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Divergent functions of the myotubularin (MTM) homologs AtMTM1 and AtMTM2 in *Arabidopsis thaliana*: evolution of the plant MTM family

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SUMMARY

Myotubularin and myotubularin-related proteins are evolutionarily conserved in eukaryotes. Defects in their function result in muscular dystrophy, neuronal diseases and leukemia in humans. In contrast to the animal lineage, where genes encoding both active and inactive myotubularins (phosphoinositide 3-phosphatases) have appeared and proliferated in the basal metazoan group, myotubularin genes are not found in the unicellular relatives of green plants. However, they are present in land plants encoding proteins highly similar to the active metazoan enzymes. Despite their remarkable structural conservation, plant and animal myotubularins have significantly diverged in their functions. While loss of myotubularin function causes severe disease phenotypes in humans it is not essential for the cellular homeostasis under normal conditions in *Arabidopsis thaliana*. Instead, myotubularin deficiency is associated with altered tolerance to dehydration stress. The two Arabidopsis genes *AtMTM1* and *AtMTM2* have originated from a segmental chromosomal duplication and encode catalytically active enzymes. However, only AtMTM1 is involved in elevating the cellular level of phosphatidylinositol 5-phosphate in response to dehydration stress, and the two myotubularins differentially affect the Arabidopsis dehydration stress-responding transcriptome. AtMTM1 and AtMTM2 display different localization patterns in the cell, consistent with the idea that they associate with different membranes to perform specific functions. A single amino acid mutation in AtMTM2 (L250W) results in a dramatic loss of subcellular localization. Mutations in this region are linked to disease conditions in humans.

Keywords: Arabidopsis MTM1, MTM2, plant myotubularins, evolution.

INTRODUCTION

The myotubularin (MTM) and myotubularin-related (MTMR) proteins display the signature CX_5R motif of the members of the large family of dual-specificity serine–threonine phosphatase (DSP)-class I Cys-based protein tyrosine phosphatases (PTPs). However, they dephosphorylate lipids *in vivo* and have not been shown to dephosphorylate proteins (Blondeau *et al.*, 2000; Taylor *et al.*, 2000; Tronchere *et al.*, 2004). The MTMs are phosphoinositide 3-phosphatases using phosphatidylinositol 3-phosphate (PtdIns3P) and phosphatidylinositol (3,5)-bisphosphate [PtdIns(3,5)P_2] as substrates. Myotubularins carry the consensus sequence (CxDxxDR) at their catalytic site and utilize a unique mechanism during catalysis (Begley *et al.*, 2003, 2006).

Despite being present at low levels in cells, regulated levels of PtdIns3P and PtdIns(3,5)P_2 are critical for cellular and organismal homeostasis (Michell *et al.*, 2006). Using PtdIns(3,5)P_2 as a substrate MTMs can generate PtdIns5P, which until recently has been considered only as a source for the much more abundant PtdIns(4,5)P_2 (Rameh *et al.*, 1997; Carlton and Cullen, 2005). However, research linking severe muscular and neurodegenerative diseases in humans with mutations in genes encoding PtdIns5P regulatory proteins

has implicated PtdIns5P in these disease conditions. PtdIns5P is an intermediate in the cell osmoprotective response pathway (Sbrissa *et al.*, 2002), in the etiology of severe muscular/neuronal pathologies, and in host-cell response to infection with the pathogen *Shigella flexneri* (Niebhur *et al.*, 2002; Laporte *et al.*, 2003; Pendaries *et al.*, 2005). It is also implicated in the Akt pathway (Carricaburu *et al.*, 2003; Pendaries *et al.*, 2005; Coronas *et al.*, 2007) and a role for PtdIns5P in regulating transport from late endosomal compartments to the plasma membrane of mammalian cells has been suggested (Lecompte *et al.*, 2008).

Although the cellular localization and functions of PtdIns5P are still not well understood, its ability to bind the plant homology domain (PHD) of the tumor suppressor ING2 (inhibitor of growth family 2) to promote p53-dependent apoptosis under cellular stress (Gozani *et al.*, 2004; Jones *et al.*, 2006) has defined PtdIns5P as a ligand in nuclear signaling pathways (Jones and Divecha, 2004; Gozani *et al.*, 2005).

All these mono- and bi-phosphorylated phosphoinositides have also been identified in plants and implicated in the responses to salinity, drought, temperature stresses or pathogenic invasion (reviews in Wang, 2004; Boss *et al.*, 2006, Munnik and Vermeer, 2010; Vallutu and Van den Ende, 2011). Despite sharing common aspects, features unique to plants for PtdIns3P, PtdIns(3,5)P_2, and PtdIns5P signaling have been recognized. Phosphatidylinositol 3-phosphate modulates stomatal closing and actin reorganization in guard cells (Jung *et al.*, 2002; Choi *et al.*, 2008) and might mediate oscillations in Ca^{2+} levels (Jung *et al.*, 2002) as well as the generation of reactive oxygen species (ROS) in response to abscisic acid (Park *et al.* 2003). The function of PtdIns(3,5)P_2 in plants is still completely unknown except for its accumulation in cells under hyperosmotic stress, suggesting a role in the water stress response (Meijer and Munnik, 2003; Zonia and Munnik, 2004).

Plants contain only trace amounts of PtdIns5P, but elevated PtdIns5P has been reported within minutes of salt stress in *Chlamydomonas* and in cultured carrot cells, as well as in tomato, pea, and alfalfa plant tissues (Meijer *et al.*, 2001). Transcriptome analysis has revealed that PtdIns4P and PtdIns5P trigger distinct specific responses of *Arabidopsis thaliana* genes (Alvarez-Venegas *et al.*, 2006a). However, studies of PtdIns5P in plants have been hampered by its low endogenous levels under non-stressed conditions and by the difficulty in separating PtdIns5P from PtdIns4P by either TLC or HPLC (Pical *et al.*, 1999; Meijer *et al.*, 2001; Ndamukong *et al.*, 2010). Using a radioactive mass assay (Jones *et al.*, 2009), we have positively identified PtdIns5P in *A. thaliana* cells and have demonstrated that endogenous PtdIns5P increases upon both dehydration and hypotonic stresses (Ndamukong *et al.*, 2010). The ARABIDOPSIS HOMOLOG OF TRITHORAX 1 (ATX1) specifically binds PtdIns5P through its PHD domain. The ATX1 protein is a histone modifier responsible for the histone H3 lysine 4 trimethylation (H3K4me3) of its target genes, a modification linked to actively transcribed genes (Avramova, 2009; Cazzonelli *et al.*, 2009). Elevated PtdIns5P negatively affects ATX1 activity at a co-regulated set of genes (Alvarez-Venegas *et al.*, 2006b) by restricting access of ATX1 to chromatin (Ndamukong *et al.*, 2010).

The emergence of PtdIns5P as a messenger in a signaling pathway that links lipid signaling with chromatin (epigenetic) regulation in plant cells underlies our interest in activities generating cellular PtdIns5P. The canonical pathway to its production is through dephosphorylation of PtdIns(3,5)P_2 by the MTMs (Tronchere *et al.*, 2004; Ding *et al.*, 2009). Two other phosphatases can produce PtdIns5P from PtdIns(4,5)P_2 (Ungewickell *et al.*, 2005) in human cells, but this route is thought to be exclusive to metazoans (Lecompte *et al.*, 2008).

Myotubularins form a family of about 15 members in humans (Vergne and Deretic, 2010). A remarkable feature is that about half of the family members have conserved mutations in the amino acids in the catalytic site rendering them enzymatically inactive. Inactive MTMs also have physiological roles, most likely as regulators of the active enzymes (Begley and Dixon, 2005). Inactive MTMs are found early in eukaryotic evolution, present in multicellular as well as unicellular forms of life, like *Dictyostelium discoideum*, *Entamoeba histolytica* and *Monosiga brevicollis* (Kerk and Moorhead, 2010). The catalytically active MTMs show identical substrate specificity *in vitro*, high sequence homology (especially within a subgroup) and an overall ubiquitous expression, but, nonetheless, loss-of-function of a particular MTM leads to specific disease phenotypes indicating that they possess specific roles. Thus, mutations in MTM1 lead to myotubular myopathy, a severe X-linked congenital myopathy characterized by the abnormal positioning of nuclei within muscle fibers (Laporte *et al.*,1996), while mutations in the highly similar MTMR2 affect the peripheral nervous system causing the Charcot–Marie–Tooth neuropathy type 4B1 (CMT4B1) (Bolino *et al.*, 2000; Kim *et al.*, 2002).

Despite the high evolutionary conservation of the MTM-encoding genes in metazoans and in plants their functions have greatly diverged. Plants do not encode inactive MTMs (see further below) and it is impossible to predict a role for the plant homologs based on the phenotypes (muscular dystrophy, neuronal malfunction and leukemia) caused by MTM mutations in humans. Two genes, *ARABIDOPSIS MYOTUBULARIN1* (*AtMTM1*) and *ARABIDOPSIS MYOTUBULARIN2* (*AtMTM2*), originating from a segmental chromosomal duplication encode MTM homologs in Arabidopsis. Structurally, the genes are highly similar with conserved biochemically active catalytic sites. According to current theoretical models, the two genes may either have redundant functions or may have entered a path of divergence that could lead to subfunctionalization (Kondra-

shov *et al.*, 2005). Here, we analyze the functions of the two genes in parallel and demonstrate that AtMTM2, in contrast to AtMTM1 (Ding *et al.*, 2009; Ndamukong *et al.*, 2010), is not involved in the plant's resistance to dehydration stress and is not essential for the endogenous elevation of PtdIns5P under dehydration stress. In addition, AtMTM1 and AtMTM2 display different affinities for the two common substrates, show different distribution patterns in the cell and differentially affect the Arabidopsis dehydration stress-responding transcriptome.

Thereby, despite preserving identical catalytic sites and largely overlapping domains of expression in the plant, the two genes have functionally diverged, suggesting that each gene has evolved along a different evolutionary path. The evolution of the MTM genes in the plant lineage is also analyzed.

RESULTS

Origin of the two Arabidopsis MTM genes

The *A. thaliana* genes *At3g10550* and *At5g04540* (named *AtMTM1* and *AtMTM2*, respectively), encode myotubularin (AtMTM) homologs (Figure 1a). The two proteins are 77% identical, 85% similar to each other and to the human MTMR2 (34% identical, 49% similar, 4×10^{-81}). The proteins contain a conserved PH-GRAM domain, found in a number of membrane-interacting proteins, a putative membrane-targeting motif (RID, the Rac-induced recruitment domain), as well as the catalytic domain and the SET-interacting domain (SID) (Laporte *et al.*, 2002; Begley *et al.*, 2003). The PTP/DSPs catalytic domains carry the consensus motif of DSP-class I-phosphatases but the unique presence of the SID and the RID motifs is a signature feature for the MTMs.

Detailed analysis of the locations of *At3g10540* and *At5g04510* genes on chromosomes 3 and 5, respectively, indicated that a relatively small region encompassing the *AtMTM* gene and a downstream gene encoding a 3′-phosphatidylinositol phosphate-dependent kinase is conserved on both chromosomes. The *At3g10540* gene is adjacent to *AtMTM1*, while *At5g04510* is positioned two genes downstream of *AtMTM2* on chromosome 5 (Figure 1b). The results illustrate a segmental chromosomal duplication of the DNA regions involving the *AtMTM1*/*AtMTM2* genes and the neighboring sequences encoding a kinase using the same substrate, PtdIns3P.

Duplicated genes may remain as redundant functions or may evolve along separate paths to adapt for different functions. The fate of the duplicated phosphoinositide 3-phosphatase genes in Arabidopsis was analyzed next.

Domains of *AtMTM1* and *AtMTM2* expression in *A. thaliana*

Transgenic lines expressing the β-glucuronidase (GUS) coding sequence under the *AtMTM1* or *AtMTM2* promoters (see Experimental procedures) were generated, and a dozen independently transformed lines (for each *AtMTM1* and *AtMTM2*) were examined for GUS expression at different developmental stages. By being expressed in different temporal and/or spatial manners, redundant genes may acquire functional divergence (Pickett and Meeks-Wagner, 1995).

Overall, the promoters of both genes outlined similar domains of expression activity (Figure 2a). Young seedlings displayed very strong staining throughout, and at the

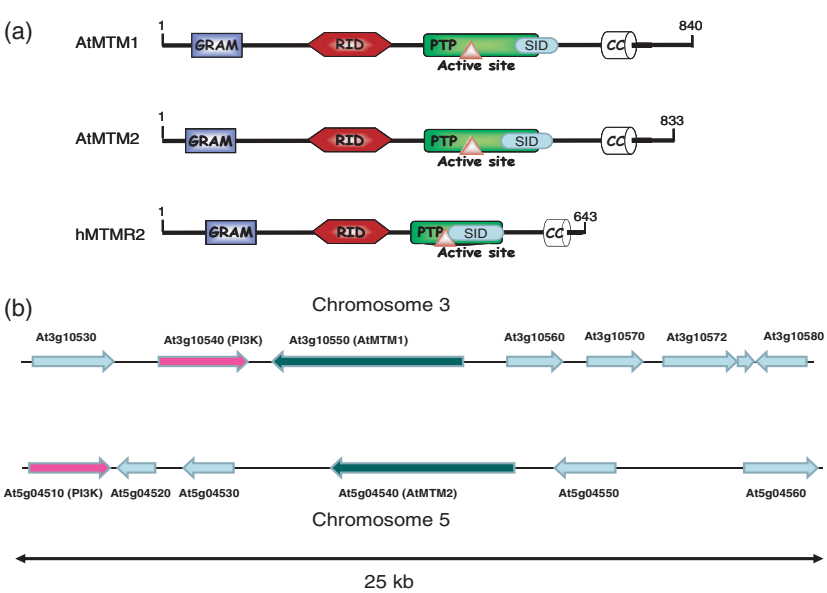

Figure 1. Structure of the *Arabidopsis thaliana* myotubularins AtMTM1 and AtMTM2, showing segmental duplication of the *At3g10550* and *At5g04540* gene containing regions on chromosomes 3 and 5.
(a) Structures of AtMTM1 and AtMTM2 and of the human homolog hMTMR2. The conserved domains, GRAM (glycosyltransferase, Rab-like GTPase activator and myotubularins), RID (Rac-induced membrane-binding domain), the phosphatase (PTP) region with the catalytically active sites and SID (the SET-interacting domain) are indicated. The Arabidopsis and the human myotubularins contain predicted coil-coil (CC) domains, albeit the sequences are not as highly conserved between the plant and the human proteins.
(b) Locations of the *At3g10550* and *At5g04540* genes on chromosomes 3 and 5, respectively. The genes encoding conserved 3′-PIP- dependent kinases are shown in pink. Shaded areas illustrate conserved DNA sequences on the two chromosomes. The two genes between the *AtMTM2* and the 3′-PtdInsP-dependent kinase on chromosome 5 (*At5g04530* and *At5g04520)* encode a KCS19 (3-ketoacyl-CoA synthase19) and a hypothetical protein, respectively.

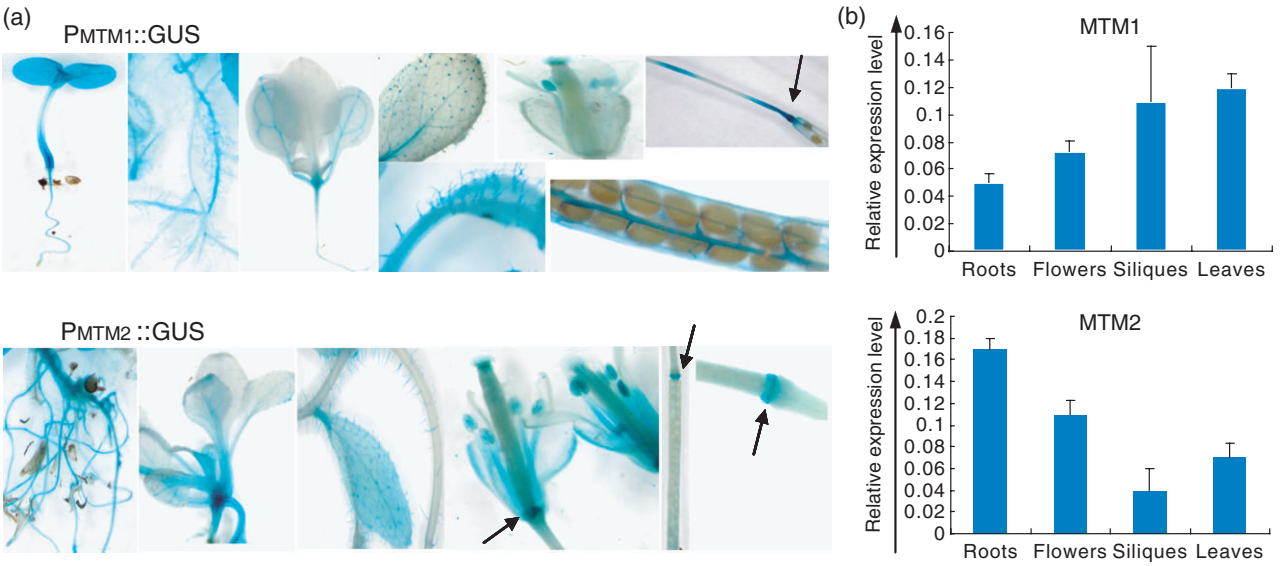

Figure 2. Domains of *AtMTM1* and *AtMTM2* expression in Arabidopsis plants.
(a) Transgenic lines expressing the β-glucuronidase (GUS) coding sequence under the promoters for the *AtMTM1* (P_{MTM1}::GUS) or *AtMTM2* (P_{MTM2}::GUS) genes, respectively. Arrows point to peduncle cells. Specific expression of P_{MTM1}::GUS in the septum and funiculi of developing siliques (see text).
(b) Tissue-specific *AtMTM1* and *AtMTM2* expression. The mRNA levels are measured by real-time quantitative RT-PCR. Bars are standard deviations from three independent measurements.

tip of the growing shoot meristems in particular. Later in development, expression was observed in roots and in aerial parts. The staining intensity was disperse and weak in the rosette leaves, but remained well-pronounced in the trichomes and in cotyledon veins. The staining in the flowers was also weak, but strong staining was displayed in cells at organ–stem junctions (Figure 2a). In particular, the activity of the *AtMTM2* promoter was restricted to the developing peduncle (Figure 2a, arrows), while active *AtMTM1* promoter domains were diffuse, appearing as patches along the stem but also concentrated at the peduncle. We note the activity of the *AtMTM1* promoter in the septum and the funiculi of the developing siliques (Figure 2a).

Collectively, GUS staining suggested largely overlapping expression patterns for the *AtMTM1* and *AtMTM2* genes *in planta*. However, as transgenic GUS expression reflects not only the strength of the promoters but is also dependent on the sites of insertion and on effects of potential regulatory sequences located in introns, we analyzed the mRNA levels of *AtMTM1* and *AtMTM2* by real-time quantitative PCR (qPCR) (Figure 2b). The results confirmed that *AtMTM1* and *AtMTM2* were ubiquitously expressed. However, in mature plants higher *AtMTM1* transcripts were found in leaves and siliques, while *AtMTM2* transcript levels were more abundant in the roots (Figure 2b). The tissue-specific intensity of each gene's expression suggested that *AtMTM1* and *AtMTM2* were differentially regulated in specific cell types.

AtMTM1 and *AtMTM2* expression in response to dehydration

Our earlier studies implicated AtMTM1 in the Arabidopsis response to dehydration stress (Ding *et al.*, 2009; Ndamukong *et al.*, 2010). Whether AtMTM2 was involved was examined by quantitative qRT-PCR assays of *AtMTM2* mRNAs produced under both watered and dehydration stress conditions (see Experimental procedures). In contrast to *AtMTM1*, the *AtMTM2* transcripts did not increase during dehydration stress (Figure 3a). Increased expression in hydathodes of P_{AtMTM1}::GUS, but not of P_{AtMTM2}::GUS (Figure 3b) illustrates cell-specific activation of the *AtMTM1* promoter upon dehydration stress.

Loss of *AtMTM1* and *AtMTM2* functions in *mtm1* and *mtm2* mutant plants

Whether the high structural similarity between *AtMTM1* and *AtMTM2* resulted in functional redundancy was examined in *Ti*-insertion lines (see Experimental procedures). Among the seven available lines only three germinated successfully and contained the expected *Ti*-insertions: SALK_029185, SALK_073312 (referred to as *mtm1-1* and *mtm1-2*, respectively) and SALK_147282 (*mtm2*) (SF1 A). Homozygous mutant lines were selected, verified by genotyping, and tested for producing mRNAs (specific primers are shown in Table S1 in the Supporting Information).

No full-size *AtMTM1*mRNA was detected in the *mtm1-1* or *mtm1-2* backgrounds, but low-level *AtMTM1*mRNA was

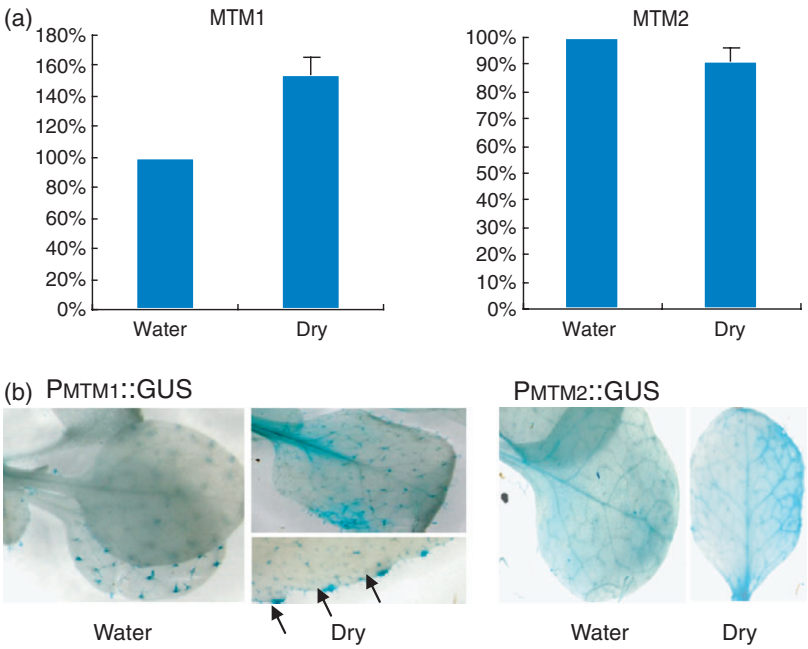

Figure 3. Expression of *AtMTM1* and *AtMTM2* in response to dehydration stress.
(a) Relative *AtMTM1* or *AtMTM2* transcripts under non-stressed (water) and stressed (dry) conditions quantified by real-time PCR shown as a percentage of the non-stressed watered levels. Bars are standard deviations from three independent measurements.
(b) Activity of the *AtMTM1* or *AtMTM2* promoters under watered or dry conditions, respectively. Increased expression of PAtMTM1::GUS upon dehydration stress in hydathodes (indicated by arrows).

produced in the SALK_073312 line (*mtm1-2*) containing a *Ti*-DNA insertion in the 3′ untranslated region (UTR) (SF1B). No *AtMTM2m* RNA was detected in the SALK_147282 line. Importantly, the *AtMTM1* expression in the *mtm2* mutant background and of *AtMTM2* in *mtm1-1* and *mtm1-2* backgrounds were similar to their respective levels in the wild type (Col-0), indicating that the *AtMTM1* and *AtMTM2* genes did not influence each other's transcriptional behavior.

Plants were grown under normal conditions and scored for possible deviations from the wild type. No phenotypes were detected in any mutant line from germination to seed-producing stages under greenhouse conditions (12 h light, 20°C, regular watering). Given that no transcripts were produced in the *mtm2* and *mtm1-1* backgrounds, these mutants were considered null. Lack of a phenotype, therefore, suggested functional redundancy for *AtMTM1* and *AtMTM2*, a possibility that was further explored.

Responses to soil-water-withdrawal dehydration stress

The different transcriptional responses from the *AtMTM1* and *AtMTM* genes during dehydration stress suggested that *mtm1* and *mtm2* mutant plants might show different sensitivity to dehydration. Three-week-old Col-0 and homozygous *mtm1-1*, *mtm1-2* and *mtm2* plants were tested for their resistance to soil-water withdrawal. After 19 days without watering, the Col-0 plants were severely dehydrated, while the *mtm1-1* mutants displayed increased resistance (Figure 4). The response of the *mtm2* plants was similar to the Col-0 plants, illustrating a major contrast with *mtm1-1* mutants.

The different involvement of the two genes in the dehydration stress response was further confirmed by generating and testing double mutants. The double *mtm1-1*$^{-/-}$/*mtm2*$^{-/-}$ mutants showed stress resistance similar to the single *mtm1-1* mutants (Figure 4). We conclude that loss of *AtMTM1* function, but not of *AtMTM2*, conferred increased resistance to soil-water-deficit stress. However, neither the *mtm1-2* mutants nor the *mtm1-2*/*mtm2* mutants showed responses significantly different from the wild type (*SF 2*) and were not included in subsequent studies.

Differential roles of *AtMTM1* and *AtMTM2* at the genome level

To distinguish the roles of *AtMTM1* and *AtMTM2* at the global genome level, we performed transcription profiling in the *mtm1* or *mtm2* backgrounds under non-stressed and during dehydration stress conditions. We assumed that

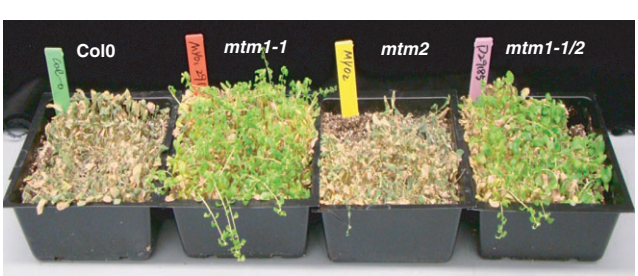

Figure 4. Responses to soil water-withdrawal stress of plants from different genetic backgrounds.
Plants from the wild type (Col-0) and from the homozygous *mtm1-1*, *mtm2* and *mtm1-1*/*mtm2* backgrounds after 19 days without watering.

AtMTM1 and AtMTM2 participate in pathway(s) that transfer gene regulatory signals to the nucleus and do not imply that AtMTM1 or AtMTM2 directly regulate gene expression. Cluster (overlap) analyses are among the best available tools for outlining common, or partially overlapping, pathways. Largely overlapping gene sets from the *mtm1* and *mtm2* data would be consistent with redundant *AtMTM1* and *AtMTM2* functions. The gene expression data are interpreted within this context.

Affymetrix gene chips (ATH1 Genome Arrays, with ~24 000 Arabidopsis genes) were used in whole-genome expression analysis of *mtm1-1* and *mtm2* homozygous mutant plants. The RNA was isolated from rosette leaves of 3-week-old mutant and Col-0 plants in two independent biological replicates. Plants from the respective backgrounds grown under watered conditions were used as controls for dehydration-stressed plants. These experiments were performed as part of a larger experiment involving two additional mutant backgrounds (*atx1* and *OX-AtMTM1* plants). Detailed conditions and microarray data analyses and validation have already been published (Ding *et al.*, 2009; see also Experimental procedures).

Under watered conditions, 27 genes significantly changed expression in the *mtm1* background, while none changed expression in *mtm2* background (ST 2). After dehydration stress, transcripts of 134 genes in *mtm1* plants deviated significantly (73 up-regulated, 61 down-regulated) from the dehydration-stressed Col-0 plants. The majority of these genes included biotic, abiotic and heat shock stress-response genes (26 genes) and transcription factors (23, six belonging to the Myb family), in addition to metabolic and membrane-wall associated functions (ST 3). In contrast, only four genes were differentially expressed (down-regulated) in the stressed *mtm2* plants: three of these four genes were also present in the down-regulated *mtm1* fraction including the *ACS7* gene (*At4g26200*) involved in ethylene biosynthesis and in responses to abscisic acid (ABA) (Wang *et al.*, 2005), a gene (*At2g02060*) encoding a transcription factor from the Myb family, and the *At5g12030* gene encoding a cytosolic small heat shock protein with chaperone activity that is induced by heat and osmotic stress. The fourth gene identified in the *mtm2* deregulated gene-set was the *AtMTM2* gene, capturing the lost signal from *AtMTM2* transcripts in the SALK_147282 (see Figure S1b).

Collectively, the results illustrated differential roles for AtMTM1 and AtMTM2 in the dehydration stress-responding transcriptome in *A. thaliana*, suggesting that AtMTM1 and AtMTM2 participate in separate cellular signaling pathways.

Contributions by AtMTM1 and AtMTM2 to the endogenous PtdIns5P pool

To determine whether the lesser involvement of AtMTM2 in the dehydration stress response influenced the endogenous PtdIns5P level in Arabidopsis cells differently from AtMTM1 during the response to dehydration stress, we quantitatively determined PtdIns5P, as previously described (Jones *et al.*, 2009; see Supplementary Methods for details).

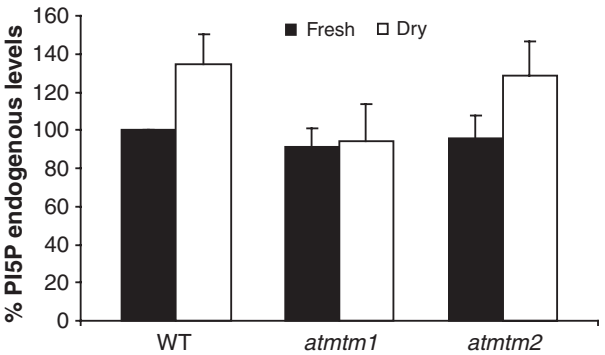

Figure 5. Contributions by AtMTM1 and AtMTM2 to the cellular phosphatidylinositol 5-phosphate (PtdIns5P) level.
The PtdIns5P levels in the wild type (WT) (Col-0) and in homozygous mutant *mtm1* or *mtm2* plants under non-stressed (black columns) or after dehydration stress conditions (white columns). Data are expressed as mean ± SD. Statistical analysis by a two-way ANOVA (ANOVA-N) confirmed no difference in the stress responses of *mtm2* and the wild type ($P = 0.3064$); the difference in PtdIns5P production in wild-type and *mtm1* mutant cells under stress is significant ($P = 0.0185$).

Under non-stressed (watered) conditions, the loss of AtMTM2-function did not significantly affect the PtdIns5P level in *mtm2* mutant cells. Upon dehydration, PtdIns5P in the *mtm2* background increased to a degree comparable to the wild-type cells, indicating that AtMTM2 depletion did not significantly affect the production of PtdIns5P (Figure 5). Thereby, loss of MTM2 function did not cause a response different from the wild type. Statistical analysis by a two-way ANOVA (ANOVA-N) confirmed no difference in the stress responses of *mtm2* and wild type ($P = 0.3064$). In contrast, *mtm1* mutant cells showed lack of PtdIns5P increase in response to dehydration. The difference in PtdIns5P production in wild-type and *mtm1* mutant cells under stress is significant ($P = 0.0185$), indicating that AtMTM1 mediates the increase in PtdIns5P during the response to dehydration stress. These results are in full agreement with our earlier results showing that *AtMTM1* overexpression elevated the endogenous PtdIns5P (Ndamukong *et al.*, 2010). Neither MTM was affecting the low basal level of endogenous PtdIns5P in hydrated cells. However, their differential involvement in PtdIns5P levels under dehydration stress provided strong evidence that AtMTM1, but not AtMTM2, function was required for the PtdIns5P response. The question of whether AtMTM2 has preserved its enzyme activity was explored next.

Phosphatase activities of *AtMTM1* and *AtMTM2*

The RID, the PTP/DSPs and SID define the catalytic domain of MTMs, and all essential amino acids determined to be critical

for MTMR2 function (Begley *et al.*, 2003; Robinson and Dixon, 2006) are conserved in the two Arabidopsis homologs. AtMTM1 has 3′-phosphatase activity with both PtdIns3,5P_2 and PtdIns3P substrates (Ding *et al.*, 2009). Here, we determined that AtMTM2 was also catalytically active. A recombinant glutathione *S*-transferase (GST)-tagged construct containing the AtMTM2 RID-PTP-SID domain regions was tested for enzyme activity by the malachite green assay (Martin *et al.*, 1985; Schaletzky *et al.*, 2003). A recombinant GST-tagged AtMTM1 protein expressed, purified and tested in parallel was used as a positive control. The kinetic parameters of the phosphatase activity of both AtMTM1 and AtMTM2 were measured with both substrates. Based on the Lineweaver–Burk curves (Figure 6a–d), for AtMTM2 we estimated a slightly higher affinity (K_m = 158.2 μM) and activity (V_{max} = 28.4 pmol min^{-1} mg^{-1}) with Ptdins3,5P_2 than with Ptdins3P (K_m = 216.5 μM, V_{max} = 15.4 pmol min^{-1} mg^{-1}). A preference for Ptdins3,5P_2 as a substrate versus Ptdins3P was also shown by AtMTM1 (K_m = 146 μM and V_{max} = 142.6 pmol min^{-1} mg^{-1} with Ptdins3,5P_2; K_m = 201.7 μM and V_{max} = 94.3 pmol min^{-1} $mg^{-1}$with Ptdins3P). Thereby, AtMTM2 is a catalytically active phosphatase that shows similar substrate-binding specificity as AtMTM1 (the K_m values for each substrate are comparable for the two enzymes). Important differences, however, are the lower dephosphorylation rates of both substrates by AtMTM2 compared with AtMTM1 (Figure 6e). Thereby, although enzymatically active AtMTM2 shows a much lower activity than AtMTM1.

Subcellular localization

Next, we compared the subcellular localization of the two plant MTMR proteins. Fluorescently-tagged tMTM1 is seen mostly as granular particles of varying abundance and size at the cell periphery and throughout the cytoplasm (Figure 7a). This AtMTM1 distribution pattern is highly reproducible, as we have seen in our earlier studies (Ndamukong *et al.*, 2010). The nature of these particulate structures remains unclear but, interestingly, cytoplasmic 'punctate elements' of unknown origins have also been reported for mammalian MTMs (Kim *et al.*, 2002; Laporte *et al.*, 2002; Nandukar *et al.*, 2003).

A GFP-AtMTM2 fusion protein transiently expressed in tobacco leaf cells displayed a visibly different distribution pattern: a diffuse green signal appeared throughout the cytoplasm but the signal was highly concentrated at the peripheral lobes of the epidermal cells (Figure 7b,c). Interestingly, no granular particulate structures were observed in AtMTM2 in transformed cells, suggesting that AtMTM1 and AtMTM2 associate with different substructures inside cells.

Loss of AtMTM2 subcellular localization resulting from a single amino acid mutation

One of our AtMTM2-GFP constructs showed a different intracellular distribution similar to the distribution of GFP alone (Figure 7d,e). The signal distribution suggested to us that AtMTM2 in this particular construct had lost its usual localization in the dense regions at the periphery. The subsequent sequencing of the construct revealed a single amino acid substitution (L250W). This mutation appears to be linked to this different localization and/or a lost ability of AtMTM2 to associate with specific cellular substructures.

Evolution of plant MTMs

Myotubularin-encoding genes have proliferated in the animal lineage as five copies have been identified in the

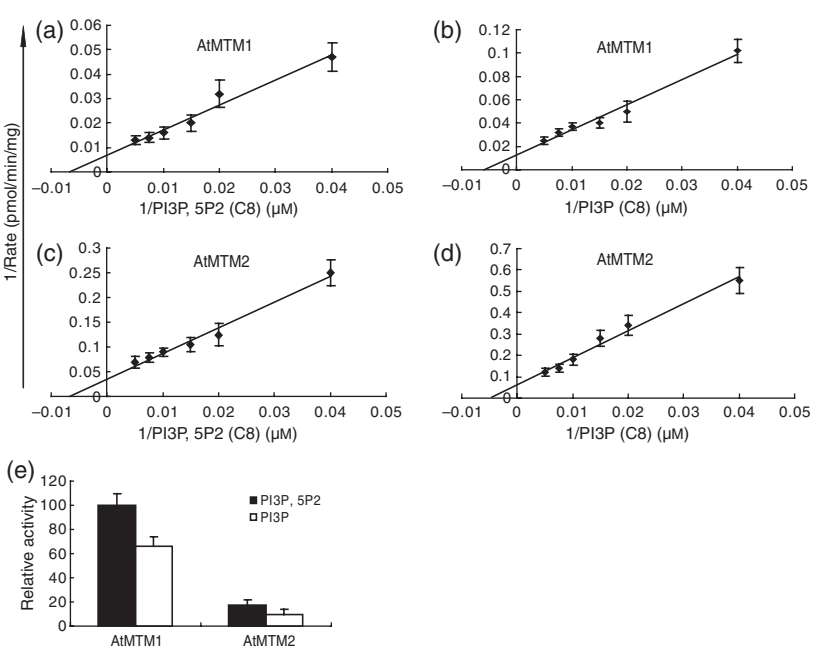

Figure 6. Enzyme activities of AtMTM1 and AtMTM2 with phosphatidylinositol (3,5)-bisphosphate [PtdIns(3,5)P_2] and phosphatidylinositol 3-phosphate (PtdIns3P) substrates.
Phosphoinositide 3′-phosphatase activity of the two proteins with PtdIns3P and PtdIns(3,5)P_2 as substrates.
(a–d) Lineweaver–Burk curves for PtdIns(3,5)P_2 and PtdIns3P used as substrates with AtMTM1 (a, b) and with AtMTM2 (c, d). See text for kinetic parameters.
(e) Relative activity of AtMTM2 with both substrates versus AtMTM1.

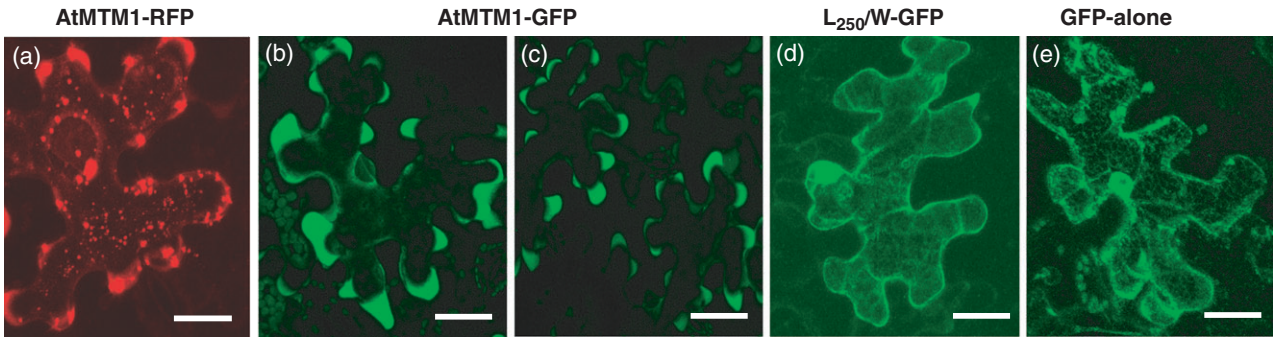

Figure 7. Subcellular localization of fluorescently tagged Arabidopsis myotubularins (MTMs).
(a) Red fluorescent protein (RFP)-AtMTM1 fusion protein expressed in tobacco cells associated with numerous granular structures.
(b, c) GFP-tagged AtMTM2 expressed in tobacco cells seen under different exposures. The strongly concentrated signal localizes at the cell periphery. No particulate structures were seen associated with AtMTM2.
(d) An altered AtMTM2-GFP distribution of the L250W AtMTM2-GFP construct.
(e) Distribution of a control (empty) GFP. Bars are 20 nm.

genome of the unicellular metazoan relative *M. brevicollis* (Kerk and Moorhead, 2010). Interestingly, there are no MTMR genes in the genomes of the green algae, including *Chlamydomonas*, which shares a common ancestor with green plants (Merchant *et al.*, 2007; Herron *et al.*, 2009). To trace the evolution of MTM genes in the plant lineage, we analyzed several fully sequenced plant genomes.

The moss *Physcomitrella patens* is an extant relative of the earliest land plants considered half-way between algae and angiosperms (Quatrano *et al.*, 2007). The lycophyte *Selaginella moellendorffii*, with no true roots and leaves, occupies an important node in the plant evolutionary tree (Hedges *et al.*, 2004). There are two MTM-type genes in the genome of the moss (Kerk and Moorhead, 2010) while one, weakly related, gene in *Selaginella* is fused with additional domains not found in any other known myotubularin (*SF 3*). The two *Physcomitrella* myotubularins have similar structures to the animal and plants proteins. The C-terminal regions of the moss proteins, however, are only weakly related to the coil–coil domain sequences of the other MTMs and belong to the Flagellar family of proteins found in eukaryotic paraflagellar rod component proteins. The moss proteins are most highly related to each other, suggesting that the two copies have resulted from moss-specific gene duplication. Different genes flank the MTM gene on the respective chromosomes restricting the duplication to the MTM sequence.

There is only one copy of a MTM-encoding gene in the genomes of mono- and dicotyledonous plants tested here, with the notable exception of Arabidopsis (see above). The gene encoding a MTM in rice (*Os08g05567*) is homologous to two adjacently positioned genes (SORDIDRAFT_07g024440 and SORDIDRAFT_07g024450) on the sorghum chromosome, encoding the N-terminal and C-terminal halves of the MTM protein. Together, the two genes encode a full-length MTM, highly related to the rice and to all plant MTMs. Distribution of the coding sequences into two sorghum genes, thereby, might reflect an annotation problem. The chromosomal regions upstream of the rice and sorghum MTM genes are divergent, sharing only one common gene. However, there is a remarkable collinearity of the regions downstream of the MTM genes conserved in the evolution of the two grass genomes (Figure 8a).

As in monocots, single genes encode MTM homologs in representatives of the eudicots, *Populus trichocarpa* (poplar), *Vitis vinifera* (grapevine) and *Ricinus communis*. Analyses of the poplar and the grapevine genomes have suggested that these species have captured traits common to all eurosids (Tuskan *et al.*, 2006; Jaillon *et al.*, 2007). Furthermore, the collinearity downstream from the respective MTM gene is well-preserved between the chromosomes of these three species (Figure 8b). The need to thrive in fixed locations over centuries under changing environmental conditions and biotic and abiotic stresses sets these species apart from the shorter-lived herbaceous (*Ricinus* and *Arabidopsis*) plants.

DISCUSSION

The *A. thaliana* MTM genes *AtMTM1* and *AtMTM2* have originated from a segmental duplication involving the MTM-encoding and the adjacent PIP3K-encoding sequences on chromosomes 3 and 5. Accordingly, the two *A. thaliana* MTMs are most highly related among themselves. Analysis of the MTM-related genes in the closely related *Arabidopsis lyrata* revealed that homologs of both *AtMTM1* and *AtMTM2* genes are present in conserved collinear regions in the respective chromosomes. The two Arabidopsis species split about 13 million years ago (Beilstein *et al.*, 2010) and the conserved gene duplication and collinearity that are different from the collinear regions in *Populus*, *Vitis* and *Ricinus* suggests that the duplication and rearrangement at the MTM locus occurred after separation of the Arabidopsis lineage

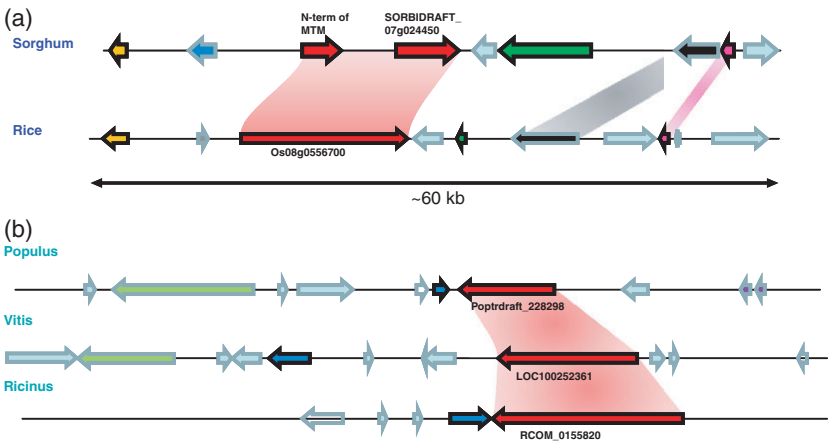

Figure 8. Collinearity of the myotubularin (MTM) gene-containing regions in plants.
(a) Single copies of myotubularin-encoding genes are found in largely collinear regions (shaded area) on the sorghum and rice chromosomes. The two adjacent genes (SORDIDRAFT_07g024440 and SORDIDRAFT_07g024450) on the sorghum chromosome encode the N-terminal and C-terminal halves of the myotubularin protein. The collinearity here also extends to regions upstream of the myotubularin gene including a gene for a putative Peptidase_C1A protein. Homologous genes at the collinear regions are shown in the same colors.
(b) Single genes encode myotubularin homologs in genomes of the dicotyledonous *Populus trichocarpa*, *Vitis vinifera* and *Ricinus communis*. Collinearity downstream from the respective myotubularin gene is well preserved between the chromosomes of the three species. No data are available for the genes in the neighborhood of the myotubularin gene in *Ricinus* preventing a more extended analysis of the chromosomal region. Homologous genes at the collinear regions are shown in the same colors.

from the other dicots. The collinearity downstream of the single sorghum and rice MTM genes (Figure 8a), as well as the extended collinear regions containing the myotubularin genes in *Populus*, *Vitis* and *Ricinus* (Figure 8b) illustrate a remarkably stable arrangement of these regions inherited from the ancestral monocot or dicot chromosomes, respectively.

Plants do not carry genes encoding inactive MTMs. In contrast, genes encoding both active and inactive MTMs have proliferated in the basal metazoan group. There are five genes in *M. brevicollis* and eight in *Trichoplax adhearens* (Kerk and Moorhead, 2010). It is quite surprising, then, that in species ancestral to the green plants (*Chlamydomonas reinhardtii*, *Volvox carterii*, *Ostreococcus tauri*, *Ostreococcus lucimarinus*) there are no MTM-related genes. However, the appearance of MTM-encoding genes in the moss suggests that a primordial gene present in the last eukaryotic ancestor (LECA) has been lost at the separation of the algal lineages but has survived in the ancestor of the extant green plant lineage. Furthermore, in the moss and in Arabidopsis the MTM gene has undergone species-specific duplications.

Despite the high degree of structural similarity between the MTMs from animal and plant origins, the genes in each lineage have evolved along different paths and the encoded proteins have acquired distinct functions. The different requirements for MTM activities in the plant and animal systems is illustrated by our results here, showing that, in contrast to MTM deficiency in humans (Laporte *et al.*, 2003; Pendaries *et al.*, 2005), loss of MTM function does not cause severe phenotypes in Arabidopsis under regular, non-stressed, conditions.

Recognized as a force in the evolution of biological diversity, duplicated genes may remain as redundant functions or may acquire specialized roles. In contrast to single copies in angiosperms, there are two Arabidopsis genes that have diverged in function. The following results support this conclusion: First, despite being expressed in the same tissues, *AtMTM1* and *AtMTM2* mRNAs display tissue-specific accumulation (Figure 2a,b). Second, *AtMTM1* and *AtMTM2* show distinct transcriptional responses and different roles in the response to drought exposure (Figures 3a and 4). The higher resistance of *mtm1* mutants to water-withdrawal stress is mirrored by the decreased resistance of plants overexpressing AtMTM1 (Ding *et al.*, 2009) providing independent support to the conclusion that AtMTM1 is involved in the dehydration stress response in Arabidopsis. One possible scenario is through effects on the endogenous PtdIns3P levels, which was shown to stimulate stomatal closures (Choi *et al.*, 2008). Third, critical evidence for functional divergence between *AtMTM1* and *AtMTM2* emerged from the transcriptome analysis of the respective mutants under watered and dehydration stress conditions. While AtMTM1 loss of function affected transcription from 134 dehydration-response genes, only three genes were misregulated in *mtm2* cells. These results are important, as they illustrate very different roles at the global genome level. Fourth, different contributions of AtMTM1 and AtMTM2 to the endogenous PtdIns5P level provided yet further evidence for distinct functions: in contrast to AtMTM2, only AtMTM1 significantly affected the cellular PtdIns5P level under dehydration stress (Figure 5). Both Arabidopsis proteins are enzymatically active, but the lower AtMTM2 activity

(Figure 6a–e) could account partially for this result. Lack of *AtMTM2* induction by dehydration stress is another factor. It will be interesting to establish whether *AtMTM2* transcription would be stimulated by a different type of a stress. Different PtdIns5P levels produced under stress could be related with an association of AtMTM1 and AtMTM2 with different subcellular membranes so that each phosphatase could be accessing and working on different lipid pools. This possibility has been used to explain the unique functional roles of human MTMs despite identical (*in vitro*) substrate specificity, sequence homology and ubiquitous expression (Pendaries *et al.*, 2003; Laporte *et al.*, 2003; Cao *et al.,* 2008). Their highly specific roles are thought to be determined by the nature of the membranes they are attached to and by the nature of the phosphoinositide anchored on specific membranes, as PtdInsP isomers are viewed as docking sites that 'attract' signaling proteins to specific membranes 'guiding' them to their substrates (Robinson and Dixon, 2006). In plants, a phosphoinositide transiently increasing under hyperosmotic stress represents a physiological pool different from the constitutive phosphoinositide pools of non-challenged plants (König *et al.*, 2007), and thus stress-inducible and constitutive phosphoinositide pools may involve different enzyme activities (König *et al.*, 2007, 2008). Fifth, AtMTM1 and AtMTM2 show distinct localization patterns inside cells, as only AtMTM1 appears in granulate 'punctate structures'. It would be of great interest to determine their nature as well as that of the structures associating with AtMTM2.

For the most part, the biological functions of MTMs are still poorly understood, but association with specific membranes is considered critical for their function and loss of this association is linked to various diseases (Skwarek and Boulianne, 2009). The RID domain, conserved in all MTMs, is responsible for the membrane location of MTM1 in human cells (Laporte *et al.*, 2002), and this region is particularly rich in mutations found in various diseases (Begley and Dixon, 2005). In this regard, it is interesting to note the L250W substitution in the RID domain of AtMTM2 resulting in a changed cellular localization of the protein (Figure 7b–e) as it may provide an example for further studies of the role of RID in MTM function.

Typically, PtdInsPs are studied in the context of their classical roles as second messengers in signal transduction. However, increasing evidence is pointing to an involvement of PtdInsPs in regulating nuclear events as well (Irvine, 2003; Jones and Divecha, 2004; Gozani *et al.*, 2005; Jones *et al.*, 2006). Phosphatidylinositol 5-phosphate binds to the PHD domain of the epigenetic factor ATX1 and negatively regulates its function (Alvarez-Venegas *et al.*, 2006a,b; Ndamukong *et al.*, 2010). Production of PtdIns5P by an active AtMTM1 is required for the cellular localization of ATX1 and a mutation in the AtMTM1 active site that affects its phosphatase activity towards PtdIns(3,5)P_2 failed to retain the ATX1-ePHD in the cytoplasm (Ndamukong *et al.*, 2010). It was found that ATX1 loss-of-function and AtMTM1 over-expressing plants responded similarly to water-deprivation stress. The ATX1 and AtMTM1 proteins co-regulate a common set of genes (Ding *et al.*, 2009), linking ATX1, AtMTM1 and PtdIns5P in a biologically relevant pathway. Importantly, there were only four misregulated genes, and no genes co-regulated with ATX1 during the Arabidopsis response to the stress.

Collectively, our results demonstrate that the two monophyletic, highly conserved Arabidopsis MTM genes have evolved along different functional paths. AtMTM1, but not AtMTM2, participates in the dehydration stress response, regulating the plant's transcriptome and the endogenous levels of PtdIns5P. The role of AtMTM2 remains unclear.

EXPERIMENTAL PROCEDURES

Plant material and selection for *AtMTM1* and *AtMTM2* insertion lines

Wild-type and mutant plants were grown in soil under the same controlled daylight environmental conditions (12 h light, 20°C, regular watering). For soil-dehydration stress, watering of 3-week-old plants was terminated for 19 days. Four *Ti*-insertion lines (José *et al.*, 2003) were analyzed for *AtMTM1* function: SALK_135710, SALK_ 018481, SALK_ 073312 and SALK_029185. Three were analyzed for *AtMTM2*: SALK_147338, SALK_082030 and SALK_147282. Homozygous mutant lines were selected, verified by genotyping and tested for producing mRNAs (see Table S1 for specific primers).

Constructs

Transgenic plants were generated by transformation with binary vectors. Binary plasmids were transformed into chemically competent *Agrobacterium tumefaciens* strain C58C1 by incubating DNA with agrobacteria on ice for 5 min, freezing in liquid nitrogen for 5 min and heat shock at 37°C for 5 min. The cells were allowed to recover in growth medium with shaking for 2 h at 20°C and plated on selection medium containing rifampicin, gentamycin and a third antibiotic for plasmid selection. Agrobacteria selected for transformation were used to transform Col-0 plants using a floral dipping method as described (Clough and Bent, 1998). For cloning approaches, vectors, and primers see the Appendix S1 (Supporting Methods).

Tobacco transient assays

Transient expression of fluorescent tagged proteins was carried out as described before (Ndamukong *et al.*, 2010). Detection of expressed proteins was determined 40 h after *A. tumefaciens* mediated transformation, by laser scanning confocal microscopy using 488- and 633-nm excitation and two-channel measurement of emission, 522 nm (green/GFP) and 680 nm (red/chlorophyll). Red fluorescent protein (RFP) was detected by excitation at 540 nm and emission at 590 nm.

Real-time quantitative RT-PCR analysis

The RNA for quantitative RT-PCR was isolated with TRIzol (Invitrogen, http://www.invitrogen.com/) and purified with a RNeasy Plant Mini Kit (Qiagen, catalogue number 74903, http://www.qiagen.com/). For first-strand cDNA synthesis 8 μg total RNA was treated with DNase I, extracted with phenol and chloroform, pre-

cipitated with ethanol, followed by the addition of oligo (dT) and superscript III reverse transcriptase (Invitrogen). The RT-PCR analysis was performed using the iCycleriQ real-time PCR instrument (Bio-Rad, http://www.bio-rad.com/) and iQ SYBR Green Supermix (Bio-Rad). The relative expression of specific genes was quantified using $2^{-\Delta\Delta C_t}$ calculation, where ΔC_t is the difference in the threshold cycles of the test and housekeeping gene *ACTIN7*. The mean threshold cycle values for the genes of interest were calculated from three experiments.

The PtdIns5P mass assay

The specificity of the reactions and relevant controls were exactly as described earlier for the AtMTM1-overexpressing cells (Ndamukong *et al.*, 2010). Detailed description of the method is in Appendix S1, Supporting Methods.

Phosphatase activity

Phosphoinositide 3-phosphatase assays were performed using the malachite green assay (Martin *et al.*, 1985; Schaletzky *et al.*, 2003) with a standardized phosphatase kit (Echelon K1500 JJ-052208, http://www.echelon-inc.com/) according to the manufacture's protocol. Recombinantly expressed and affinity-column purified GST-tagged proteins were reacted with the Mono- and Di-C8 phosphoinositides (Echelon) as substrates. Phosphatase and tensin (PTEN) lipid phosphatase (Echelon, E-3000) was used as a positive control. Inorganic phosphate release was measured by a standard curve of KH_2PO_4 in distilled water (Ding *et al.*, 2009).

Microarray analysis

Affymetrix ATH1 Genome Arrays (Affymetrix, http://www.affymetrix.com/) were used for the analysis of expression of ~24 000 Arabidopsis genes in watered and in dehydration-stressed samples of the Col-0 (wild type), *mtm1*-1 and *mtm2* mutant backgrounds. All microarray analyses were performed at University of Nebraska at Lincoln's Center for Biotechnology Bioinformatics and Genomics Core Research Facilities (see Appendix S1, Supporting Methods for details).

The gene expression data from the analysis of *mtm1* and *mtm2* have been deposited at the NCBI Gene Expression Omnibus with series number GSE15577.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. *Ti*-insertion lines for the *AtMTM1* and *AtMTM2* loci.

Figure S2. Response to withdrawal of soil water of *mtm1-2* mutant plants.

Figure S3. Structure of the two myotubularins from the moss (*Physcomitrella*) and from *Selaginella*.

Table S1. Primers used for the various cloning and analytical procedures.

Table S2. Genes with up-regulated or down-regulated transcription in the mtm1 mutant background versus Col0 in the watered state.

Table S3. Genes with up-regulated or down-regulated transcription in the mtm1 mutant background in response to dehydration stress

Appendix S1. Methods: Detailed description of the constructs used, of the phosphatidylinositol 5-phosphate (PtdIns5P) mass assay and the microarray analyses.

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