Plant Vacuolar Sorting: An Overview

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Contents

Abstract Eukaryotic cells have developed membrane-bound organelles, connected between themselves in a complex and tightly regulated network – the endomembrane system. Despite being less well understood when compared to the animal and yeast models, plant cells have begun to reveal an intricate and dense network of endomembranes. Particularly diverse is the network of pathways revolving around the vacuole, especially when comparing plant and non-plant models. This dynamic, pleiomorphic and multifunctional organelle is essential for correct plant growth and development, compartmentalizing different components, from proteins to secondary metabolites. In this review we will provide an historical perspective of what has been discovered relating vacuolar sorting, and the potential biotech applications of such findings.

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List of Abbreviations

AP	Aspartic proteinase
BFA	Brefeldin A
BP-80	80 kDa proaleurein-binding protein
CCV	Clathrin coated vesicle
CPY	Carboxypeptidase Y
ctVSD	C-terminal vacuolar sorting determinant
Cvt	Cytosol-to-vacuole targeting pathway
DCB	Dichlorobenzonitrile
EM	Electron microscopy
ER	Endoplasmic reticulum
ERvt	ER to the vacuole targeting pathway
EST	Expressed sequence tag
GEF	Guanine nucleotide exchange factor
LV	Lytic vacuole
M6P	Mannose-6-phosphate
PA Domain	Protease associated domain
PB	Protein body
PI3P	Phosphatidylinositol 3-phosphate
PM	Plasma membrane
PSI	Plant-specific insert
PSV	Protein storage vacuole
psVSD	Physical structure vacuolar sorting determinant
PVC	Prevacuolar compartment
RMR	Receptor-homology-region-transmembrane-domain-RING-H2
SAPLIP	Saposin-like protein
SNARE	Soluble NSF attachment protein receptor
ssVSD	Sequence-specific vacuolar sorting determinant
TGN	*trans* Golgi Network
TIP	Tonoplast intrinsic protein
VSR	Vacuolar sorting receptor

1 Introduction

Though often unnoticed, plants play vital roles in our lives. Whole industrial sectors, as varied as food and feed, textile, construction, fuels and even the pharmaceutical sectors, derive their raw materials from plants. In more recent years, these versatile organisms have also been exploited as biofactories for the

recombinant production of vaccines, antibiotics and vitamins. Plant biology has been advancing rather rapidly, and plant biologists find themselves at the point where they must study plants from a holistic point of view, integrating physiology and metabolism with the subcellular architecture and their associated dynamics underlying each individual cellular component.

Understanding plants at the molecular level – how are their proteins synthesized? What functions do they serve? When and where are these proteins accumulated to serve their function? And in which way are these proteins interacting with each other, for the ultimate purpose of homeostatic maintenance of the whole organism is becoming fundamental in our quest to further explore these organisms as the rich sources of materials and wealth that they have already proven to be.

In essence, a eukaryotic cell is an integrated system comprised of several membrane-delimited compartments. These endomembranes serve the function of delimiting different physical and chemical sub-regions within the cell, allowing for much more diversified metabolic reactions to occur. Thus, understanding the functional organization of the endomembrane system, and how these cells create, maintain and erase their chemical landscapes at the most fundamental levels might allow for future manipulation of such processes by the biotechnological sector.

At present, most studies focusing on the plant endomembrane system attempt to either functionally characterize these compartments, identifying key pathways, vesicle carriers or molecular machinery responsible for the selective transport of cargo molecules between compartments, allowing for the arrival of all proteins at their site of action in a timely fashion. Our group is no exception, and our focus during the last decade has been on the study of two aspartic proteinases. Originally isolated from *Cynara cardunculus* L. (cardoon), cardosins share substantial sequence similarity (approximately 70%), but accumulate in different subcellular compartments in the native system. This curious dynamic led us to try and comprehend the subtleties of cardosin trafficking in plant systems, which have proven themselves a very rich model to study vacuolar sorting in plant cells.

Plants are sessile organisms, and thus must be capable of incredible adaptability in order to survive an ever-changing environment. By studying the plant endomembrane system, we have started to better understand the molecular bases behind plant plasticity and adaptation to all sorts of stressors, be they biotic or abiotic in nature. Such knowledge may also prove useful for the future design and engineering of tailor-made plants, better prepared to both face specific environmental challenges, or better optimized for obtaining different sets of end-products (food vs fuel strains, for example).

Focusing on the plant vacuole and the sorting of its proteins, this paper will review what is currently known about this subject, from an historical perspective and up to the latest findings in the study of the plant endomembrane system, namely in terms of RAB GTPases, SNAREs and sorting receptors and determinants. We will explore some of the chemical tools currently under development and the rise of "chemical genomics" in plant biology, present a study case, and explore how the

application of this body of knowledge through biotechnology might one day benefit all of mankind.

2 Plant Vacuoles and Function

The vacuole is an integral part of the plant endomembrane system, occupying a large percentage of these cells' volume (up to 90%). Delineated by a membrane (the tonoplast), these organelles are incredibly dynamic. Vacuolar morphology has been a hot topic for some time, and still attracts the attention of many researchers worldwide, as it changes continuously during a cell's life cycle and developmental cycles (Zhang et al. 2014). Vacuoles play several functions in plant cell physiology, most notably as a storage point for solutes, ions and water. Due to the osmotic water uptake, these organelles serve as a hydrostatic skeleton, which combined with the rigid cell wall, drives cell growth and cell volume regulation. The plant vacuole is also a key player in plant defence, whether by facilitating cytoplasm detoxification from harmful molecules, or by accumulating toxic compounds to be released against predators. The plant vacuole may also serve as a plant lysosome, hydrolysing proteins received either by endocytic or autophagic processes. This lytic vacuole (LV) is mostly present in plant vegetative tissues. In seeds, most vacuoles are of a different type – protein storage vacuoles (PSVs) accumulate reserve proteins for the embryo to mobilize during the early phases of seed germination. Both types of vacuoles may co-exist, particularly during seed germination, when LVs fuse with PSVs, for the degradation of the storage proteins, and the mobilization of free amino acids.

The tonoplast is impermeable to water, ions and other metabolites. For transporting these compounds, the vacuolar membrane is adorned with transmembrane enzymes, transporters and channels. These proteins' activities are modulated by the chemical landscape of the cell's cytosol, regulating water and solute exchange between the two compartments, maintaining cellular homeostasis. Classically, LVs and PSVs have been distinguished by the aquaporin isoforms that ornament their tonoplast – tonoplast intrinsic proteins (TIPs). Lytic vacuoles would have a γ-TIP enriched membrane, whereas protein storage vacuoles would instead accumulate α-TIP in their tonoplasts (Vitale and Raikhel 1999; Paris et al. 1996). We have since then come to realize the real picture to be somewhat more complex, as overlapping of both markers and vacuolar remodelling has been observed (Olbrich et al. 2007; Bolte et al. 2011). Vacuolar remodelling is particularly evident during seed germination, due to the massive amount of morphological changes that can be observed in a short amount of time. During this remodelling, PSV tonoplasts are converted into the central vacuole membrane (Maeshima et al. 1994), but this system is far from being perfectly understood, as evidenced by the complexity unearthed in recent studies of *Arabidopsis thaliana* vacuome remodelling during seed germination (Bolte et al. 2011).

3 Plant Vacuolar Sorting: Receptors and Determinants

From a simplified perspective, the transport of cargo molecules between any two organelles can occur via two main mechanisms – either by protein–protein or protein–lipid interactions. These interactions are responsible for the formation and shaping of either vesicular or tubular structures that interconnect different organelles, mediating the transfer of cargo between them (Bonifacino and Glick 2004). This exchange of material may result in the gradual change of the compartments' biochemical compositions, forming the basis of organelle maturation and differentiation events (Luini 2011). The aforementioned protein and lipid bases of the sorting machinery are not linearly transported in a single direction; they must be continuously recycled back and forth between any two adjacent organelles, in order to maintain the basic cellular architecture from collapsing during normal cellular metabolism. This means that some components of the endomembrane system must be capable of bidirectional movement between organelles (De Marcos Lousa et al. 2012).

The principles behind protein trafficking and vesicular transport were historically laid out by a mix of electron microscopy (EM) and analytical biochemistry. One such discovery was the identification of the mammalian mannose-6-phosphate (M6P) receptors, responsible for the lysosomal transport of soluble proteins (Hoflack and Kornfeld 1985).

Lytic vacuoles are the plant equivalents to the mammalian lysosomes, accumulating a wide range of hydrolytic enzymes. Early studies quickly led to the understanding that, unlike the mammalian systems, plants produced no mannose 6-phosphate residues. Instead, vacuolar sorting information appeared encoded by short peptide sequences, rather than modified glycan structures (Shinshi et al. 1988; Voelker et al. 1989; Bednarek et al. 1990; Matsuoka and Nakamura 1991; Neuhaus et al. 1991; Holwerda et al. 1992). It is by interacting with these peptides that proteins contained in the endomembrane system are segregated from the default passive bulk-flow pathway, towards their final destinations.

The observation that a series of vacuolar proteins were enriched in pea (*Pisum sativum*) clathrin coated vesicles (CCVs) inspired the very first studies with plant vacuolar sorting receptor (VSR) proteins (Harley and Beevers 1989; Kirsch et al. 1994). With the exponential growth of available expressed sequence tags (ESTs) and sequenced genomes, it became apparent that these VSR proteins were encoded by large gene families. Arabidopsis alone has seven such genes, distributed by three different classes, all of them predicted to have very similar topologies (Fig. 1), and a possibly highly redundant biological function, as single mutants for most of these VSR proteins fail to produce any discernible phenotypes during plant development and growth (De Marcos Lousa et al. 2012). Nonetheless, Shimada et al. (2003) detected partial secretion of vacuolar cargo in *vsr1* mutant lines. Further work some years later, yielded confirmatory results that VSRs have a broad specificity in terms of vacuolar cargo recognition and sorting (Craddock et al. 2008). This interaction with sorting determinants is conditional, with pH and Ca^{2+} concentrations playing

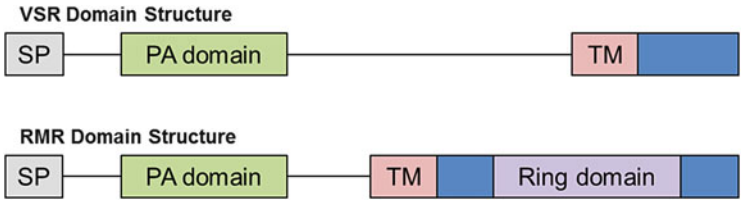

Fig. 1 Graphical representation of the domain structure of plant VSR and RMR proteins. Both receptor proteins are characterized by the presence of an amino-terminally located signal peptide (SP), responsible for inserting the proteins into the endomembrane system, a conserved protease associated domain (PA domain), which has been shown to be important for VSR–ligand interaction, and a transmembrane (TM) domain, which anchors the proteins to the lipid membranes. The RMR proteins are also characterized by the presence of a RING domain, which is inserted into a highly conserved area (represented by the *violet boxes*)

an important role in the regulation of ligand binding (Kirsch et al. 1994; Cao et al. 2000; Watanabe et al. 2002, 2004; Suen et al. 2010).

Luo et al. (2014) have been trying to understand the molecular mechanisms behind VSR-VSD interaction. By resolving the crystallographic structures of the protease-associated (PA) VSR1 domain alone and complexed with a VSD-containing peptide ($_{1}$ADS**NPIR**PVT$_{10}$), they were able to identify the occurrence of large structural changes of the VSR upon ligand binding. The main conclusions drawn by this study were that (1) hydrogen bonds were formed at the PA binding cavity in order to accept the ligand peptide; (2) binding of cargo induced a 180° flip of the VSR PA C-terminus region, which became stabilized by a novel hydrogen bond between His-181 and Glu-24. Also very relevant is that (3) the residues preceding the NPIR motif are important for cargo recognition and trafficking – the peptide's Ser-3 residue was identified as particularly relevant in this recognition process, as mutational analysis of this residue resulted in considerable missorting in vivo. (4) The invariant Arg-95 residue of the VSR PA domain is crucial for cargo binding, as it has been shown to interact with the previously identified peptide Ser-3 and (5) the swivel motion of the C-terminal tail is needed for receptor-cargo recognition (Luo et al. 2014).

The authors didn't fail to point out the observable homology between the VSR PA domain and the luminal region of the receptor-homology-region-transmembrane-domain-RING-H2 (RMR) proteins. The sequence of the binding loop appears conserved among both VSR and RMR proteins, with the RGxCxF consensus sequence, raising the possibility that RMR receptor–cargo interaction could be similar to what was observed for VSR1. How the NPIR domain interacts with the VSR receptor remains elusive; however, as this study shows a clearly disordered state of these residues upon binding between the PA domain and the preceding peptide residues. It remains possible that the PA and central domains of the VSR molecule each recognize a different sequence motif within vacuolar VSDs – the PA domain would be responsible for binding the NPIR-preceding residues, whereas the NPIR motif itself could interact with the central domain (Luo et al. 2014). Despite the open questions about how VSR–VSD interactions occur at the biophysical

level, this work appears as an important landmark for the understanding of vacuolar sorting in plant systems.

The similarity between the VSR amino-terminal 100 amino acids and the lumenal RMR protein sequence has led to the initial hypothesis that these novel receptor proteins could be involved in the sorting of vacuolar proteins (Cao et al. 2000). The first two plant RMR genes were identified due to this homology (Jiang et al. 2000), but it is currently known that the Arabidopsis genome contains a total of five such genes (AtRMR1-5). They all share high sequence similarity, and are characterized as type I integral membrane proteins, with a typical N-terminal signal peptide and a PA domain, in similarity to VSR topology (Fig. 1) (Wang et al. 2011). Although the conservation of the PA-TM-RING region is highly conserved not only amongst plant proteins, but also between Arabidopsis and animal proteins, raising the possibility of a highly conserved biological function for these receptors, the fact remains that RMR RING-H2 domain functions remain to be properly elucidated in plant systems (Wang et al. 2011).

In order to further understand these plant proteins, the intracellular localization and trafficking of RMRs have been studied in different plant cells and tissue types (Jiang et al. 2000; Park et al. 2005, 2007; Hinz et al. 2007). Jiang et al. (2000) observed tomato seed PSVs and detected the presence of RMR proteins within these organelles, particularly at the crystalloid. Further analysis revealed that this intravacuolar structure presented a high lipid to protein ratio, fuelling the idea that these crystalloids are composed of integral membrane and soluble proteins, packed within an array of lipid bilayers (Jiang et al. 2000). Curiously, even plants without a well-defined crystalloid structure inside the PSV were shown to still contain an internal network of cross-linked integral membrane proteins, including RMR proteins (Gillespie et al. 2005). Dissection of the trafficking pathway followed by these receptors showed them to possess complex glycans, a strong indication of passage through the Golgi Complex, but the receptor's final destination differs from that of the VSR reporter BP-80, which is known to accumulate in the prevacuolar compartment (PVC) (Jiang et al. 2000).

Regarding potential cargoes, Park et al. (2007) determined that the AtRMR2 receptor bound specifically to C-terminal vacuolar sorting signals, but only when these residues were exposed at the C-terminal region of the peptide. Contrary to what is observed for VSRs, this binding was found to not be pH dependent (Park et al. 2007). These observations strongly suggest that these RMR receptor proteins could be involved in the sorting of cargo to the protein storage vacuole. It is important to note that the accumulation of the RMR proteins at the PSV crystalloid would effectively remove these receptors from the endomembrane system after just one round of sorting – the aggregation model of storage protein sorting, by which a single receptor protein would interact with an aggregate of several storage protein molecules during dense vesicle-mediated sorting, could explain how a "one-round receptor" would remain efficient (Hillmer et al. 2001). In fact, Wang et al. (2011) postulated that such trapping of the RMR proteins could in fact be necessary, should the plant RING-H2 domain possess ubiquitin ligase activity, as has been

demonstrated for the mammalian homologues (Bocock et al. 2009; Kriegel et al. 2009; Wang et al. 2011).

An initial classification of vacuolar sorting determinants (VSDs) separated these signals into N-terminal, C-terminal and internal signals. Some of these determinants are easily swapped between proteins, whereas others seem to be context-dependent. Sequence-specific sorting signals (ssVSDs) allow little variation of the conserved Asn-Pro-Ile-Arg (NPIR) sequence. This VSD is typically located at the amino-terminal region of the protein, directing it towards the lytic vacuole, and can be found in proteins such as barley (*Hordeum vulgare*) proaleurain or sweet potato (*Ipomoea batatas*) sporamin. C-terminal signals (ctVSDs) are generally responsible for sorting of proteins towards the protein storage vacuole and must be exposed at the carboxyl terminus of the peptide in order for them to function properly. ctVSDs can be found in proteins such as cardoon's (*Cynara cardunculus*) cardosins, tobacco (*Nicotiana tabacum*) chitinase or Brazil nut (*Bertholletia excelsa*) 2S storage albumin. No homologous sequence or size has been so far identified for these determinants, but one common characteristic among them is that they are rich in hydrophobic amino acids. The third group of VSDs is slightly more complicated to characterize, as these signals are dependent on their tertiary structure – physical structure VSDs (psVSDs). Typically found in storage proteins, such as common bean (*Phaseolus vulgaris*) phytohemagglutinin, or broad bean (*Vicia faba*) B-type legumin, these sorting signals are present in the protein sequence and the residues that comprise them might be scattered along the protein's primary structure, coming together after correct three-dimensional folding is completed (Neuhaus and Rogers 1998; Jolliffe et al. 2005; Zouhar and Rojo 2009). Traditionally, in order for a sequence of residues to be considered a true VSD, two conditions must be met – the peptide must be both necessary and sufficient for vacuolar sorting. Many examples of VSDs that comply with these prerequisites have been described in the past (Nakamura and Matsuoka 1993; Vitale and Raikhel 1999; Robinson et al. 2005; Zouhar and Rojo 2009). However, some proteins have been identified carrying more than one VSD, often of different types. The C-terminus of soybean's β-conglycinin α-subunit withholds two functional sorting signals – a typical ctVSD and a sequence-specific sorting signal (Nishizawa et al. 2006). Cardosin A is another such example – this aspartic proteinase (AP) also possesses a typical ctVSD at the carboxy terminus, and an internal signal sequence – the plant-specific insert (PSI) – both perfectly capable of efficiently redirecting a secretory mCherry marker, towards the vacuole (Pereira et al. 2013). This PSI sequence has revealed itself as an unusual VSD sequence – approximately 100 residues-long and present in all typical plant APs, this domain is removed during the proteolytic maturation of these enzymes. Highly similar to mammalian saposins, this member of the saposin-like proteins (SAPLIPs) has been proposed to play a similar role in AP vacuolar sorting as the one described for mammalian saposin C and cathepsin D. The formation of a saposin C – cathepsin D complex is thought to be behind the M6P-independent lysosomal sorting of this mammalian enzyme (reviewed in Simões and Faro 2004).

The simultaneous presence of two VSDs in the same protein could be a strategy developed by plant cells to regulate vacuolar targeting of proteins under different physiological conditions, or tissue types. Another not entirely incompatible explanation is that two sorting signals might have an additive effect, resulting in a much greater sorting efficiency (Holkeri and Vitale 2001; Nishizawa et al. 2006). Studies with phaseolin have shown that the C-terminal peptide undergoes homotypic interactions and could be partially substituted by a C-terminal Cys residue (Pompa et al. 2010). This study has raised the question about the importance of multiple VSDs in assembled homo-oligomeric complexes. The ratio between number of VSDs and protein size is also suggested to play a role in the issue of sorting efficiency (Nishizawa et al. 2006), but so far this question is far from being clearly understood.

4 Plant Vacuolar Sorting: SNAREs

SNAREs (*N*-ethylmaleimide-sensitive factor adaptor protein receptors) are small proteins capable of forming a coiled-coil structure, interacting with other SNARE proteins via hetero-oligomeric interactions, forming highly stable SNARE complexes responsible for membrane fusion (Fig. 2) (Di Sansebastiano and Piro 2014). Particularly relevant in vesicle trafficking regulation, these proteins are nonetheless important regulators of many other signalling networks, due to their direct role in the exo- and endocytosis of all sorts of membrane proteins, such as receptor and channel proteins (Di Sansebastiano and Piro 2014). SNAREs have been classified in

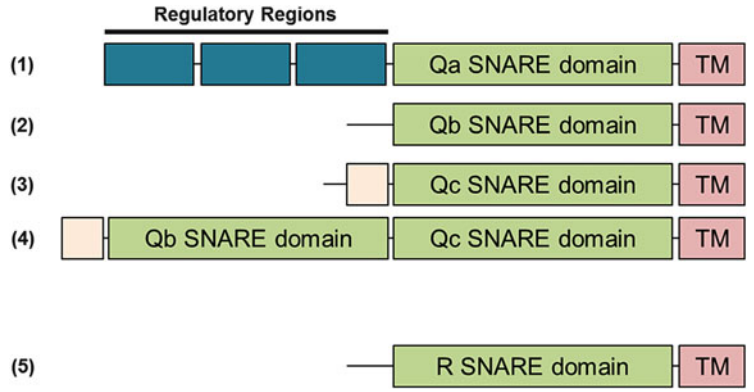

Fig. 2 Graphical representation of the domain structure of the different SNARE classes. (*1–4*) t-SNAREs are comprised by a transmembrane (TM) domain and a SNARE domain. (*1*) Qa-SNAREs are classified as "syntaxin-like" and possess a series of regulatory domains, prior to the SNARE domain (*dark blue boxes*). (*4*) SNAP25-like t-SNARE is comprised by two SNARE domains, belonging to the Qb and Qc groups. (*5*) Graphical representation of a typical R-SNARE

several ways, but the most commonly employed classification nowadays takes into account the amino acid present at the centre of the SNARE motif. Q-SNAREs have a conserved glutamine residue at this location, whereas R-SNAREs possess a conserved arginine residue instead (Fasshauer et al. 1998). There are three types of Q-SNAREs: Qa-, Qb- and Qc-SNAREs, whereas R-SNAREs can have either a short N-terminal regulatory region (designated as brevins), or a long regulatory region (designated as longins). Curiously enough, plants seem to only possess longins (Uemura et al. 2005).

A typical plant SNARE complex will generally consist of Qa-, Qb- and Qc-SNAREs at the target membrane, and R-SNAREs on the vesicle membrane (Sutter et al. 2006). The large number of plant SNAREs (estimated at 65 for Arabidopsis) may very well be the basis of the distinct secretory and vacuolar trafficking steps of plant systems (Sanderfoot 2007), and some authors argue that the multiplication of SNAREs and other membrane trafficking key component proteins is a prerequisite for the support of increasingly more complex body plans and life cycles (Fujimoto and Ueda 2012). Some of these SNARE proteins have been localized to post-Golgi compartments – particularly relevant for vacuolar sorting, SYP21/PEP12 and SYP22/VAM3 Qa-SNAREs have been found to localize at the late endosome (Sanderfoot 2007). Knock-out *syp22* mutants show pleiotropic phenotypes, such as semi-dwarfism, late flowering and an underdeveloped leaf vascular network (Ohtomo et al. 2005; Ueda et al. 2006; Shirakawa et al. 2009; Ebine et al. 2012). Although *syp21* lines fail to develop any observable phenotype, overexpression of SYP21 results in the homotypic fusion of PVC bodies and the partial secretion of vacuolar proteins (Foresti et al. 2006), perhaps by inhibiting anterograde trafficking at the PVC-vacuole route. Double mutants *syp21 syp22* are gametophytic lethal, but knocked-down SYP21 levels in a *syp22* background have been shown to result in impaired vacuolar transport of 12S globulin and 2S albumin, pointing to a possibly redundant function between the two SYP2 members in vacuolar transport of proteins (Shirakawa et al. 2010; Uemura et al. 2010).

The Qb-VTI11 SNARE is a SYP22 and SYP21 binding partner. This Qb-SNARE is essential for phosphatidylinositol 3-phosphate (PI3P)-mediated vacuolar biogenesis, and loss of its function in the *zig-1* mutant line results in defective gravitropic responses, zig-zag shaped inflorescences and altered vacuolar morphologies (Sanderfoot et al. 2001; Morita et al. 2002; Hashiguchi et al. 2010; Saito et al. 2011). SYP5 members are Qc-SNARES that interact with SYP22 and are localized to the tonoplast and TGN. Despite their degree of similarity, their function differs, in that SYP51 is required for GFP-chitinase transport, whereas SYP52 is involved in transporting Aleurain-GFP (De Benedictis et al. 2013). SYP51/52 has also been reported to act as interfering SNAREs (i-SNAREs) when overexpressed, accumulating at the tonoplast level (Uemura et al. 2004). i-SNAREs are a novel functional class of SNAREs, which are thought capable of inhibiting membrane fusion by direct competition with fusogenic SNARE pins, effectively forming nonfusogenic complexes (Di Sansebastiano 2013). Sorting processes would then be modulated by the different levels of these SNAREs – an example is that VSR proteins appear diverted to the plasma membrane in germinating pollen tubes (Wang et al. 2011),

where SYP5 members are found in particularly elevated levels (Lipka et al. 2007; De Benedictis et al. 2013).

Members of the VAMP71 family (AtVAMP711-AtVAMP714) have been associated with stomatal aperture control during drought stress (Leshem et al. 2010). This would implicate this protein family in membrane fusion events at the tonoplast or vesicle budding from the vacuole, processes responsible for the control of vacuolar size and tonoplast surface area. The R-SNARE AtVAMP727 has been found to dual-localize at the endosome/PVC and plasma membrane (PM) (Uemura et al. 2004; Ebine et al. 2008, 2011). VAMP713 has already been localized to the tonoplast (De Benedictis et al. 2013), and it is known that VAMP713 is also a member of the SYP22 complex (Ebine et al. 2011). The R-SNARE VAMP727 has also been shown to associate with the SYP22, VTI11 and SYP5 complex (Ebine et al. 2008). The *atsyp22*/*atvamp727* double mutant secretes storage vacuolar cargo such as 2S albumin, pointing towards an important role of these proteins in vacuolar trafficking. Vacuolar morphology was also severely affected in this mutant (Ebine et al. 2008). It is interesting to note that VAMP727 is present and well conserved in seed plants, but absent from the currently sequenced mosses, raising the hypothesis that vacuolar trafficking pathways might have diversified in the higher plants. Further studying this R-SNARE might prove valuable in understanding not only the point of evolutionary divergence of vacuolar sorting pathways, but also to better understand higher plants' vacuolar plasticity and trafficking routes. The results herein described have been summarized in Table 1, for easier reading.

5 Plant Vacuolar Sorting: RAB GTPases

RAB GTPases are the largest family of proteins in the Ras superfamily, and their main biological function is to serve as molecular switches, regulating the targeting and tethering of transport carriers to target membranes (Fig. 3). To do this, they cycle between a GTP-bound active state and a GDP-bound inactive state. Activation of RAB GTPases begins with membrane attachment, and the exchange of the bound GDP for a GTP molecule, effectively priming the RAB protein for action. This exchange is catalysed by guanine nucleotide exchange factors (GEFs). Once activated, GTP-bound RAB GTPases will interact with effector molecules, inducing downstream reactions such as tethering of transport carriers to target membranes (Grosshans et al. 2006). Similarly to the SNARE proteins, RAB GTPases are also incredibly diverse proteins, and are also thought to be the basis behind endomembrane system diversification events (Dacks and Fields 2007; Gurkan et al. 2007; Elias 2010). Some authors also believe that RAB GTPases, in tandem with certain SNARE proteins at specific subcellular localizations, may provide specificity to the membrane fusion events (Stenmark and Olkkonen 2001; Zerial and McBride 2001; Rehman et al. 2008), as GTP hydrolysis enables the SNARE complex syntaxin to bind the vesicles during docking. In this way, it would be feasible to assume that processes involving particular SNARE complexes

Table 1 Overview of the different SNARE proteins explored in this review

SNARE type	SNARE sub-type	Name	Subcellular localization	Described phenotypes (KO/OE/KD)	References
Q-SNARE	Qa-SNARE	SYP21/ PEP12	Late endosome	**SYP21 OE** Homotypic fusion of PVC bodies Partial secretion of vacuolar proteins **SYP21 KD/SYP22 KO** Impaired vacuolar transport of 12S globulin and 2S albumin **SYP21 KO/SYP22 KO** Gametophytic lethal	Foresti et al. (2006), Sanderfoot (2007), Shirakawa et al. (2010), and Uemura et al. (2010)
		SYP22/ VAM3	Late endosome	**SYP22 KO** Semi-dwarfism Late flowering Underdeveloped leaf vascular network **SYP21 KD/SYP22 KO** Impaired vacuolar transport of 12S globulin and 2S albumin **SYP21 KO/SYP22 KO** Gametophytic lethal **SYP22 KO/VAMP727 KO** Secreted storage vacuolar cargo Aberrant vacuolar morphology	Ebine et al. (2012), Ohtomo et al. (2005), Sanderfoot (2007), Shirakawa et al. (2009), and Ueda et al. (2006)
	Qb-SNARE	VTI11		**VTI11 KO (*zig-1* mutant)** Defective gravitropic responses Zig-zag shaped inflorescences Altered vacuolar morphologies	Hashiguchi et al. (2010), Morita et al. (2002), Saito et al. (2011), and Sanderfoot et al. (2001)
	Qc –SNARE/ i-SNARE	SYP51	Tonoplast/ TGN	–	De Benedictis et al. (2013) and Uemura et al. (2004)
		SYP52	Tonoplast/ TGN	–	De Benedictis et al. (2013) and Uemura et al. (2004)

R-SNARE	VAMP713	Tonoplast	–	De Benedictis et al. (2013)
	VAMP727	Endosome/ PVC Plasma membrane	**SYP22 KO/VAMP727 KO** Secreted storage vacuolar cargo Aberrant vacuolar morphology	Ebine et al. (2008), Ebine et al. (2011), and Uemura et al. (2004)

In the "Described Phenotypes" Section, KO stands for "Knock out", OE stands for "Overexpressed" and KD stands for "Knock down"

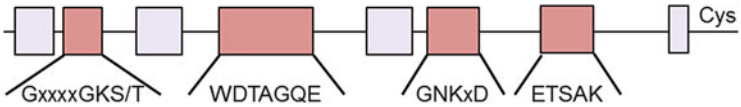

Fig. 3 Graphical representation of a typical RAB GTPase. *Brown boxes* represent the sequence motifs conserved in RAB GTPases. These motifs are indicated below the boxes, in the single-letter amino acid code, and are involved in nucleotide binding and hydrolysis. Some of these residues have been mutated in order to generate constitutively active (GTP-bound) or inactive (GDP-bound) mutant RAB GTPases, that have been employed for the functional study of these proteins. *Light violet boxes* represent the residues conserved in each functional subclass. They differ between subclasses and are used for the identification of all RAB GTPases into the different subgroups. At the carboxy-terminus, the cysteine (Cys) residues are indicated. These Cys residues are involved in membrane attachment of the RAB GTPases and are highly conserved

(comprising specific syntaxins), could be capable of coordinating specific RAB GTPases (Rehman et al. 2008).

The *Arabidopsis thaliana* genome contains 57 RAB GTPases, classified into eight different groups (RABA-RABH). These plant groups are highly similar to the animal RAB1, RAB2, RAB5-8, RAB11 and RAB18 groups (Rutherford and Moore 2002; Vernoud et al. 2003). When comparing land plants to other eukaryotic organisms, it becomes easy to realize that the RABA/RAB11 group is particularly enriched in genes, with 26 out of the 57 Arabidopsis genes, belonging to this group. This RABA group can be further sub-divided into 6 sub-groups: RABA1-RABA6 (Rutherford and Moore 2002). These RABA members have been shown to localize around the *trans* Golgi network (TGN) in the past (Ueda et al. 1996; de Graaf et al. 2005; Chow et al. 2008; Szumlanski and Nielsen 2009). The great diversity of RABA members in land plants might suggest this group to be responsible for plant-unique functions, such as cell-plate formation (Chow et al. 2008), or normal tip growth in pollen tubes and root hairs (Preuss et al. 2004; de Graaf et al. 2005; Szumlanski and Nielsen 2009).

In terms of endosomal and vacuolar trafficking, RAB5 and RAB7 are characterized as particularly relevant in yeast and animal cells, while also being present in plant systems. *Arabidopsis thaliana* possesses orthologous genes for all these RAB GTPases, plus a plant-specific RAB5 member – ARA6/RABF1 (Rutherford and Moore 2002). RABF1, RABF2a and RABF2b are all localized to distinct endosomal populations, despite being considerably overlapping, and they regulate separate trafficking pathways (Ueda et al. 2004; Haas et al. 2007; Ebine et al. 2011). Both RABF2a and RABF2b are involved in the vacuolar trafficking pathway, whereas RABF1 is responsible for mediating the flow of transport towards the plasma membrane (Kotzer et al. 2004; Viotti et al. 2010; Ebine et al. 2011). Despite its role in the trafficking of proteins towards the plasma membrane, RABF1 has also been implicated in the vacuolar sorting of soluble cargo and the recycling of vacuolar sorting receptor molecules from the late endosome to the TGN, implying multifunctional roles for this RAB in different plant species or tissues (Bottanelli et al. 2011, 2012). Some of the eight RAB7-related GTPases found in Arabidopsis (RABG1, RABG2 and RABG3a-f) have been detected at the tonoplast and the

multivesicular body (Rutherford and Moore 2002; Saito and Ueda 2009; Cui et al. 2014). These RABs have been implicated in plant responses against biotic and abiotic stress conditions. Overexpression of RABG3e results in increased vacuolar concentrations of sodium, conferring tolerance to salt and osmotic stress. This RAB GTPase has also been shown to become upregulated upon superoxide or salicylic acid treatment, or during infection (Mazel et al. 2004). Utilizing a dominant-negative mutant of RABG3f (RABG3f [T22N]), Cui et al. (2014) were successful in causing phenotypic alterations of the multivesicular body and vacuole – these organelles became enlarged and deformed, soluble vacuolar cargoes became missorted to the extracellular space, and storage proteins failed to be properly degraded during germination. These observations all point towards a critical role that RABG3 plays during the vacuolar transport of soluble cargo in Arabidopsis. At this point in time, an Arabidopsis sextuple *rabg3a,b,c,d,e,f* mutant has been generated and analysed – this mutant exhibits semi-dwarfism in the earliest developmental stages (Ebine et al. 2014). RABG activity is therefore important for the proper development of the Arabidopsis model.

The similarity of phenotypes obtained upon impairment of RABF or RABG proteins suggests they might both be active in the same trafficking pathway. However, vacuolar morphology appears more sensitive to mutations in RABG-related molecular components, whereas the RABF-related machineries appear to affect particularly the transport of soluble cargo to the protein storage vacuole (Ebine et al. 2014). It is also relevant to note that *rabf* and *rabg* mutations result in antagonistic effects in a *vti1/zig-1* mutant background. Overexpression of dominant-negative mutants of either RABF or RABG proteins also results in differential transport of membrane proteins in tobacco (Bottanelli et al. 2011). All these observations seem to point towards a differential function for these two GTPases in the plant vacuolar pathways, against what has been previously observed in non-plant systems. An overview of all the described RAB GTPases, their subcellular localizations, as well as their putative physiological functions in plant cells has been compiled in Table 2.

6 Chemical Genomics as a Novel Tool for the Study of the Plant Endomembrane System

Historically, drug discovery has been a very long and hard process, with most of the lead compounds being discovered by methodical biochemical and molecular biology analysis – as most biological targets were not known, luck played a huge role in the discovery of a chemical structure capable of producing a positive phenotypic effect. One such example is that of aspirin, which was employed by the medical community for its biological effects, long before the scientific community could understand its molecular mechanism (Robert et al. 2009). Nowadays, the strategy has been vastly improved, and the drug discovery process is no longer such a hit-

Table 2 An overview of the different RAB GTPases explored in this review, as well as their subcellular localizations and putative physiological functions

RAB group	Mammalian homologues	RAB sub-group	Subcellular localization	Putative physiological functions	References
RABA	–	RABA1	TGN	Cell-plate formation Tip growth (pollen tubes and root hairs)	Chow et al. (2008), de Graaf et al. (2005), Preuss et al. (2004), Szumlanski and Nielsen (2009), Ueda et al. (1996), and Vernoud et al. (2003)
	RAB11	RABA2			
	–	RABA3			
	–	RABA4			
	–	RABA5			
	–	RABA6			
RABF	–	RABF1/ ARA6	Endosomes	Sorting of proteins towards the plasma membrane Vacuolar sorting of soluble cargo Recycling of vacuolar sorting receptors	Bottanelli et al. (2011), Bottanelli et al. (2012), Ebine et al. (2011), Haas et al. (2007), Kotzer et al. (2004), Ueda et al. (2004), Vernoud et al. (2003), and Viotti et al. (2010)
	RAB5	RABF2a-b	Endosomes	Vacuolar sorting	
RABG	–	RABG1	Tonoplast and MVB	–	Bottanelli et al. (2011), Cui et al. (2014), Ebine et al. (2014), Mazel et al. (2004), Rutherford and Moore (2002), Saito and Ueda (2009), and Vernoud et al. (2003)
	–	RABG2		–	
	RAB7	RABG3a-f		Vacuolar transport of soluble cargo	

and-miss strategy. Chemists will rely on general rules, such as the Lipinski rule of five (Lipinski et al. 1997) when designing lead compounds. These leads will then be structurally altered in the slightest ways, creating a vast library of chemical compounds based upon the same lead compound. These libraries can then be employed for high-throughput screens, allowing the researcher to choose the structural scaffold with the highest biological activity, the most precise point of action, the lowest toxicity or any other desirable characteristic (Robert et al. 2009). Chemical genomics is based on different principles, when compared with drug design. For chemical genomics studies, researchers are no longer interested in the screening of drugs, but rather they search for compounds capable of eliciting an observable biological effect that may be used for understanding that organism's basic physiology. An obvious difference is that the chemical libraries are not collections of similar compounds, but rather a collection of widely different chemical structures and lead scaffolds. Furthermore, plant biologists are less interested in

certain chemical characteristics, such as high potency or metabolic turnover. Reversibility of effects is desirable, but not mandatory for all applications (Robert et al. 2009).

The most widely employed probe reagents are small organic molecules, and despite its insipience, plant chemical genomic screen projects have been returning some very interesting molecules for basic physiological research. Some of these molecules affect cell wall biogenesis, such as the herbicides dichlorobenzonitrile (DCB), isoxaben (*N*-[3(1-ethyl-1-methylpropyl)-5-isoxazolyl]) and CGA325_615 (Peng et al. 2001).

Classical genetic approaches have proven limited for the study of the plant endomembrane system, particularly the study of vesicular trafficking. The *vacuoless 1* (*vcl1*) Arabidopsis mutant is one of the rare cases of limited success, leading to defective vacuole biogenesis, but also embryo lethality, indicating the importance of this organelle for plant organisms (something not observed in yeast, for example). Gene-redundancy is also a hurdle that usually results in no observable phenotype for many T-DNA insertion lines. Chemical genomics can, therefore, be a powerful tool for overcoming these common limitations, and some work has been done in this area already (Robert et al. 2009). In 2004 Zouhar et al. screened a library of 4800 compounds in yeast. Of these compounds, 14 resulted in the aberrant secretion of the commonly vacuolar carboxypeptidase Y (CPY) enzyme, effectively mimicking the *vps* phenotype (Bowers and Stevens 2005). Of these 14 compounds, 2 (Sortin1 and Sortin2) were shown to have an effect in Arabidopsis – reversible effects were detected on vacuole biogenesis, root development and CPY secretion, with no detectable phenotype in any other organelles (Zouhar et al. 2004). More recently, Rosado et al. (2011) have further expanded these results, coming to the understanding that sortin1-hypersensitive Arabidopsis mutants exhibited not only drastic vacuolar morphological alterations, but also defects in flavonoid accumulation, thus linking both vacuolar-trafficking defects and flavonoid metabolism, all the while proving the usefulness of chemical compounds in the elucidation of plant biological responses that have been difficult to dissect by conventional genetics (Rosado et al. 2011). Other examples of already characterized compounds, well accepted by the community as useful chemical tools include wortmannin, a phosphoinositide 3-kinase inhibitor, brefeldin A (BFA), an inhibitor of vesicular transport, coronatine, a bacterial phytotoxin and other variations of the E-64 protease inhibitor (Murphy et al. 2005; Samaj et al. 2006; Kolodziejek and van der Hoorn 2010; Wasternack and Kombrink 2010). Despite the enormous potential of the chemical biology methods for screening basic plant physiology, some processes remain unprobed by these methodologies – current strategies employ either single-celled (pollen or suspension cultures) or seedlings grown in microplates, making the study of end-of-cycle events, such as flowering and seed formation hard, if not impossible, to investigate. Alternative model organisms such as the duckweeds Lemneae and Wolffieae sp. have been suggested, as they include some of the smallest flowering plants and can be easily cultivated in liquid cultures in microplates, and might be the key for the chemical biological study of subjects as diverse as endomembrane trafficking or flowering control (Serrano et al. 2015).

7 Alternative Sorting Routes

Recent advances in the study of plant autophagy have led to the discovery of alternative pathways for proteins to reach the vacuole. Macroautophagy is a process generally associated with stress and starvation-induced bulk degradation of cellular components, though it may also be a highly selective process, capable of targeting malfunctioning organelles such as mitochondria, peroxisomes, protein aggregates, pathogens or even specific proteins (Floyd et al. 2012; Li and Vierstra 2012). Selective autophagy has thus been identified as the major mechanism behind the delivery of several resident vacuolar proteins (Michaeli et al. 2014). Of these processes, the cytosol-to-vacuole targeting pathway (Cvt) of yeast is the most well studied and better understood (Scott et al. 1996), being responsible for the transport of at least two hydrolases (aminopeptidase 1 and α-mannosidase) from the cytosol to the vacuole. The selectivity of this mechanism is determined by the core autophagy machinery, namely Atg19, which bridges between both hydrolases, and the Atg11 and Atg8 autophagic proteins mediating the formation of the Cvt vesicle (Lynch-Day and Klionsky 2010).

Another alternative vacuolar sorting route is present mainly during seed development, during the biosynthesis of storage proteins. These storage proteins may be transported directly from the endoplasmic reticulum (ER) to the vacuole (ERvt route), bypassing the Golgi Complex entirely, an hypothesis which was formulated due to the careful study of these storage proteins through electron microscopy observations of wheat seeds (Levanony et al. 1992; Galili et al. 1993). This sorting pathway begins with the aggregation of the proteins inside the ER, budding and formation of ER-derived protein bodies (PBs) and their subsequent internalization into the storage vacuoles by an autophagy-like mechanism (Hara-Nishimura et al. 1998; Robinson et al. 2005; Herman 2008; Ibl and Stoger 2011; Wang et al. 2011), although it is still currently unknown, whether this process utilizes the same molecular machinery.

8 Plant Aspartic Proteinases: Cardosins as a Study Case

Cardosins are aspartic proteinases (APs) belonging to the A1 family of APs, that have been identified in *Cynara cardunculus* L. The two main isoforms of these enzymes – cardosins A and B are found in the floral tissues of this plant, and despite their high sequence similarity, cardosin A is found to accumulate in the protein storage vacuoles of the stigmatic papillae, whereas cardosin B is detected in the extracellular matrix of the stigma and style transmitting tissue (Ramalho-Santos et al. 1997; Vieira et al. 2001; Duarte et al. 2008). Curiously enough, when expressed in heterologous systems, cardosin A accumulated in different vacuole types, in a tissue-dependent manner – lytic vacuoles in vegetative tissues, or protein storage vacuoles in *Arabidopsis thaliana* seedlings. Alternatively, cardosin B is no

longer secreted in tobacco leaf epidermal cells, accumulating in the large central vacuole instead (da Costa et al. 2010). These enzymes are synthesized as zymogens (preprocardosins) – inactive precursors that suffer a series of proteolytic cleavages during their vacuolar sorting, resulting in their timely activation when reaching the vacuolar lumen (Duarte et al. 2008). In cardoon seeds however, preprocardosin A can be detected in protein bodies (PBs), its mature form appearing at a later time, in the central lytic vacuole, as it engulfs the remaining PBs. Cardosin A tissue-specific dual localization could be explained by the presence of two distinct vacuolar sorting determinants in the enzyme's primary structure – a typical ctVSD, and an internal signal, the PSI. Cardosin A's PSI domain is capable of the COPII independent vacuolar sorting of a fluorescent mCherry reporter in *Nicotiana tabacum* leaf epidermal cells, as demonstrated in 2013 by Pereira and collaborators (Pereira et al. 2013). The authors postulate that a PSI-mediated COPII independent vacuolar sorting mechanism could be relevant in metabolically active organs, such as floral and seed tissues, where protein storage vacuoles are the predominant vacuole type. These direct ER-vacuole routes have been postulated to be important for the plants' rapid adaptation to the ever-changing environment around them (Xiang et al. 2013). Adding to the complexity of the cardosin model, Duarte and collaborators observed the presence of endoglycosidase-H-sensitive intermediate forms of cardosin A, and an inability for dominant-inhibitory RAB GTPases to completely inhibit this enzyme's processing events. Furthermore, secretion assays in tobacco protoplasts revealed the secretion of an intermediate form of cardosin A, raising the hypothesis that this enzyme might be transiently secreted and quickly re-internalized, prior to accumulation in the vacuolar lumen (Duarte et al. 2008). By analysing all the results obtained so far, we have developed a model of cardosin trafficking, which might follow different routes in a tissue-specific manner (Fig. 4) (Reviewed in Pereira et al. 2014).

9 Bringing Vacuoles to the Marketplace: Biotechnological Implications

Plants are the basis for the production of many different economically and socially relevant products, from food and feed, to bioactive secondary metabolites with medicinal applications. These organisms have been classified as ideal bioreactors for the production of relevant macromolecules, but the main efforts have been directed at dissecting enzymatic machineries and metabolic pathways – the transport mechanisms are generally overlooked and might be responsible for significant losses in productivity. In this regard, Di Sansebastiano et al. (2014) have been working on the classical problem of artemisinin production. Artemisinin is a sesquiterpene lactone endoperoxide with anti-malarial properties that occurs in the plant *Artemisia annua*. A wide array of strategies have been employed to increase the plants' production of this compound, such as overexpression of

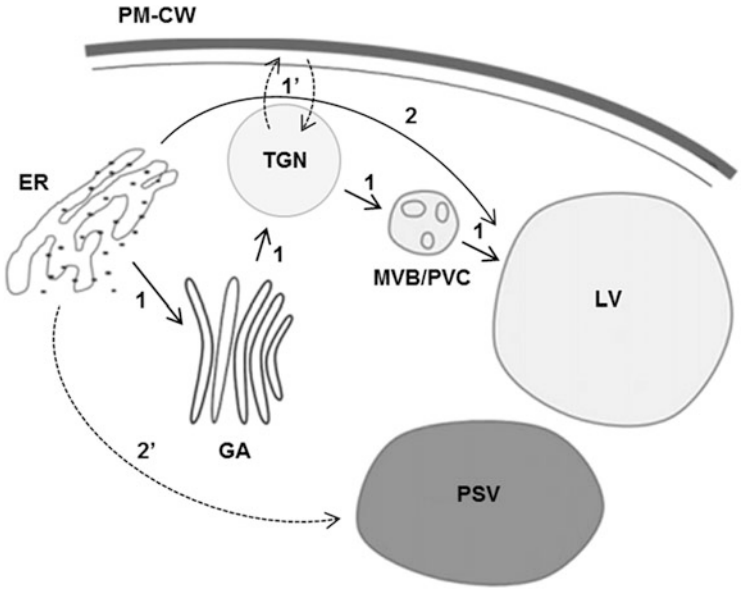

Fig. 4 A putative model of cardosin trafficking. (*1*) COPII-dependent sorting between the ER and Golgi has been demonstrated for both cardosins A and B in *Nicotiana tabacum* leaf epidermis, mediated by the enzymes' C-termini VSDs. RABF2b [S24N] blockage of post-Golgi trafficking results in secretion of both cardosins and their C-terminal peptides, suggesting these proteins must pass through the PVC, prior to vacuolar accumulation. Secretion assays with tobacco protoplasts have shown an intermediate, not fully matured form of cardosin A becoming secreted, raising the hypothesis that these enzymes might be transiently secreted during their transit to the vacuole (*1′*). This hypothesis remains insufficiently substantiated at this time, and further work will have to be done in order to confirm or disprove this idea. (*2*) Cardosin A (but not cardosin B) PSI-mediated sorting appears both COPII and RABF2b-independent, hinting at the possibility that this VSD shuttles proteins directly from the ER towards the lytic vacuole. (*2′*) It is hypothesized that this PSI-mediated GA-bypass is tissue specific and might be prevalent in seeds, as unprocessed cardosin A has been detected at the protein storage vacuole in dormant seeds, becoming fully maturated during germination upon vacuolar lumen acidification due to LV-PSV fusion events (adapted from Pereira et al. 2013)

enzymes (Nafis et al. 2011; Tang et al. 2014), inhibition of competitive biosynthetic pathways (Chen et al. 2011), transcription factor regulated modulation (Lu et al. 2013; Tang et al. 2014) and biomass (Banyai et al. 2011), or specialized cell density increments (Maes and Goossens 2010). Success has been limited, in part because the accumulated artemisinin is unstable and quickly degraded. Knowing that plant cells will sometimes generate MVB-derived small membranous compartments for the accumulation and delivery of phenylpropanoids to the cell periphery, Di Sansebastiano employed a truncated AtSYP51, whose N-terminal portion had been removed, to artificially induce the formation of a stable compartment generated by ER- and endocytosis-derived membranes, where the artemisinin metabolite became stabilized and accumulated for longer periods (Di Sansebastiano et al. 2014). By applying what is now known about vacuolar sorting and biogenesis

mechanisms, such an engineering approach might allow for plant organisms to become even more versatile biofactories, especially when complemented with the more classical approaches of gene overexpression or silencing. The same approach might also be applicable to the production of recombinant protein products, overcoming the problems associated with traditional strategies that rely on ER accumulation of recombinant protein, usually inducing ER stress due to protein overload, or the accumulation in less stable environments, such as the protease-enriched extracellular space or vacuoles.

Another important area of plant biotechnology is the development of improved crops and plant species, better capable of resisting environmental stresses. In fact, a study with Arabidopsis expressing a RABF1/ARA6 [Q93L] GTP-bound mutant transgene, found these plants to be resistant to salt stress, as tested by supplementing their growth media with NaCl. In fact, these plants showed no phenotype even when in the presence of 100 mM NaCl (Ebine et al. 2011), pointing towards an important function of these proteins in the plant stress response. This process is not limited to Arabidopsis, as RABF1 induction has also been demonstrated during the salt stress response of the halophyte *Mesembryanthemum crystallinum* plant (Bolte et al. 2000). Also in rice (*Oryza sativa*) and *Pennisetum glaucum,* RAB7 accumulated upon cold, salt and dehydration stresses, and also upon ABA treatment, hinting at important functions for this GTPase in a variety of stress responses (Nahm et al. 2003; Agarwal et al. 2008). Overexpression of Arabidopsis RAB7 also resulted in increased sodium content in the shoots, with Na^+ ions becoming trapped in the vacuole as a means of reducing the associated toxicity effects (Mazel et al. 2004). These reports are representative of how, by better understanding the molecular mechanisms behind vacuolar sorting, we may end up capable of manipulating cellular responses, in order to better engineer different plant species optimized for different ends.

10 Conclusions and Future Perspectives

In 2013 the Nobel Prize for Medicine was awarded to James E. Rothman, the researcher responsible for understanding the way SNARE proteins interact and form complexes. This prize was shared with two other scientists – Randy W. Scheckman and Thomas C. Südhof, for their contributions to the body of knowledge in vesicle trafficking. This prize is indicative of the relevance behind this area of study. In plant models, these mechanisms are behind a huge variety of different responses – from vacuolar biogenesis and protein accumulation, to plasticity towards different environmental stressors. In this regard, a better understanding of the plant endomembrane system and of their vacuolar systems in particular will undoubtedly result in novel ways to design better, more productive and hardier plants better suited for feeding an ever increasing population in a world undergoing climate change. But this knowledge will unlock even more possibilities, as plants are the providers of not only food, but also more valuable compounds, with

industrial and medicinal applications. Subcellular engineering is an exciting new possibility for increasing yields of both recombinant protein and secondary metabolite production in plants bred for the sole purpose of being used as bioreactors. In order for these exciting possibilities to be achieved, more will have to be known at the most fundamental levels – the high-throughput discovery of chemical compounds through chemical genomics projects will no doubt prove invaluable in accelerating further discoveries in this field, particularly by better defining targets worth studying in greater depth, or by allowing the transient exploration of otherwise lethal phenotypes. Better understanding the cross-roads where both RAB GTPases and SNAREs intersect in terms of stress sensing and response, as well as how they interact with each other for generating the subcellular identity of different compartments is sure to be important for our understanding of plant cell physiology and development, as well as to open up the door for novel applications in the realm of subcellular engineering. This is definitely an exciting time to be working in this subject, as the near future is sure to show us.

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