCACAACGTA

TACATCATGGCCGACAAGCAAAAGAACGGCATCAAAGCCAACTTCAAGACCCGCCACAACATCGA

AGACGGCGGCGTGCAACTCGCTGATCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCC

TTTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGA

GACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAACTATA

CAAAGATCTTTTAGTCGATACTATGTAAGGATCC
D L L V D T M Stop

**sequence of the GFPChi fragment inserted in pET22b(+) from BamHI to BamHI.**
The tobacco chitinase A CtVSD is represented in red.

GGATCCAATGAAGACTAATCTTTTTCTCTTTCTCATCTTTTCACTTCTCCTATCATTATCCTCGG

CCGAATTCAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGT

GATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCAACATACGGAAAACT

TACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTT

TCTCTTATGGTGTTCAATGCTTTTCAAGATACCCAGATCATATGAAGCGGCACGACTTCTTCAAG

AGCGCCATGCCTGAGGGATACGTGCAGGAGAGGACCATCTTCTTCAAGGACGACGGGAACTACAA

GACACGTGCTGAAGTCAAGTTTGAGGGAGACACCCTCGTCAACAGGATCGAGCTTAAGGGAATCG

ATTTCAAGGAGGACGGAAACATCCTCGGCCACAAGTTGGAATACAACTACAACTCCCACAACGTA

TACATCATGGCCGACAAGCAAAAGAACGGCATCAAAGCCAACTTCAAGACCCGCCACAACATCGA

AGACGGCGGCGTGCAACTCGCTGATCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCC

TTTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGA

GACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAACTATA

CAAGTCGACCAAGGACGAGCTCTGAGGATCC
K D E L Stop

**sequence of the GFPKDEL fragment inserted in pET22b(+) from BamHI to BamHI**
The KDEL sequence is represented in red.

# II. Alignement of RMR protein sequences

```
AtRMR1                                MNRAL------VLLLYVCTVSCLASSKVILMRNNITLSFDDIEANI
AtRMR2                                MRLVVS---SCLLVAAPFLSSLLRVSLATVVLNSISASFADLPAKF
AtRMR3                                MNLVVL---LILTLLLFIVSYVVDAGQVILVDSNITRSFVDMEADF
AtRMR4                                MIRSS------IVILSLLLISHLVSAKVLLIGNSTSLSFDDVEATF
AtRMR5                                MNYSW------ITIMSLLVICKLASAKVVLIGKNTILSFDDVEATF
AtRMR6                                MNGSW------ITIVSLLVISQLASSKVTLIGKNTFLSFDDVEANF
Medica                                MGSSNL-----CFFFFSLVSLFAMAAAKVVLIGNNITLSFDDIEANF
Lotus                                 MGTSNL-----VLLFFSLLCFSALAAAKVVLIGNNITLSFDDIEANF
Soya 1                                MGTSNL-----LLFFSLVSLCAMAASKVVLIGNNITLSFDDIEANF
Poplar1                               MKGIVV------LLLSLLSCCLVASANVVLIGNNVTMAFNDIEANF
Poplar2                               MEGVVV------LLLSLLSCCLVASGNVLLIGNNVTMAFDDIEANF
Sorghum                               MKSSSF-----LFLCAMFCLMARLGAANVVLMGNNLTLSFDDIEASF
Rice A                                MNRRRTMLLLICLCATFCLMTQLGAANVVLMGTNLTLSFDDVEASF
Rice B                                MNCTKGGGFPLLFCAVICLMAQQGACNVVLIANNTTLSFDDVETTF
Moss a      MRQSRQWRMVPFTDVCGCGRGLVSRIMNHRELMISLAGLSLVLLTLLLGRVNSAVILLTESNESWSFPDTEASF
Moss b                MPSVVAEAGFASRIMSYREIMISLAGLCLVLLTLLIGRVNSAVILLAGTNETWSFPDVESRF

        |1 Intron  / PA Domain                                                      /
AtRMR1  APSVKGTGEIGVVYVAEPLD-ACQNLMN---KPEQSSNET--SPFVLIVR-GGCSFEEKVRKAQRAGFKAAIIYDNEDRGTLIA
AtRMR2  DGSVTKNGICGALYVADPLD-GCSPLLH---AAASNWTQHRTTKFALIIR-GECSFEDKLLNAQNSGFQAVIVYDNIDNEDLIV
AtRMR3  SPSV-TTVETGVVYVAEPLN-ACRNLRN-----KPEQSPYGTSPLVLIIR-GGCSFEYKVRNAQRSGFKAAIVYDNVDRNFLSA
AtRMR4  TPMIKRSDQGGVLYVAEPLD-ACSDLVN---TVNVKNGTTVSSSYVLIIR-GGCSFEDKIRNAQKAGYKAAIVYDYEDFGFLVS
AtRMR5  TPIVRNSGECGILYVAEPLE-ACSDITN---MAEKRSKYR--SSYVLIVL-GGCSFEEKVRKAQKAGYKAAIVYNDGYDELLVP
AtRMR6  TPVVRRSGEYGLLYAAEPLD-ACSYLTN---MAEKGSKFR--PSYVLIVR-GGCSFEEKIRNAQEAGYKAAIVYNDRYEELLVR
Medica  APSVKGSGEYGALFLAEPLD-ACTELTN-----KARTLSNASSPFVLMVR-GGCSFEDKVRIAQSAGYKAAIVYDSEDGGILVA
Lotus   APAVKGSGEAGVLYLAEPLD-ACTELTN-----KV
Soya 1  APTVKGSGEYGILYLAEPLD-ACTELTN-----KVEQLPNASSPFALVVR-GGCxLEDKVRRAQKAGFKAVIVYDNEDGGILVA
Poplar1 APAIKGSGECGVLSLAEPID-ACTDLTN-----KAEKGLNSSSPYVLIIR-GGCSFEHKVRRAQKAGFKAAIVFDNEEG-VLVA
Poplar2 APAIKGSGECGVLYLAEPID-ACSDLTN-----QAEKGSNCSSPFVLIIR-EGCSFEDKVRRAQKAGYKAAIIYDNEEG-ILVA
Sorghum SPGVKGSGVNGVVYASEPLD-ACSPLTI-----KAVK--GPPSPFALIIR-GGCTFDEKVKNAQDAGFKAAIVYDNENSGVLVSKM
Rice A  APGVKGSGFEGVVYTAEPLD-ACSPLTS-----KAEK--GPPSPFALIIR-GGCTFDEKVKNAQDAGFKAAIVYDNENSGVLIS
Rice B  TPEVKDSGVNGAIYAVEPLD-ACSPLRK-----KAAN--GPVSPFALVIR-GGCQFDDKVRNAQNAGFKAVIVYDDEDSGVLVS
Moss a  SPRIPTTGIVGVLHASNPLD-ACSPLTN-VSRQGQTLF----SDFLLVER-GVCNFEVKVWNAQEAGFEAVIIYNNQNDHELVT
Moss b  APRVPTAGVGGVLYASNPLD-ACSPLLN-VSTPGKGSA----PAFLLVQR-GVCNFEIKVRLAQEAGFAAVIVYNDQDDRELVT

        |1 Intron
AtRMR1  MAGNSGGI-----RIH-----AVFVTKETGEVLKEYAGFPDTKVWLI-PSFEN
AtRMR2  MKVNPQDI-----TVD-----AVFVSNVAGEILRKYARGRDGECCLN-PPDRG
AtRMR3  MGGDSDGI-----KIQ-----AVFVMKRAGEMLKKYAGSEEMEVMLVPPNTED
AtRMR4  MAGNPSGV-----LIY-----GTFVSKATGEVLKEYAGRTDFEVWLM-PSFET
AtRMR5  MAGNSSGV-----DIH-----GLLVTRASGEVLKGYADQDEMKLWLI-PGFGI
AtRMR6  MAGNSSGV-----YIH-----GVLVTRTSGEVLKEYTSRAEMELLLI-PGFGI
Medica  MAGNSAGV-----SIH-----AVFVSKASGEILKEYTGLINVETWLI-PTFEN
Soya 1  MAGNSAGI-----KIH-----AVFVSKASGEILSKYAGLTNVEIWLI-STFEN
Poplar1 R--NSVGV-----KIH-----AVFVSKKSGETLTKYAGLTGLELWLI-PSFEN
Poplar2 R--NSAGV-----TIP-----AVFVSKTSGETLKKYAGLTDLELWII-PSFEN
Sorghum LQWLEAQVA---YIY-----MLFVSKASGEVLKNFSGHTDVEVWIL-PTVEN
Rice A  MAGSSGGI-----HIY-----AVFISKASGEVLKKFSGHTDVEVWIL-PAFEN
Rice B  MAGSSSGI-----YIY-----AVFLSKASGEVLKKYSGQSDVEVWIL-PVYEN
Moss a  MSG-SSN-----DIH---AYSVFVSKVTGEFLLKYADDKGATCYIM-PAFEN
Moss b  MSG-NPV-----NIH---AYAVFVSKYSGEFLLKYAGDVGATCHIM-PAFEN

AtRMR1  SAWSIMA-VSFISLLAMSAVLATCFFVRRHRIRRRTSRSS-RVREFHGMSRRLVKAMPSLIFS-SFHEDN
AtRMR2  SAWTVLA-ISFFSLLLIVTFLLIAFFAPRHWTQWRGRHT--RTI---RLDAKLVHTLPCFTFT-DSAHHK
AtRMR3  SVWSLYASIALILSLAIFCVMVTCVFFYRYCST---IRN--STSQFNGMCRRTVKAMPSVTFT-CAKIDN
AtRMR4  SAWSIMA-ISFISLLAMSAVLATCFFVRRHRVRRRRILAL-NGNDFHRMPKSMIIRMPTTIFN-GICDEA
AtRMR5  SSWSIMG-ITFISLLAMSAILATCFVVRRHQIRQSVRDLPHGGQGLSCMPRDLLQSMPTEVYS-GVLEES
AtRMR6  SSWSIMA-ITFVSLLVISAVLASYFSVRRHRIRQHVRDLHHGGQGHSRMPKDLLQSMPTEVYT-GVLEEG
Medica  SAWSIMA-ISFISLLAMSAVLATCFFVRRHRIRRERPRTSSHVREFHGMSSRLVKAMPSLIFT-SALEDN
Soya 1  SAWSIMA-ISFISLLAMSAVLATLFFVRKHHRIRRER-P
Poplar1 SAWSIMA-ISFISLLAMSAVLATCFFIRRHRIRREHSHSS-RVREFHGMSSRLVKAMPSLTFT-SVLEDN
Poplar2 SAWSIMA-ISFISLLAMSAVLATCFFVRRHRIRRERPRSS-RVREFHGMSSRLVKAMPSLTFT-SALEDN
Sorghum SAWSIMG-ISFISLLAMSAVLATCFFVRRHRIRRDHPRIP-EARREFQGMSSQLVKAMPSLIFT-KVQEDN
Rice A  SAWSIMA-ISFISLLAMSAVLATCFFVRRHHIRRDRPRIP-EAREFHGMSSQLVKAMPSLIFT-KVQEDN
Rice B  SAWSIMA-ISFTSLLAMAAVLATCFFVRRHQIRRDRGRIP-VTREFHGMSSQLVKAMPSLIFT-KVQEDN
Moss a  TAWSVMx-VSFISLLAVSSVLVTFFFV.    IQHLSARF--LPKEPAGMSVKEVNTLPSFVF--KHIEDGK
Moss b  TAWSVMA-VSFISLLAVSSVLATFFFVRQHRLRHLSARY--LLREPAGMSVKEVNALPSLIF--KCVEDGK



ring-H2 finger      *  *              * *|1*  *          *  *              /0 (RMR2)
AtRMR1  TTAFTCAICLEDYTVGDKLRLLP-CCHKFHAACVDSWLTSWRTFCPVCKRDARTSTGEPPASESTPLLSS------AASSFTSS
AtRMR2  A-GETCAICLEDYRFGESLRLLP-CQHAFHLNCIDSWLTKWGTSCPVCKHDIRTET---MSSEVHKRESPRTD--TSTSRFAFQ
AtRMR3  TTGFSCAICLEDYIVGDKLRVLP-CSHKFHVACVDSWLISWRTFCPVCKRDARTTADEPLATESTPFLSS---------SIATA
AtRMR4  TTSILCCICLENYEKGDKLRILP-CHHKFHVACVDLWLGQRKSFCPVCKRDARSISTDKPPSEHTPFLSRTPS-MTPTSSFLLS
AtRMR5  STSVTCAICIDDYCVGEKLRILP-CKHKYHAVCIDSWLGRCRSFCPVCKQNPRTGNDVPPASETTPLISPSPNSITSLQSFYDL
AtRMR6  STSVTCAICIDDYRVGEILRILP-CKHKYHAVCIDSWLGRCRSFCPVCKQNPRTGNDVPPASETTPLISPGPNSITSLQSFYDL
Lotus   STSGTCAVCLEDYCVGEKLRILP-CCHKFHAACVDSWLTSWRTFCPVCKRDARTGLSEPPPSESTPLLSSTPAS--VAS-----
Medica  CTSRTCAICLEDYCAGEKLRILP-CCHKFHAACVDSWLTSWRTFCPVCKRDARTGLADPPPSESTPLLSSTPSS--AASSF---
```


**Sequence alignement of the plant RMR proteins.**
The lines quoted “insert” refer to sequences in the Soya 4, Rice A and Rice B sequences that do not align to any other RMR sequences.

# III. Genevestigator expression data

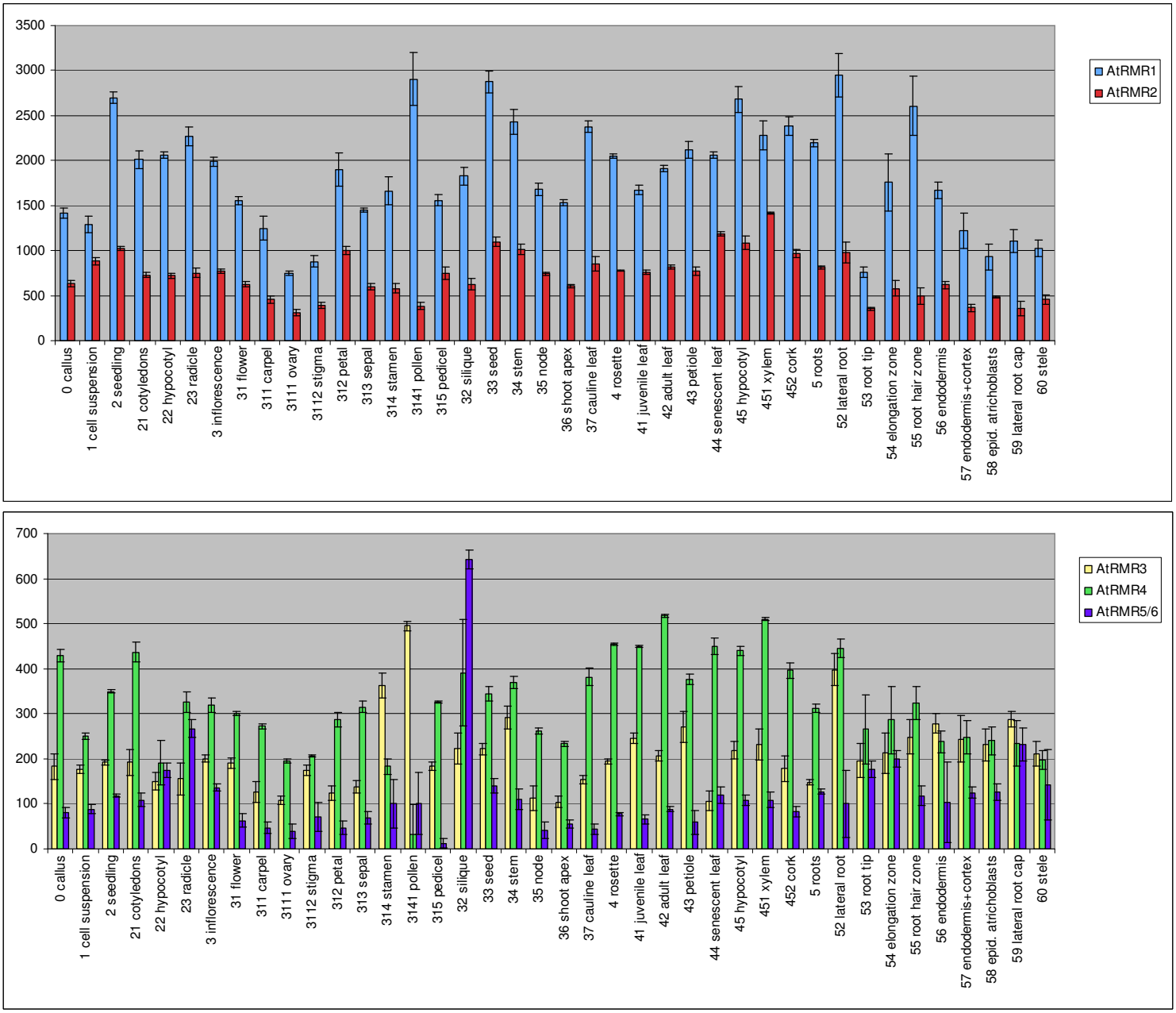



**Fig. 37: expression levels of AtRMR genes (data obtained with the "Gene Atlas" applet of Genevestigator, Zimmermann et al. 2004).**

| | AtRMR1 | AtRMR2 | AtRMR3 | AtRMR4 | AtRMR5/6 |
|---|---|---|---|---|---|
| Biotic: B. cinerea | - | | | | |
| Biotic: E. cichoracearum | - | - | - | | |
| Biotic: M. persicae | | | - | - | |
| Biotic: nematode | - | - | - | | |
| Biotic: P. infestans | | + | + | + | |
| Biotic: P. syringae | - | | | | |

**Table XIX: Summary of the influence of biotic stresses on the expression levels of AtRMR genes (data obtained with the "Response Viewer" applet found on Genevestigator Zimmermann et al. 2005).**

| | AtRMR1 | AtRMR2 | AtRMR3 | AtRMR4 | AtRMR5/6 |
|---|---|---|---|---|---|
| Chemical: 2,4,6 T | - | - | | | |
| Chemical: 6-benzyl adenine | + | + | | + | |
| Chemical: AgNO3 | - | | - | + | |
| Chemical: AVG | | | + | + | |
| Chemical: brz220 | | | - | | |
| Chemical: cycloheximide | - | - | - | + | |
| Chemical: daminozide | | | - | + | |
| Chemical: hydrogen peroxide | - | | + | | - |
| Chemical: ibuprofen | - | | | | |
| Chemical: MG13 | - | | | | |
| Chemical: norflurazon | | - | - | + | |
| Chemical: NPA | - | | - | + | |
| Chemical: ozone | - | - | | | |
| Chemical: paclobutrazole | | | - | | |
| Chemical: PCIB | | + | + | | |
| Chemical: PNO8 | | - | - | | |
| Chemical: propiconazole | | | - | | |
| Chemical: syringolin | - | + | | - | |
| Chemical: uniconazole | | - | - | - | |
| Chemical: zearalenone | | - | | + | - |
| Hormone: ABA | | + | | - | |
| Hormone: ACC | | + | | | |
| Hormone: BL | | | | + | |
| Hormone: BL / H3BO3 | + | + | | | |
| Hormone: ethylene | | | | - | |
| Hormone: IAA | - | + | | | |
| Hormone: MJ | - | | | + | + |
| Hormone: salicylic acid | | + | - | - | |
| Hormone: zeatin | | | | - | |

**Table XX: Summary of the influence of various chemicals on the expression levels of AtRMR genes (data obtained with the "Response Viewer" applet found on Genevestigator Zimmermann et al. 2005).**

| | AtRMR1 | AtRMR2 | AtRMR3 | AtRMR4 | AtRMR5/6 |
|---|---|---|---|---|---|
| Light intensity: light | - | | | | |
| Light quality: blue | - | - | + | | |
| Light quality: far red | - | | + | | |
| Light quality: red | - | | | | |
| Light quality: white | - | | | | |
| Nutrient: glucose | | - | | | |
| Nutrient: Nitrate low | - | + | | | |
| Nutrient: sucrose | - | - | + | - | + |
| Stress: cold | - | - | | (+-) | |
| Stress: drought | | + | | | |
| Stress: genotoxic | | + | | | |
| Stress: heat | | + | | | |
| Stress: osmotic | + | + | | | |
| Stress: salt | - | | | + | |

**Table XXI: Summary of the influence of various abiotic stresses on the expression levels of AtRMR genes (data obtained with the "Response Viewer" applet found on Genevestigator Zimmermann et al. 2005).**

The Genevestigator Response Viewer application was used to screen 1434 microarray chips (22K) of experiments where wt col0 plants were subjected to various growth conditions.

The AGI of the different AtRMR were used as queries. The conditions where the gene is induced are marked by a (+), when the gene is repressed, the cell is marked by a (-). The empty cells represent conditions where the gene is equally expressed in control and tested conditions or experiments where the gene expression level was so low that the change in expression may not be considered significant. The cell marked by (+-) means that AtRMR4 has been found to be induced in a set of experiments testing the cold stress and repressed in a replicate of the experiment.

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