Journal of Plant Physiology 200 (2016) 45–52

Contents lists available at ScienceDirect

Journal of Plant Physiology

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

Subcellular localizations of Arabidopsis myotubularins MTM1 and MTM2 suggest possible functions in vesicular trafficking between ER and *cis*-Golgi

Akanksha Nagpal[a], Ivan Ndamukong[b], Ammar Hassan[a], Zoya Avramova[b,**], František Baluška[a,*]

[a] *IZMB, University of Bonn, Kirschalle 1, 53115 Bonn, Germany*
[b] *School of Biological Sciences, UNL, Lincoln NE, 68588, United States*

ARTICLE INFO

Article history:
Received 6 May 2016
Received in revised form 3 June 2016
Accepted 3 June 2016
Available online 18 June 2016

Keywords:
Arbidopsis
AtMTM1
AtMTM2
Vesicular trafficking
ER
cis-Golgi

ABSTRACT

The two Arabidopsis genes AtMTM1 and AtMTM2 encode highly similar phosphoinositide 3-phosphatases from the myotubularin family. Despite the high-level conservation of structure and biochemical activities, their physiological roles have significantly diverged. The nature of a membrane and the concentrations of their membrane-anchored substrates (PtdIns3*P* or PtdIns3,5P_2) and/or products (PtdIns5*P* and PtdIns) are considered critical for determining the functional specificity of myotubularins. We have performed comprehensive analyses of the subcellular localization of AtMTM1 and AtMTM2 using a variety of specific constructs transiently expressed in *Nicotiana benthamiana* leaf epidermal cells under the control of 35 S promoter. AtMTM1 co-localized preferentially with *cis*-Golgi membranes, while AtMTM2 associated predominantly with ER membranes. In a stark contrast with animal/human MTMs, neither AtMTM1 nor AtMTM2 co-localizes with early or late endosomes or with TGN/EE compartments, making them unlikely participants in the endosomal trafficking system. Localization of the AtMTM2 is sensitive to cold and osmotic stress challenges. In contrast to animal myotubularins, Arabidopsis myotubularins do not associate with endosomes. Our results suggest that Arabidopsis myotubularins play a role in the vesicular trafficking between ER exit sites and *cis*-Golgi elements. The significance of these results is discussed also in the context of stress biology and plant autophagy.

1. Introduction

Phosphatidylinositol phosphates (PtdIns*P*s) are low-abundance lipid molecules that undergo rapid turnover and differential phosphorylation modifications generating seven distinct isomers: the tri-phosphate (PtdIns3,4,5P_3), the bi-phosphates (PtdIns3,4P_2, PtdIns3,5P_2, PtdIns4,5P_2) and the monophosphorylated phosphates (PtdIns3*P*, PtdIns4*P*, and PtdIns5*P*). The biological relevance of the PtdIns*P*s is in their ability to function as secondary messengers in pathways regulating various cellular processes including glucose metabolism, membrane trafficking, cytoskeletal rearrangements, cell wall assembly, signal transduction, autophagy, and gene expression in both animal and plant cells (Balla, 2013; Gillaspy, 2013; Shisheva, 2013; Heilmann and Heilmann, 2014; Krishnamoorthy et al., 2014). The capacity of PtdIns*P*s to bind specific proteins and, thus, to alter their localization and/or activity has defined the phosphoinositides as dynamic signaling molecules playing key roles in the regulatory network coordinating stress responses at the subcellular level (Balla, 2013). The PtdIns*P* isomers are viewed as docking sites 'attracting' signaling proteins to specific membranes 'guiding' them to their substrates (Robinson and Dixon, 2006). Rapid and localized changes in the concentration of membrane-bound PtdIns*P*, then, may result in specific recruitment and/or activation of signaling proteins or enzyme activities.

PtdIns5*P* was the last discovered phosphoinositide (Rameh et al., 1997), considered initially only as a precursor for the much more abundant bi-phosphate PtdIns4,5P_2 (Tolias et al., 1998). Despite its low abundance in non-stressed cells, however, PtdIns5*P* is emerging as a key component in novel stress signaling pathways regulating diverse metabolic and cellular functions (Jones and Divecha, 2004). PtdIns5*P* has been recognized as a main intermediate in the cell osmoprotective response pathway (Sbrissa et al.,

* Corresponding author.
** Corresponding author.
E-mail addresses: zavramova2@unl.edu (Z. Avramova), baluska@uni-bonn.de (F. Baluška).

http://dx.doi.org/10.1016/j.jplph.2016.06.001
0176-1617/

2002), in the etiology of severe muscular/neuronal pathologies (Tronchere et al., 2004), in various human cancers and leukemias (Balla, 2013), and in host-cell response to infection with the pathogen *Shigella flexneri* (Laporte et al., 2003; Pendaries et al., 2006). PtdIns5 *P* acts as a stress-induced second messenger that can calibrate how cells manage ROS (Keune et al., 2013).

PtdIns5 *P* was detected also in cultured plant cells increasing its concentration in response to hyperosmotic stress (Meijer et al., 2001). However, its presence in plants was positively identified only recently (Ndamukong et al., 2010). The levels of endogenous PtdIns5 P increase during dehydration and hypotonic stresses and affect the activity of the plant histone H3K4 methyltransferase ATX1 (Alvarez-Venegas et al., 2006a,b; Ndamukong et al., 2010). ATX1 and elevated PtdIns5 *P* (either exogenously provided or resulting from the over-expression of Arabidopsis MYOTUBULARIN1, AtMTM1) oppositely affected the expression from an overlapping set of dehydration stress responding genes suggesting counteracting roles in a common lipid-signaling pathway (Alvarez-Venegas et al., 2006a,b; Ding et al., 2009,2012).

Despite its potential to regulate both cytoplasmic and nuclear functions (Gozani et al., 2003, 2005; Jones and Divecha, 2004; Jones et al., 2006), PtdIns5 P remains the least-characterized and the most enigmatic member of the PtdIns family. Its localization and molecular functions in either mammalian or plant cells remains elusive. As the PtdIns*P* may anchor partners to specific membranes, a widely used approach for studying their functions is to trace the subcellular location and distribution of membrane interacting proteins.

Myotubularins (MTMs) are specific 3'-phosphatases which, *in vivo,* dephosphorylate the lipids PtdIns3 *P* and PtdIns3,5P_2, providing the main route for the generation of cellular PtdIns5 *P* from PtdIns3,5P_2 (Blondeau et al., 2000; Begley et al., 2003; Schaletzky et al., 2003; Ndamukong et al., 2010; Ding et al., 2012; Marat and Haucke, 2016). Myotubularin deficiency leads to decreased levels of cellular PtdIns5 *P* and increased levels of PtdIns(3,5)P_2 and PtdIns3P, respectively. There are eight catalytically active and six inactive members of the myotubularin family in humans (Laporte et al., 2003). It is remarkable that all catalytically active MTMs are highly similar in protein structure and domain architecture, display identical substrate specificity (*in vitro*); and are ubiquitously expressed but, nonetheless, are not redundant functionally. Each myotubularin has a unique role as evidenced by the specific types of diseases occurring in their absence. Disease phenotypes have been associated with misregulated levels of the cellular PtdIns3*P,* PtdIns5*P,* and PtdIns3,5P_2 implicating these phospholipids in the regulation of vital signaling pathways (Cao et al., 2008; Skwarek and Boulianne, 2009; reviewed in Balla, 2013).

In *Arabidopsis thaliana* there are two genes (AtMTM1 and AtMTM2) originating from a short segmental duplication in the Arabidopsis lineage (Ding et al., 2012). Both genes encode enzymatically active phosphatases that are 77% identical in protein structure and that are expressed in the same plant tissues (Ding et al., 2009, 2012). Despite these similarities, the AtMTM1 and AtMTM2 genes are differentially expressed under dehydration stress and play also different roles in the plant's responses to drought. In particular, the transcriptomes of *atmtm1* and *atmtm2* mutants under dehydration stress conditions revealed that only AtMTM1 affected transcript levels of stress responding genes (Ding et al., 2012). Biochemically, the two phosphatases displayed different affinity towards the substrates – PtdIns3 *P* and PtdIns3,5P_2 – (Ding et al., 2012) and only AtMTM1 contributed significantly to the endogenous level of PtdIns5 *P* in response to dehydration stress (Ndamukong et al., 2010). Collectively, the results from our studies so far have indicated that despite the highly conserved structure and biochemical activity, the proteins encoded by *AtMTM1* and *AtMTM2* have functionally diverged after their duplication.

Both animal and plant myotubularins share the core architectural domains GRAM, RID, PTP/DSP and SID (reviewed in Kerk and Moorhead, 2010). The multidomain structure facilitates interactions with different ligands and with diverse membranes and/or proteins. Consequently, the nature of the membrane and the concentrations of PtdIns5 *P* and/or of the substrate phosphoinositides (PtdIns3 *P* or PtdIns3,5P_2) anchored on these membranes are thought to determine the functional specificity of each myotubularin (Robinson and Dixon, 2006). In support, mutations in the membrane-binding domains of MTMs or lost association with specific membranes have been linked to different disease phenotypes (Laporte et al., 2002; Robinson and Dixon, 2006; Skwarek and Boulianne, 2009).

As the nature of the membrane is considered critical for the specific function of the myotubularin associated with it, it was of great interest to determine the identity of the subcellular structures that bind AtMTM1 and AtMTM2 in plant cells. Transiently expressed fluorescently tagged AtMTM1 and AtMTM2 displayed distinct cellular localization patterns: AtMTM1 signaled from numerous granulate 'punctate structures', while AtMTM2 appeared as dense patches in lobes of leaf epidermal pavement cells (Ding et al., 2012). Here, we further expanded these interesting data and investigated in detail the nature of these different subcellular localizations of AtMTM1 and AtMTM2, as well as identities of associated structures in transiently expressed *Nicotiana benthamiana* leaf cells.

2. Material and methods

2.1. Plant material and growth conditions

Tobacco plants were grown in growth chamber under a defined light and temperature regime, i.e., 16 h in the light at a light irradiance of 200 $\mu E\, m^{-2}\, s^{-1}$ and a temperature of 27 °C; and 8 h in the dark at 24 °C. Commercially available peat moss based soil after treating with insecticide was used to grow the tobacco plants on soil.

2.2. Transient expression in tobacco

Transient expression of the fluorescent tagged proteins in *N. benthamiana* was carried out via the *A. tumefaciens* leaf infiltration method (Ron and Avni, 2004). After 40 h of infiltration of bacterial suspension into the abaxial surface of the leaf of 6–8 week old tobacco plants, the protein expression was observed under the confocal microscope using a 40 x oil immersion lens (UPLAPO40XOI3, NA1.0). Time-lapse series were captured from single optical sections of tissues which were acquired at defined time intervals. Serial confocal optical sections were acquired for Z-stack projections at different step sizes. Various fluorescent markers were used in order to identify the subcellular localization of myotubularins. The following Tans Golgi Network (TGN) markers were used: syntaxins of plants (SYP61-GFP) which is mainly localized to TGN (Sanderfoot et al., 2000, 2001). VTI12-YFP is one of the Arabidopsis homolog of the yeast SNARE Vti1p (Zheng et al., 1999); RabA1d-GFP is a member of the RabA subfamily of small Rab GTPases which is one of the reliable marker used for studying secretory/recycling vesicles (Berson et al., 2014). G-YK is a *cis*-Golgi marker based on the targeting sequence of soybean alpha-1,2 mannosidase I (a *cis*-Golgi enzyme) (Nelson et al., 2007). C-terminal HDEL (tetrapeptide sequence) fused to the DsRed gene is ER marker, and 2xFYVE construct is a marker for late endosomes in plants (Voigt et al., 2005).

2.3. FM dye staining

For FM-Dye staining, tobacco leaves were infiltrated with FM4-64 solution (5 μM in the Milli-Q water), which was pre cooled for