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ORIGINAL PAPER

A fluorescent reporter protein containing AtRMR1 domains is targeted to the storage and central vacuoles in *Arabidopsis thaliana* and tobacco leaf cells

Camila María Scabone · Lorenzo Frigerio · Silvana Petruccelli

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Abstract To develop a new strategy to target recombinant proteins to the vacuolar storage system in transgenic plants, the ability of the transmembrane and cytosolic domains of Arabidopsis receptor homology-transmembrane-RING H2-1 (AtRMR1) was evaluated. A secreted version of RFP (secRFP) and a fusion of it to the transmembrane and cytosolic domains of AtRMR1 (RFP-TMCT) were produced and studied both in transient and stable expression assays. Transient expression in leaves of *Nicotiana tabacum* showed that secRFP is secreted to the apoplast while its fusion to TMCT of AtRMR1 is sufficient to prevent secretion of the reporter. In tobacco leaves, RFP-TMCT reporter showed an endoplasmic reticulum pattern in early expression stages while in late expression stages, it was found in the vacuolar lumen. For the first time, the role of TM and CT domains of AtRMR1 in stable expression in *Arabidopsis thaliana* is presented; the fusion of TMCT to secRFP is sufficient to sort RFP to the lumen of the central vacuoles in leaves and roots and to the lumen of PSV in cotyledons of mature embryos. In addition, biochemical studies performed in extract from transgenic plants showed that RFP-TMCT is an integral membrane protein. Full-length RFP-TMCT was also found in the vacuolar lumen, suggesting internalization into destination vacuole. Not colocalization of RFP-TMCT with tonoplast and plasma membrane markers were observed. This membrane vacuolar determinant sorting signal could be used for future application in molecular pharming as an alternative means to sort proteins of interest to vacuoles.

Keywords Membrane proteins · RMR · Vacuolar sorting signal · PSV · Plant expression · Seed expression

Abbreviations

VSS	Vacuolar sorting signals
ctVSS	C-terminal VSS
VSR	Vacuolar sorting receptor
RFP	Monomeric red fluorescent protein
PSV	Protein storage vacuole
RMR	Receptor homology-transmembrane-RING H2
TM	Transmembrane
CT	Cytosolic tail
TIP	Tonoplast intrinsic protein
CLSM	Confocal laser scanning microscopy

Introduction

Numerous efforts have been made over the last decades to develop and optimize plant expression platforms for the large-scale production of both industrial and clinically useful proteins (Fischer et al. 2004; Ma et al. 2005; Stoger et al. 2005), with a few successful examples (Faye and

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C. M. Scabone · S. Petruccelli (✉)
Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), CCT-La Plata CONICET, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de la Plata, CC553, 1900 La Plata, Argentina
e-mail: silvana@biol.unlp.edu.ar

L. Frigerio
School of Life Sciences, University of Warwick, Coventry CV4 7AL, UK

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Gomord 2010). Recombinant protein degradation in plant expression systems is one of the main factors that causes low final yield and heterogeneity of the resulting protein product, therefore limiting efficacy of plant platforms. Several strategies have been proposed to avoid proteolytic processing, and a promising option is the accumulation of the recombinant proteins in storage vacuoles, which constitute a naturally safe environment (Benchabane et al. 2008; Petruccelli et al. 2006). Vacuolar sorting is, however, a very complex process since different types of vacuolar sorting pathways have been reported in diverse plant species and tissues (Herman and Larkins 1999; Vitale and Hinz 2005; Zouhar and Rojo 2009). In addition, the molecular mechanisms involved in protein recognition and sorting are not fully understood (Hinz et al. 2007; Jolliffe et al. 2005).

Transport of soluble proteins to vacuoles is a saturable process (Frigerio et al. 1998) that depends on the presence of vacuolar sorting signals (VSS) in their structure (Denecke et al. 1990; Vitale and Raikhel 1999). Three different types of VSS have been described: located at the N- or C-terminus, or within internal sequences of the vacuolar proteins. The sequence-specific VSS (ssVSS) NPIXL/NPIR motifs usually reside within propeptides located at N-terminus (but also can work if present in internal or C-terminus positions) and an aliphatic Ile or Leu residue is crucial for sorting (Matsuoka and Nakamura 1999). In contrast, C-terminal VSS (ctVSS) do not have similarity in amino acid composition and length, but are generally rich in hydrophobic residues and must be accessible at the C-terminus (Matsuoka and Neuhaus 1999; Vitale and Hinz 2005). Internal sorting signals are less well defined and it has been suggested that they could act as physical determinants triggering aggregation, which would then result in sorting (Holkeri and Vitale 2001; Maruyama et al. 2006; Neuhaus and Rogers 1998; Petruccelli et al. 2007).

Two families of vacuolar sorting receptors have been shown to interact with VSS: VSR/BP-80/AtELP (80 kDa membrane protein first found in clathrin-coated vesicles of developing pea seeds and then cloned in Arabidopsis and named AtELP for *Arabidopsis thaliana* epidermal growth factor-like protein) (Ahmed et al. 1997; Kirsch et al. 1994; Paris et al. 1997, 2002) and RMR (receptor-transmembrane sequence-RING-H2) (Jiang et al. 2000). The role of these putative receptors in sorting cargo proteins to vacuoles is still the subject of current research (Hinz et al. 2007; Craddock et al. 2008; Zouhar et al. 2010; Wang et al. 2011). VSR are characterized for having a signal peptide, a luminal domain (LU) that recognizes the VSS, a transmembrane domain (TM) and a cytosolic tail (CT) which is involved in the regulation of receptor trafficking (DaSilva et al. 2006; Saint-Jean et al. 2010). AtRMRs are composed of an N-terminal LU region consisting of a single protease associated (PA) domain (but lacking EGF-repeats present in VSRs), a TM domain predicted to be a type I integral membrane protein, and a C-terminal CT region that has a RING-H2 domain and a serine-rich region (Jiang et al. 2000). Seven AtVSRs and six AtRMRs protein genes have been described in *Arabidopsis thaliana* (Shimada et al. 2003; Park et al. 2005). VSR and RMR proteins recognized different targets, e.g. aleurain is recognized by AtVSR, but not by AtRMR, and ctVSS of barley lectin, bean phaseolin and tobacco chitinase are recognized by AtRMR but not by AtVSR (Park et al. 2005, 2007). The lack of a conserved sequence in ctVSS of different vacuolar proteins suggests that the interaction between the VSS and the LU domain of RMR could be a low-affinity one. In this work, we studied a direct fusion of the cargo protein to non-luminal domains of AtRMR.

In relationship with the sorting information of AtRMR, Park et al. (2005) demonstrated that full-length AtRMR1 protein mainly localized in DIP (dark induced tonoplastic intrinsic protein) organelle, but a minor portion also localized to the Golgi complex in transient expression *A. thaliana* leaf protoplasts. The DIP-positive organelle, according to Jiang et al. (2000) is the pre-vacuolar compartment (PVC) for the protein storage vacuole (PSV); and described AtRMR1 as a cargo receptor for protein trafficking to the protein storage vacuole. In addition, AtRMRs proteins were localized as components of the membrane-containing crystalloid in tobacco and tomato seed PSV (Jiang et al. 2000) and seed PSV of *A. thaliana* (Hinz et al. 2007). The AtRMR1 gene is expressed in most *A. thaliana* tissues regardless of the developing stage of the plant (Park et al. 2005).

In transient expression experiments performed in *A. thaliana* leaf protoplasts, to dissect functionally of the different domains of AtRMRs, it was shown that the TM and CT expressed without its LU domain, gave a punctate staining pattern that overlapped with Golgi marker ST-GFP suggesting that AtRMR1 without LU domain mainly localized at the Golgi complex (Park et al. 2005). In contrast, a reporter protein that contains proaleurain fused to the TM and CT from AtRMR2 traffic via Golgi apparatus to PSV-like compartment in transient expression of tobacco suspensions culture cells (Jiang et al. 2000). Unlike VSRs, the cytosolic tail of RMR protein is internalized into central vacuoles of tobacco suspension culture protoplast as part of the sorting process (Park et al. 2007; Wang et al. 2011).

In this study, we localized a fluorescent reporter fused to TM and CT from AtRMR1 protein using transgenic *A. thaliana* plants. A complete localization pattern in leaves, roots and seeds of transgenic Arabidopsis is presented. The development of a fluorescent reporter consisting of the

monomeric red fluorescent protein, with ability to fluoresce at the low pH of vacuoles facilitates live cell imaging studies. With this reporter, the sorting process of this membrane bound fluorescent protein was studied by colocalization studies with markers of the secretory pathway and also by biochemical assays. Finally, we investigated the hypothesis that TM and CT domains are a sufficient signal to deliver proteins to storage vacuoles in transgenic tissues for future application in molecular pharming.

Materials and methods

Recombinant DNA and generation of transgenic plants

A schematic representation of the constructs used in this study is shown in Fig. 1. All primer sequences are shown in Supplementary Table S1. To generate secRFP and RFP-TMCT, the monomeric RFP coding region was amplified by PCR with DNA polymerase (Invitrogen Argentina, SA, Buenos Aires, Argentina) from pcDNA3.1-mRFP (Snapp et al. 2006), using primers 1 and 2, that were designed to fuse RFP in frame with the murine heavy chain immunoglobulin secretory signal peptide (sp) in the pRTL202 (Carrington et al. 1991) by *Kpn*I/*Xba*I ligation to yield secRFP. This was used as a template for subsequent constructs. The secRFP and TMCT were spliced together by fusion PCR. The coding sequence TMCT from pUni51-AtRMR1 (Arabidopsis stock) was amplified using primers 4 and 6 and secRFP with the primers 3 and 5, overlapping PCR was used to produce RFP-TMCT. In addition, secRFP control was amplified using primers 3 and 2. All PCR products were cloned into binary vector by Gateway (Invitrogen Argentina, SA, Buenos Aires, Argentina) recombination system with entry vector pENTR D-Topo and destination vector pGWB2 (Nakagawa et al. 2007). All the constructs were introduced into strain GV3101 of *Agrobacterium tumefaciens*. *Arabidopsis thaliana* col-0 plants were then transformed using the floral dip method (Clough and Bent 1998).

Agrobacterium-mediated transient protein expression

Leaf abaxial epidermal cells of *Nicotiana tabacum* cv. SR1 were transformed using the Agrobacterium infiltration technique (Batoko et al. 2000; Sparkes et al. 2006). In all cases, for expression of each construct alone or in combination, the final level of inoculum was adjusted to $DO_{600} = 0.1$ and observed 2–3 days after agroinfiltration by CLSM. Post-infiltration, the light-incubated plants were maintained at 20°C with 16 h light and 8 h dark. The constructs used in this study were as follows: ST-YFP (Brandizzi et al. 2002), spGFP-HDEL (Boevink et al. 1996), γ-TIP-YFP (Hunter et al. 2007), Lti6b-eGFP (Cutler et al. 2000).

Confocal analysis and image processing

About 30 seeds from at least 4 independent transgenic lines per construct were germinated onto agar plates containing half-strength Murashige and Skoog (MS) Basal Medium (Sigma-Aldrich) and grown at 22°C, in a 16:8 light:dark regime. 8-day roots and 15-day leaves were excised, mounted on to 0.5× liquid MS medium and immediately

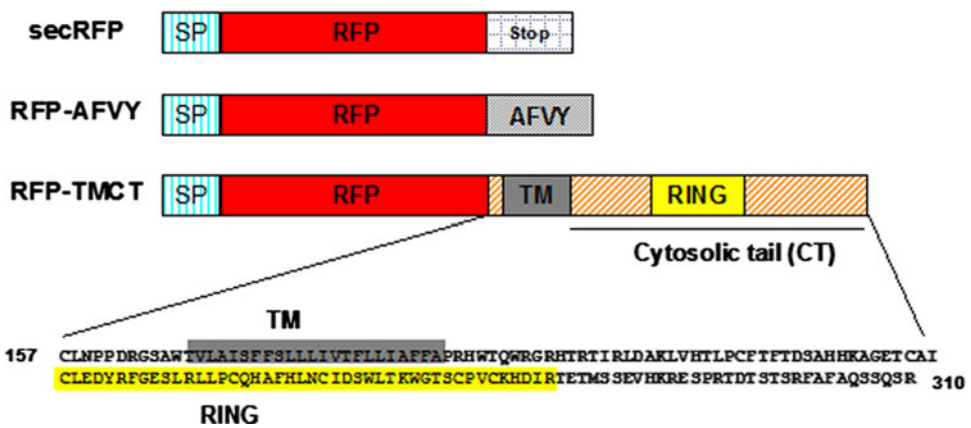

Fig. 1 Schematic representation of the constructs analyzed. *secRFP* secretory RFP; *RFP-AFVY* RFP fused to the ctVSS of phaseolin storage protein, *RFP-TMCT* RFP fused to the transmembrane (TM) and cytosolic tail (CT) of AtRMR1. The polypeptide sequence of TMCT fused to RFP is shown (amino acid residues 157–310 of AtRMR1, AAF32325). The TM was predicted by the server http://www.cbs.dtu.dk/services/TMHMM (residues 168–190 of full-length AtRMR1); RING: RING-finger Domain was predicted with Prosite (residues 232–274 of full-length AtRMR1) *SP* signal peptide, *RFP* monomeric red fluorescent protein

observed with a Leica TSC SP5 confocal laser scanning microscope, using a 63× (NA 1.4) oil immersion objective. RFP was excited at 561 nm and detected in the 570–630 nm range. YFP was excited at 514 nm and detected in the 525–559 nm range. GFP was excited at 488 nm and detected in the 496–532 nm range. Simultaneous detection of RFP and YFP or GFP was performed by combining the settings indicated above in the sequential scanning facility of the microscope, as instructed by the manufacturer.

Total cell protein extracts, microsomes and vacuoles isolation from Arabidopsis transgenic leaves and Western blot analysis

Arabidopsis transgenic leaves expressing RFP-TMCT, RFP-AFVY and *wild type* as control were homogenized using a cold mortar and pestle in Suc buffer (100 mM Tris [tris(hydroxymethyl)aminomethane]–HCl, pH 7.5, 10 mM KCl, 1 mM EDTA, and 12% [*w*/*w*] Suc). The homogenate was centrifuged for 5 min at 1,000*g* to remove debris and intact cells. The supernatant was loaded on top of a 17% (*w*/*w*) Suc pad and centrifuged for 30 min at 150,000*g* in an SW41 Ti rotor (Beckman Instruments, Fullerton, CA) 4°C. The supernatant, the soluble fraction, containing cytosolic and vacuolar proteins was removed and the microsomal pellet was resuspended in Suc buffer, in Suc buffer containing 1 M NaCl, or in 0.1 M Na_2CO_3. After 30 min of incubation on ice, the microsome suspensions were reloaded on top of a 17% (*w*/*w*) Suc pad and centrifuged for 30 min at 150,000*g*. Supernatants and pellets were homogenated in 100 mM Tris–Cl, 100 mM NaCl, 1 mM EDTA, and 1% (*v*/*v*) Triton X-100, pH 7.5, supplemented with protease inhibitor cocktail (Complete, Boehringer Mannheim). Samples were denatured with Laemmli buffer, loaded on to reducing SDS-PAGE (CBS System) and transferred to a polyvinylidene difluoride (PVDF) membrane (Polyscreen®, Perkin–Elmer, Boston, MA, USA). Blot was first incubated with rabbit serum specific to RFP (1:2000 dilution, Rabbit Polyclonal IgG, ABR) and then with peroxidase conjugate secondary rabbit immunoglobulin G specific antibody (1:2000 dilution, Sigma). Immunodetection was performed using the enhanced chemiluminescence (ECL) system (Amersham) following the manufacturers protocol.

Transgenic Arabidopsis plants expressing RFP-TMCT were grown on soil and rosette leaves were digested using cell wall-digesting enzymes, and intact protoplasts were isolated according to the method of Wu et al. (2009) and vacuoles were isolation from protoplasts according to Robert et al. (2007). The vacuolar fraction quality has been examined using immunoblot analysis indicating that the fraction was not contaminated.

Results

RFP-TMCT reporter labels the lumen of central vacuoles in transient expression in *N. tabacum* leaves

To test if the sequence encoding the TM and CT domains of AtRMR1 is sufficient to direct a reporter protein to vacuoles, a fusion of this sequence to mRFP (monomeric red fluorescent protein) was constructed. The reporter mRFP was chosen because of its ability to fluoresce at the low pH of vacuoles (Shaner et al. 2005; Samalova et al. 2006). To guarantee translocation into the lumen of the endoplasmic reticulum, a sequence encoding an immunoglobulin heavy chain signal peptide (sp) was fused to the N-terminus of RFP (RFP-TMCT, Fig. 1). An control consisting of a secretory version of RFP (secRFP, Fig. 1) composed by a signal peptide and RFP alone was also generated. The constitutive enhancer cauliflower mosaic virus 35S (CaMV 35S) promoter was used in all cases.

Transient expression of these constructs by agroinfiltration (Sparkes et al. 2006) was performed in tobacco (*Nicotiana tabacum*) leaf epidermal cells (Fig. 2). Leaves were observed by confocal laser scanning microscopy (CLSM). Fluorescence in the apoplast was observed for secRFP (Fig. 2b) indicating secretion and confirming that

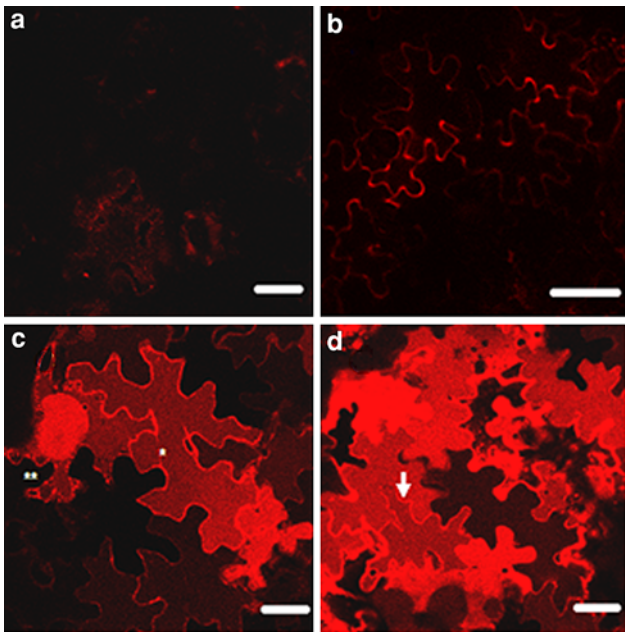

Fig. 2 RFP-TMCT reporter labels the vacuolar lumen in leaf epidermal cells of *Nicotiana tabacum.* **a** Negative control: agroinfiltration medium alone. **b** secRFP reporter showed an apoplastic pattern. **c** RFP-AFVY reporter highlights the vacuolar lumen and cortical ER (**), and transvacuolar strands (*). **d** RFP-TMCT reporter showed a vacuolar lumen and cortical ER pattern (*arrow*). *Scale bars* 50 μm

the heterologous signal peptide is working properly. As a positive control, the vacuolar reporter RFP-AFVY that contains a ctVSS (Frigerio et al. 1998) was used (RFP-AFVY, Fig. 1). RFP-AFVY was found mainly in the lumen of the central vacuole (Fig. 2c) as described previously (Hunter et al. 2007). In addition, RFP-AFVY was also detectable in the cortical ER, especially in high-expressing cells (Fig. 2c, double asterisks). This result evidences the traffic of RFP-AFVY through the endomembrane system to reach the final destination in the vacuole. The expression of RFP-TMCT reporter gave rise to strong labeling of the lumen of the central vacuole (Fig. 2d), and not tonoplast label, as expected from the presence of the TM domain. In some high-expressing cells, the cell periphery was also highlighted, possibly reflecting the cortical ER (Fig. 2d, arrow; see further below).

TM and CT of AtRMR1 are able to deliver RFP in the lumen of central vacuoles in leaves, roots and embryos of *Arabidopsis thaliana* transgenic plants

The expression patterns of RFP-tagged constructs were evaluated in *Arabidopsis thaliana* transgenic plants by CLSM. To this end, leaves from 15-day-old seedlings expressing individual RFP-tagged reporters were analyzed (Fig. 3). secRFP was found in the apoplast (Fig. 3a, b). RFP-AFVY and RFP-TMCT were exclusively localized in the lumen of the large, central vegetative vacuoles (Fig. 3c–e). At a higher magnification, both RFP-AFVY and RFP-TMCT were mostly detected in the vacuolar lumen and no labeling in the tonoplast, or indeed other endomembranes, was apparent (Fig. 3d–f). Since secRFP is secreted, the label of RFP-TMCT reporter into the vacuolar lumen is indicative of a specific role of the TMCT domain in redirecting the protein from the default secretion pathway.

In root axes from transgenic RFP-TMCT and RFP-AFVY seedlings showed a strong red fluorescent signal in the vacuolar lumen (Fig. 4), while no fluorescent signal was detected in *wild type* roots. These results indicate that the TMCT vacuolar sorting signal functions both in roots and leave, as does AFVY (Hunter et al. 2007).

Transgenic embryos dissected from dry seeds of transgenic lines expressing RFP-TMCT and RFP-AFVY constructs, and *wild type* seeds as negative controls, were also analyzed (Fig. 4). Once again, in mature embryos, the common florescence pattern for RFP-AFVY and RFP-TMCT proteins was the lumen of the PSV. RFP-TMCT showed the same distribution as RFP-AFVY (Hunter et al. 2007; Craddock et al. 2008), which differs from *wild type* seeds and it is clearly a PSV pattern. This finding indicates that TMCT domains of AtRMR1 can also sort reporter proteins to seed PSVs and suggest that, in principle, these domains could be used to deliver proteins of interest to PSV lumen in seeds.

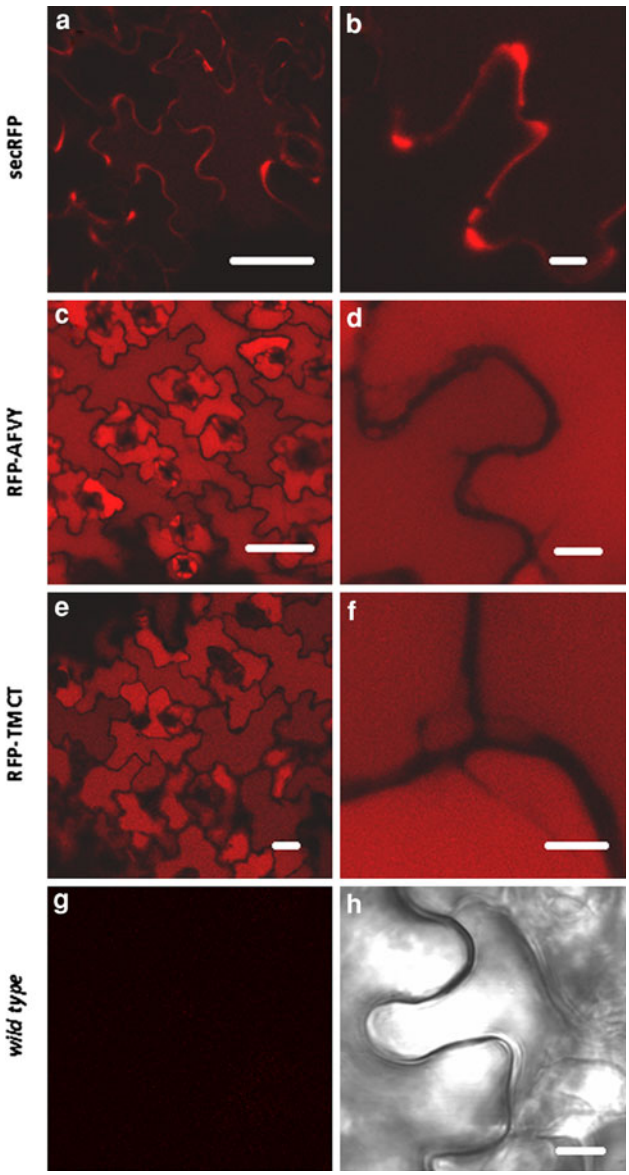

Fig. 3 RFP-TMCT reporter labels the vacuolar lumen in transgenic leaf epidermal cells of *Arabidopsis thaliana*. Leaves from transgenic plants expressing the indicated constructs were analyzed by confocal microscopy. RFP was excited at 561 nm. All images shown were acquired using the same photomultiplier gain and offset settings. For each sample, two magnifications are shown. *Scale bars* 30 µm (**a**, **c** and **e**) and 5 µm (**b**, **d**, **f**, **g** and **h**)

Because RFP-TMCT and RFP-AFVY reporter have the same distribution pattern in seeds and vegetative tissues of transgenic *Arabidopsis thaliana*, we conclude that the transmembrane domain and cytosolic tail of RMR are able to deliver RFP to the vacuolar lumen in all cell types analyzed, in a manner similar to the soluble ctVSS, AFVY.

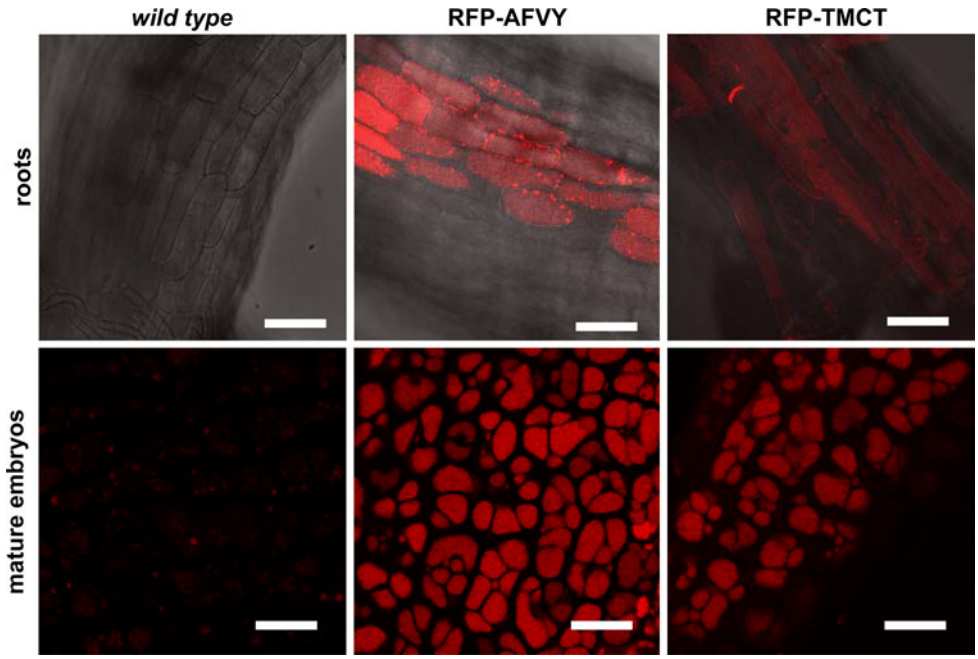

Fig. 4 RFP-TMCT reporter labels the vacuolar lumen in roots and mature embryos of transgenic *Arabidopsis thaliana* plants. Two vacuole-targeted RFP reporters localize to the same vacuole in Arabidopsis roots and embryos. RFP was excited at 561 nm. All images shown were acquired using the same photomultiplier gain and offset. *Scale bars* 50 μm in roots and 10 μm in embryos

RFP-TMCT behaves as an integral membrane protein associated to microsomes membranes

Sorting of membrane proteins is still an open question in plant cells (Barrieu and Chrispeels 1999). To verified whether the TM domain used in the reporter RFP-TMCT is inserted into the ER membrane during its synthesis, microsomes were prepared from transgenic *A. thaliana* leaves expressing RFP-TMCT (Fig. 5) as previously described (Frigerio et al. 2000). Immunoblots of total protein extracts from transgenic Arabidopsis leaves expressing RFP-AFVY and RFP-TMCT with RFP antiserum presented a band of 27 and 38 kDa, respectively (Fig. 5, lanes 2 and 3). The 38 kDa protein has the expected size for the fusion RFP (23 kDa)-TMCT (15 kDa). The soluble fraction (Fig. 5, lane 4), that contains cytosolic proteins and the content of vacuoles which is released during homogenation under these conditions, also shows a band of 38 kDa and reveals a band corresponding to full-length RFP-TMCT which may indicate that the reporter is internalized into the vacuole, rather than the RFP moiety being cleaved and separately delivered to the vacuole. To study the association of RFP-TMCT with microsome membranes, the microsomal pellet was resuspended in different buffers: Suc buffer alone as control, Suc buffer containing 1 M NaCl (to release proteins peripherally attached to the cytosolic face of microsomes) and 0.1 M sodium carbonate [to release proteins tightly bound to the membrane but not integral membranes proteins that remained associated (Fujiki et al. 1982)]. Both soluble and pellet fractions were analyzed by SDS-PAGE followed of Immunoblot with RFP antibody. Consistently, RFP-TMCT full-length protein (38 kDa) was recovered in the microsomal fraction (Fig. 5, lanes 5–10) and remained in the pellet fraction after the treatments with Suc Bf, sodium chloride and sodium carbonate. This is consistent with RFP-TMCT being an integral membrane protein for as long as it is within microsomes (Fig. 5, lane 9). These results indicate that RFP-TMCT reporter inserts into the ER membrane but is then delivered to the vacuolar lumen without undergoing proteolytic cleavage (Figs. 3, 4). The TMCT signal is therefore sufficient to sort secRFP to vacuoles. A vacuolar fraction from RFP-TMCT transgenic Arabidopsis plants also exhibit a 38 kDa recognized by RFP antibody what indicate that the full-length RFP-TMCT reached the vacuoles without undergoing proteolytic cleavage (Fig. 5, lane 11).

RFP-TMCT reporter partially colocalizes with an ER marker, but not with later secretory pathway markers

To further investigate the transport of RFP-TMCT in the secretory pathway, and resolve the localization at the cell periphery observed in tobacco epidermal cells (Fig. 2d), colocalization studies with markers of the endomembrane system were carried out (Figs. 6, 7). Leaves of *N. tabacum* were co-agroinfiltrated with RFP-TMCT reporter plus a secretory pathway marker and then doubly transformed epidermal cells were selected based on the presence of both fluorescent markers.

Co-agroinfiltration of RFP-TMCT and endoplasmic reticulum (ER) marker (GFP-HDEL) was performed

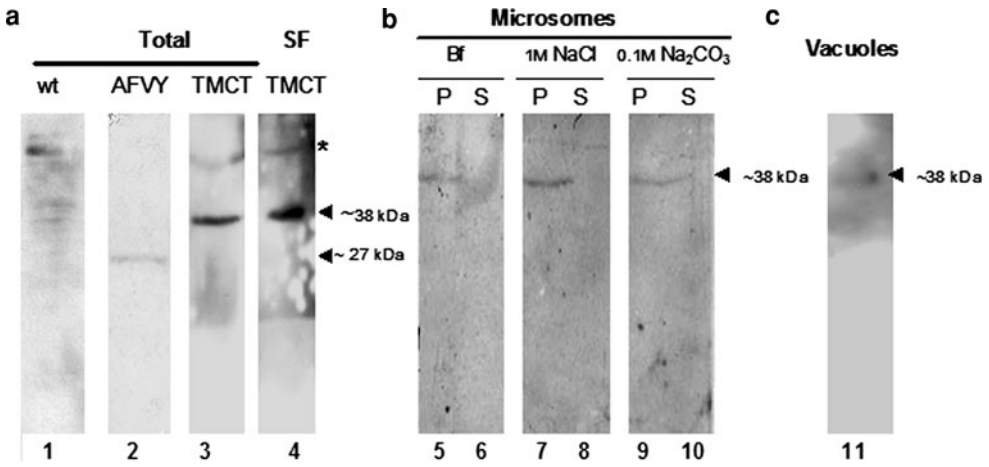

Fig. 5 RFP-TMCT reporter behaves as an integral membrane protein. Western blot with RFP antiserum (reducing SDS-PAGE): **a** Total cell homogenate from *Arabidopsis thaliana* leaves from *wild type* (*lane 1*) and transgenic expressing RFP-AFVY (*lane 2*) and RFP-TMCT (*lane 3*) plants. *SF* soluble fraction, supernatant from the first microsome preparation, containing cytosolic and vacuolar proteins from RFP-TMCT transgenic arabidopsis plants. **b** Microsomes pellet were obtained from transgenic *Arabidopsis thaliana* leaves expressing RFP-TMCT reporter and to analyze associations with membranes, microsome fraction was treated with Suc buffer, Suc buffer with 1 M NaCl or in 0.1 M Na_2CO_3. After these treatments, soluble proteins (*S*) were separated from insoluble (*P* pellets). RFP-TMCT is found in microsomes pellets (*lanes 5*, *7* and *9*) and is not releases by sodium chloride (*lane 8*) either sodium carbonate (*lane 10*) indicated its association as an integral membrane proteins. **c** Vacuole extract from RFP-TMCT arabidopsis plants (*lane 11*). *Solid arrowhead* RFP-TMCT full-length fusion protein, *asterisk* endogenous arabidopsis protein detected by RFP antiserum

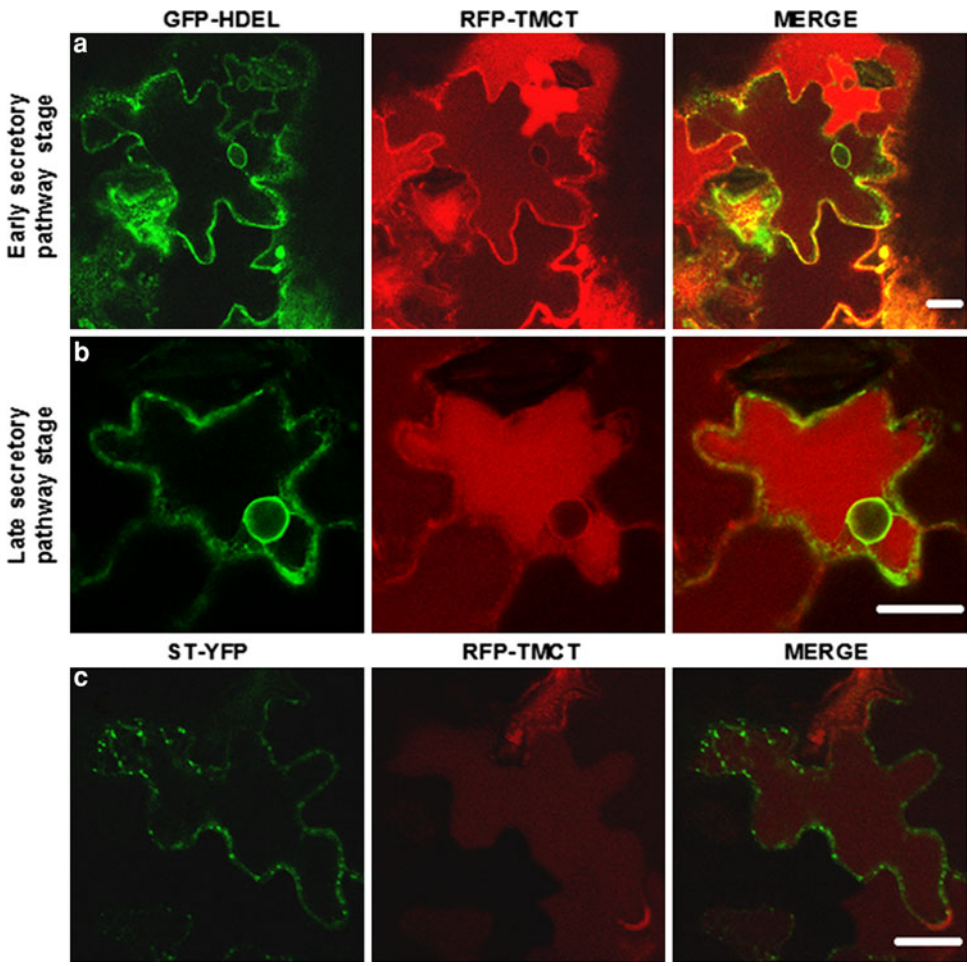

Fig. 6 RFP-TMCT colocalizes with ER but not with Golgi marker. Co-Agroinfiltration studies of RFP-TMCT with GFP-HDEL (ER marker). **a** Very few cells showed an early secretory pathway stage of red reporter and colocalization with ER marker was found, as merged images showed. **b** In most cells, were found a late secretory pathway stage where the reporter RFP-TMCT was in its final destination in the vacuolar lumen, without colocalization pattern with ER marker. **c** Co-agroinfiltration study of RFP-TMCT reporter with ST-YFP (trans-Golgi marker) and no colocalization pattern was found. *Scale bars* 25 μm

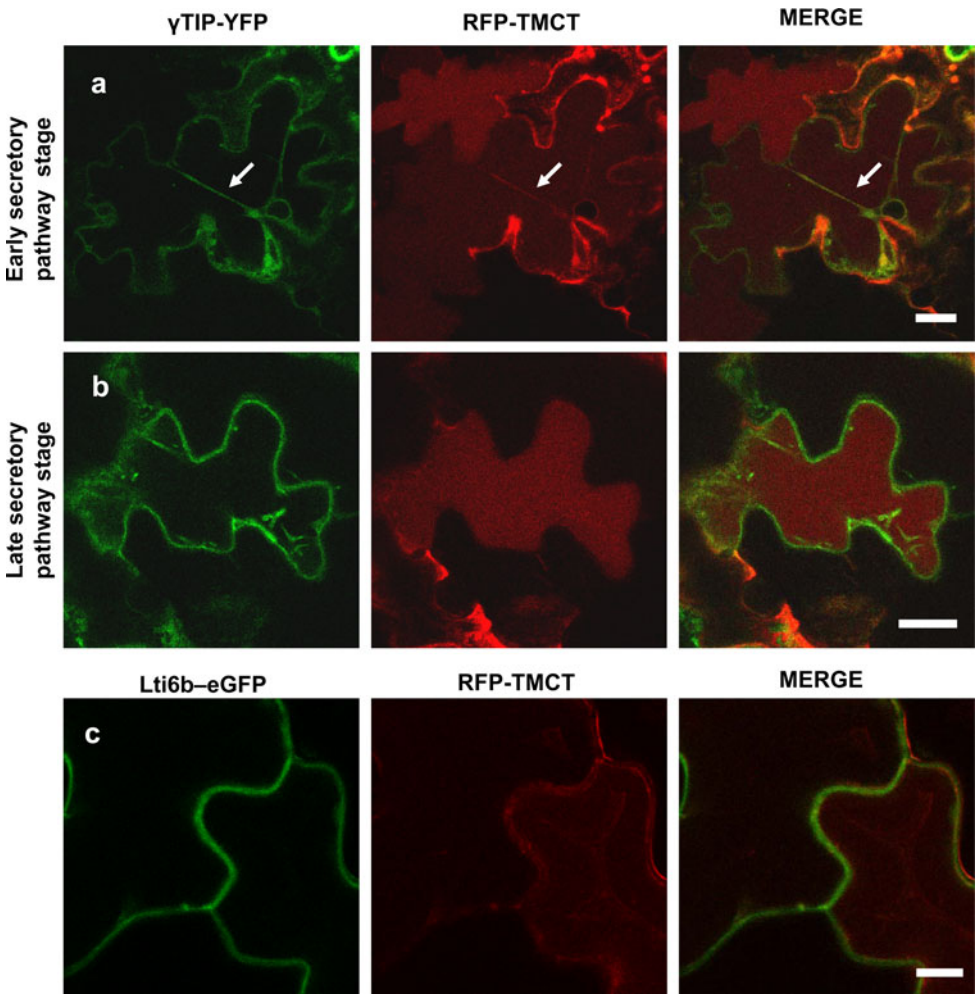

Fig. 7 RFP-TMCT does not colocalize with tonoplast and plasma membrane markers. Co-agroinfiltration studies of RFP-TMCT with γTIP-YFP (tonoplast marker). **a** Cells in early secretory pathway stage of red reporter, observing transvacuolar strand labeling (*arrow*), but no tonoplast colocalization pattern, as merged images showed. **b** Cells in late secretory pathway stage showed no colocalization of the markers, with a full localization of RFP-TMCT reporter in the vacuolar lumen. Co-agroinfiltration studies of RFP-TMCT reporter with Lti6b-eGFP (plasma membrane marker). **c** Non co-localization pattern was found. *Scale bars* 25 μm (**a**, **b**) and 10 μm (**c**)

(Fig. 6a, b). The ER marker highlighted the characteristic reticular pattern and the nuclear envelope. Colocalization with RFP-TMCT was observed (Fig. 6a, merged panel), mainly in cells with lower accumulation of RFP-TMCT in the vacuolar lumen. This likely indicates that the chimeric protein RFP-TMCT is still in transit through the early secretory pathway. Partial ER localization was also often detected for RFP-AFVY (Fig. 2c, asterisks).

When cells where observed at a later time from infiltration, the majority of red fluorescence was indeed detectable mainly in the vacuolar lumen (Fig. 6b), with little colocalization with the ER marker still visible at the nuclear envelope (Fig. 6b, merged panel).

To study if RFP-TMCT reporter is delivered via the Golgi complex, co-agroinfiltration experiments with the Golgi marker, syalyl transferase, fused to YFP (ST-YFP), were performed (Fig. 6c). No obvious colocalization was observed between RFP-TMCT and the Golgi marker.

In addition, to test if RFP-TMCT localized in tonoplast co-agroinfiltrations with the γ-TIP-YFP tonoplast marker (Hunter et al. 2007) were performed. In these experiments, two different patterns corresponding to different expression stages were observed too. The first one, cells with markers still in the early secretory pathway, showed a predominantly ER localization for γ-TIP-YFP, that co-localized with RFP-TMCT in the nuclear envelope and a transvacuolar strand, indicative of ER (Fig. 7a, arrow). RFP-TMCT transvacuolar strands were also observed with RFP-AFVY (Fig. 2c, asterisk). No colocalization of RFP-TMCT and γ-TIP-YFP was found at the tonoplast (Fig. 7a, merged panels). The second pattern, with the reporters most likely at their final destination, was found in the majority of cells. Here, no colocalization between RFP-TMCT and γ-TIP-YFP reporters was detected, with strong red fluorescence in the vacuolar lumen and green fluorescence in tonoplast (Fig. 7b). The absence of colocalization of RFP-TMCT with γ-TIP-YFP in cells in early or late secretory pathway stage suggests differences in the role of the transmembrane domain in sorting process, as discussed later.

Finally, the co-expression of RFP-TMCT with Lti6b-eGFP, a plasma membrane marker, was performed. In epidermal cells, a typical plasma membrane pattern is observed in the green channel (Fig. 7c, plasma membrane marker-GFP). Adjacent cells expressing RFP-TMCT

reporter showed red fluorescence in the vacuolar lumen (Fig. 7c, red channel) and no colocalization was found between these two makers (Fig. 7c, merged channel). This result clearly indicates that the TM of AtRMR1 does not target the RFP to the plasma membrane.

Discussion

Transmembrane and cytosolic domains of AtRMR1 by itself behaves as a vacuolar sorting signal

Using transient expression in tobacco leaves cells and stable expression in transgenic Arabidopsis plants, we showed that the fusion of secRFP to transmembrane domain and cytosolic tail (TMCT) of AtRMR1 is sufficient to redirect this reporter protein to vacuoles. The distribution pattern of RFP-TMCT in leaves, roots and embryo of transgenic Arabidopsis was similar to that obtained for the two soluble vacuolar markers RFP-AFVY and spL-RFP (Hunter et al. 2007). In contrast, fusions of spGFP to TMCT of AtVSRs mainly accumulated in prevacuolar compartments both tobacco leaf epidermal cells (Kotzer et al. 2004; DaSilva et al. 2005) and also transgenic BY2 cells (Miao et al. 2006). In addition, the RFP-TMCT distribution pattern obtained is consistent with the immunolocalization studies performed in arabidopsis embryos that shown that—although both AtRMR1 and AtVSR1 are present in prevacuolar compartment—only AtRMR protein reaches the protein storage vacuoles (Hinz et al. 2007; Wang et al. 2011). The presented results support the different steady-state localization predicted for RMR versus the VSR family.

As was previously described, AtRMR2 protein was first localized as component of the membrane-containing crystalloid in tobacco and tomato seed PSV (Jiang et al. 2000) and DIP-positive organelle in transient expression *A. thaliana* leaf protoplasts (Park et al. 2005). Temporal expression assays using tobacco suspension culture cells showed that a reporter protein (mutated proaleurain on vacuolar targeting sequence) containing the TM and CT domains of AtRMR2 is found associated with cell membranes and traffics via the Golgi apparatus to reaches vacuoles as showed on total protein extracts from isolated vacuoles (Jiang et al. 2000). Here, we show that RFP-TMCT behaved as integral membrane protein and its final destination is the lumen of central vegetative vacuoles in leaves, roots and protein storage vacuoles in seeds from transgenic tissues. Park et al. (2005) showed that in Arabidopsis leaf protoplasts, AtRMR1ΔLU-HA mutant gave only a minor portion of positive punctate stains that colocalized DIP organelle and the majority overlapped with Golgi marker and also the mutant AtRMR1ΔCT-HA, which suggest that both luminal domain and the cytoplasmic tail are important for the localization of AtRMR1-HA to the DIP-positive organelle. In contrast, RFP-TMCT in both transient assays and transgenic Arabidopsis plants is found exclusively in the lumen of central vegetative vacuoles in leaves, roots and protein storage vacuoles in seeds. Because secRFP is secreted, the TMCT is sufficient to redirect the reporter protein to vacuoles. Western blot analysis on total protein extracts and soluble fraction of transgenic leaves showed that RFP-TMCT remains intact, thus making cleavage and vacuolar sorting of soluble RFP from the reporter unlikely. Confocal observation of RFP-TMCT through the secretory pathway showed localization at ER. No obvious colocalization was observed between RFP-TMCT and the *trans*-golgi marker, but as mentioned before the reporter by Jiang et al. 2000 acquires complex modifications to asparagine-linked glycans, and could be consistent with similar localization report to AtRMR2 and cruciferin that was present at the periphery in the *cis* and medial cisternae and in DVs (Wang et al. 2011). Live cell imaging studies, revealed non tonoplast localization, consistent with findings from other studies, labeling for AtRMR2 on organelles representing PVCs was predominantly internal providing further support that these proteins are internalized during their traffic to the PSV and such internalization would remove the possibility that AtRMR2 could recycle back to the Golgi apparatus to participate in more than one round of ligand sorting (Wang et al. 2011). Finally, the results presented here show that TMCT of AtRMR1 by itself reaches the vacuoles as has been described for the full-length native receptor (Hinz et al. 2007). Nevertheless, it has been proposed that native receptor interacts with the cargo storage proteins that aggregate and trigger internalization (Wang et al. 2011). In our work, internalization seems to occur without this interaction, with a direct fusion of TMCT to a soluble reporter protein.

Taking into consideration, the limitation of transient experiments to study sorting signal because the possibilities of overload the transport machinery together with the differences in transport in the secretory pathway between different organs (Petruccelli et al. 2006; Drakakaki et al. 2006; Van Droogenbroeck et al. 2007) our results show that TMCT can sort the reporter to vacuoles in different tissues of transgenic Arabidopsis. No secretion was detected as has been observed for other vacuolar protein expressed in seeds (Petruccelli et al. 2006; Chiera et al. 2004; Drakakaki et al. 2006).

Based on the results presented here, we propose that the TMCT domain could be used to sort protein of interest to vacuoles, avoiding the requirement of interaction between the cargo protein and the receptor, which is limited by the affinity of the receptor for its target and it is susceptible of

saturation. We have previously observed a similar phenomenon when a full-length antibody fused to a KDEL signal was expressed in tobacco seeds (Petruccelli et al. 2006). We are currently testing whether the TMCT fusion strategy is more efficient than the use of VSS dependent of receptor proteins.

Conclusion

This work present a heterologous protein expression strategy based on Arabidopsis receptor homology-transmembrane-RING H2 domains. RFP with the signal of TM and CT of AtRMR1 reaches the lumen vacuolar in all tissue and species evaluated providing new experimental data with the use of a fluorescent reporter fluorescent at the low pH of vacuoles.

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