Plant Science 266 (2018) 9–18

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

RMR (Receptor Membrane RING-H2) type 1 and 2 show different promoter activities and subcellular localizations in *Arabidopsis thaliana*

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ARTICLE INFO

Keywords:
AtRMRs
AtRMR endogenous promoters
trans-Golgi network
Transmission electron microscopy
Endoplasmic reticulum
Arabidopsis thaliana

ABSTRACT

Soluble vacuolar proteins reach their compartments of final accumulation through the binding with specific transmembrane cargo receptors. In *Arabidopsis thaliana* two different families of receptors have been characterized. The AtVSRs (Vacuolar Sorting Receptor), which are known to be involved in the protein sorting to lytic vacuoles (LV), and the AtRMRs (Receptor Membrane RING-H2), for which there is less evidence for a role in the traffic to the protein storage vacuole (PSV). In this study we investigated the localization and tissue expression of two RMRs (AtRMR1 and 2) in their species of origin, *A. thaliana*. Our experiments using leaf protoplasts and transgenic plants supported previous results of subcellular localization in *Nicotiana benthamiana* that visualized AtRMR1 and 2 in the cisternae of endoplasmic reticulum (ER) and in the *trans*-Golgi network (TGN), respectively. The promoter activities of *AtRMR1* and *AtRMR2* detected in transgenic *A. thaliana* lines suggest that the expression of these two receptors only partially overlap in some organs and tissues. These results suggest that AtRMR1 and 2 are not functionally redundant, but could also interact and participate in the same cellular process in tissues with an overlapping expression.

1. Introduction

Vacuoles are unique and highly dynamic compartments of plant cells that assume a central role in many fundamental cellular processes [1–3]. Depending on the tissue type and function, vacuoles are involved in the sequestration of many compounds such as ions, pigments, secondary metabolites, storage proteins and carbohydrates [1–3]. This system is also involved in essential functions related to plants growth, development and response to environmental stresses [4–7], giving to these compartments an essential role in plant life [8].

To cover this wide number of different functions, it is not surprising that plant cells may have several vacuoles with different functions [1,9,10]. At least two different types of vacuoles, an acidic compartment similar to the animal lysosome (lytic vacuole, LV) and a compartment involved in protein storage (protein storage vacuole, PSV), have been visualized within the same plant cells using specific molecular markers [11–15]. Therefore, it is likely that plant cells possess specific mechanisms of protein sorting to these different compartments [16–18].

In plant cells, the sorting of soluble proteins to vacuoles involves the presence of specific transmembrane cargo-receptors able to bind protein motifs, known as vacuolar sorting determinants (VSDs), located at either N-terminal, C-terminal or internal sequences of vacuolar protein precursors [19–21]. By binding to these VSDs, vacuolar receptors are able to divert the traffic of soluble vacuolar proteins from the default pathway of secretion into the apoplast to their final destination of accumulation, vacuoles [16,17]. Two types of cargo receptors, probably involved in the traffic to different vacuolar compartments, have been described in plants so far.

The first family, the Vacuolar Sorting Receptors (VSRs) [20–22] is encoded by seven genes in *Arabidopsis thaliana* (from *AtVSR1* to *AtVSR7*), and has been described to be involved in sorting of vacuolar protein equipped with sequence specific VSDs (ssVSDs) to the LV [20,23,24] but also to contribute to the accumulation of storage proteins in seeds [25]. Unlike this well described pathway, less is known about the cargo receptors involved in the route that lead proteins

Abbreviations: ctVSD, C-terminal VSD; DV, dense vesicles; ER, endoplasmic reticulum; LVl, ytic vacuole; PA, protease-associated domain; PSV, protein storage vacuole; PVC, prevacuolar compartments; RMR, receptor membrane RING-H2; RING-H2, RING (really interesting new gene)-H2 domain; ssVSD, sequence specific VSD; Ser-Rich, domain rich in serines; TGN, *trans*-Golgi network; TM, transmembrane domain; VSR, vacuolar sorting receptor; VSD, vacuolar sorting determinant

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http://dx.doi.org/10.1016/j.plantsci.2017.10.007
Received 8 June 2017; Received in revised form 27 August 2017; Accepted 14 October 2017
Available online 20 October 2017
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equipped with C-terminal VSDs (ctVSDs) to the PSV. An important group of candidates is represented by a second family of type I transmembrane proteins, the Receptor Membrane RING-H2 (RMRs) [26–30] that is encoded by six genes (from *AtRMR1* to *AtRMR6*) in *A. thaliana*. These proteins have a peculiar structure: The N-terminal luminal part is mostly composed of a PA domain, also found in VSRs, where it is involved in the interaction with ssVSDs of vacuolar proteins [26,31]. The ability of the N-terminal domain of RMRs to interact *in vitro* with the ctVSDs of vacuolar proteins that accumulate in the PSV (e.g. barley lectin, bean phaseolin and tobacco chitinase), supports their role as vacuolar cargo receptors [28,29]. A C3H2C3 RING finger domain (RING-H2) of unknown function [26], along with a Ser-Rich tail domain that contains several potential phosphorylation sites [27,32], constitute the C-terminal cytosolic domains of RMRs.

Whether RMRs are the cargo receptors involved in sorting to PSV is still not clear and many questions about their trafficking and localization, together with the functions of their protein domains, are still open. Unlike the members of AtVSRs family that localize to the same TGN/prevacuolar compartments [21,33,34], AtRMR receptors display a more variable localization. Experiments performed in *A. thaliana* have shown that AtRMR1 (At5g66160) is localized in prevacuolar compartments (PVCs) [29] or in the central vacuole [35], whereas AtRMR2 (At1g71980), has been found in the late Golgi apparatus, dense vesicles (DV) and PSV in *A. thaliana* embryos analysed by immunogold electron microscopy [36]. In a recent study performed in our laboratory using agroinfiltrated *Nicotiana benthamiana* leaves, AtRMR2 was detected in the *trans*-Golgi network (TGN), whereas AtRMR1 was mainly localized in the membranes of endoplasmic reticulum (ER) [32]. Furthermore, we demonstrated that AtRMR2 can form homodimers but is also able to interact with AtRMR1 to form heterodimers, which can then be exported to the TGN [32].

In this study, we used different molecular approaches to confirm the localization of AtRMR1 and AtRMR2 in their species of origin, *A. thaliana*. DNA constructs for constitutive protein expression in plant cells, encoding full-length AtRMR1 or AtRMR2 fused at their C-terminus with YFP, were tested in both leaf protoplasts and stably transformed *A. thaliana* plants. The fluorescent patterns observed in protoplasts support the subcellular localization of AtRMR1 and 2 in the ER and TGN, respectively. Furthermore, by using a combination of confocal and immunogold electron-microscopy on *A. thaliana* root tip cells, we confirmed that AtRMR1 is mainly localized in the ER, while the failure to detect AtRMR2-YFP *in planta* prevented us to confirm its localization in the TGN.

While AtRMR1 and 2 are difficult to overexpress in *A. thaliana*, their endogenous promoters and terminators are able to direct the expression of a fluorescent reporter (YFP) in different organs and tissues of *A. thaliana* transgenic lines. These results suggest that AtRMR1 is mainly expressed in the epidermis (leaf and silique) and weakly expressed in developing seeds, whereas AtRMR2 is more broadly expressed in leaf epidermis and mesophyll, and in roots and siliques. These partially different tissue expressions suggest that AtRMR1 and 2 are not functionally redundant, but could interact and participate in the same cellular processes in tissues with an overlapping expression.

2. Materials and methods

2.1. Plant material and growth condition

Arabidopsis thaliana plants ecotype Columbia (Col-0) were grown in long-day photoperiod at light intensity of 120 $\mu E/m^2 \cdot s$ for 16 h and 8 h of dark. The plants were kept at a day temperature of 22 °C, and a night temperature of 20 °C. Humidity was kept constant at 70%. In non-sterile conditions, *A. thaliana* plants were grown using soil containing 45% sand, 10% perlite, 25% compost and 20% peat (RICOTER, Aarberg, Switzerland) in a growth chamber Mobylux GroBanks (CLF Plant Climatics, Wertingen, Germany). While in sterile conditions, the plants were grown in *Petri* dishes containing 4.47 g/l Murashige and Skoog medium (Duchefa Biochemie, Haarlem, Netherlands) supplemented with 20 g/l sucrose and 8 g/l phytagel (Sigma-Aldrich, Buchs, Switzerland) at pH 5.6 and were kept in a MLR-351 growth cabinet SANYO/Panasonic (Loughborough, UK).

2.2. Constructions and plasmids

The binary Ti vector pGREEN system [37] was used for both transient transformation of *Arabidopsis thaliana* leaf protoplasts and production of transgenic lines via *Agrobacterium tumefaciens*. The two pGREEN0229 based vectors, pGREEN-AtRMR1-YFP and pGREEN-AtRMR1-YFP encoding for either full-length AtRMR1 or AtRMR2 fused with the YFP at the C-terminus, were generated in a previous study [32]. The two vectors pGREEN-Venus-Syp61 encoding for the TGN marker Venus-Syp61, and pGREEN-AtRMR2-RFP encoding for AtRMR2 fused with RFP at the C-terminus have been described in a previous study [32]. The genomic sequences containing either the promoter (763 bp upstream) or terminator (555 bp downstream) of *AtRMR1* (TAIR reference: At5g66160) were amplified using a DNA genomic preparation from *Arabidopsis thaliana* Col-0 and the primer pairs Prom1-fw/Prom1-rev and Term1-fw/Term1-rv, respectively (Supplementary Table S1). While, the genomic sequences containing the promoter (2001 bp upstream) or terminator (258 bp downstream) of *AtRMR2* (TAIR reference: At1g71980) were amplified using the same DNA substrate and the primer pairs Prom2-fw/Prom2-rev and Term2-fw/Term2-rv, respectively (Supplementary Table S1). The two plasmids, pGREEN-P1-T1 and pGREEN-P2-T2, were generated by cloning promoters and terminators into the MCS of pGREEN0179, using the restriction sites *Xho*I/*Sal*I and *Pst*I/*Sac*I, respectively. The full-length *YFP* cDNA was then cloned into *Sal*I/*Hind*III restriction sites between the promoters and terminators, generating pGREEN-P1-YFP-T1 and pGREEN-P2-YFP-T2, respectively. The nucleotide sequences of DNA constructs used in this work are shown in Fig. S1–S4.

2.3. Preparation of Arabidopsis thaliana leaf protoplasts and PEG-mediated transformation

Rosettes from 2 to 3 week-old *A. thaliana* plants grown in sterile conditions were incubated overnight in the dark in digestion solution containing 400 mM mannitol, 5 mM MES, 8 mM $CaCl_2$, 1% (w/v) cellulase (Sigma-Aldrich, Buchs, Switzerland), 0,25% (w/v) macerozyme (Sigma-Aldrich, Buchs, Switzerland) at pH 5.6. The next day, protoplasts were released by carefully shaking, filtered on a 100 µm mesh filter and recuperated by centrifugation for 5 min at 50*g* (brake off) at room temperature. The cellular pellet was washed three times in W5 solution (154 mM NaCl; 125 mM $CaCl_2$; 5 mM KCl; 5 mM glucose; 1.5 mM MES; at pH 5.6), and then transferred on the top of a 21% (w/v) sucrose solution located in a 15 ml tube. After centrifugation for 10 min at 50*g* (brake off) at room temperature, intact protoplasts were recuperated in the interface between the W5 and the sucrose solutions. The protoplasts were washed again in W5 solution and then incubated for 30 min on ice. Finally, the protoplasts were counted (Burker chamber) and resuspended in an appropriate volume of MaMg solution (0.4 M mannitol; 15 mM $MgCl_2$; 0.5 M MES; pH 5.6), in order to obtain 1.5×10^6 cells per 300 µl of solution.

325 µl of PEG solution (40% (w/v) Polyethylene glycol-4000; 0.4 M mannitol; 0.1 M $Ca(NO_3)_2$; pH 7–8) were gently added to 300 µl of MaMg containing 1.5×10^6 of protoplasts, 25 µg of plasmid and 50 µg of DNA carrier. The solution was incubated for 30 min a room temperature, and then fresh W5 solution was gently added to fill the tube. The transformed protoplasts were then recuperated by centrifugation (as previously), resuspended in fresh W5 solution and incubated overnight in the dark at room temperature.

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