Magnesium Deficiency Phenotypes upon Multiple Knockout of Arabidopsis thaliana MRS2 Clade B Genes Can be Ameliorated by Concomitantly Reduced Calcium Supply

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Plant MRS2 membrane protein family members have been shown to play important roles in magnesium uptake and homeostasis. Single and double knockouts for two Arabidopsis thaliana genes, AtMRS2-1 and AtMRS2-5, have previously not shown significant phenotypes even under limiting Mg2+ supply although both are strongly expressed already in early seedlings. Together with AtMRS2-10, these genes form clade B of the AtMRS2 gene family. We now succeeded in obtaining homozygous AtMRS2-1/10 double and AtMRS2-1/5/10 triple knockout lines after selection under increased magnesium supply. Although wilting early, both new mutant lines develop fully and are also fertile under standard magnesium supply, but show severe developmental retardation under limiting Mg2+ concentrations.

To investigate nutrient dependency of germination and seedling development under various conditions, including variable supplies of Mg2+, Ca2+, Zn2+, Mn2+, Co2+, Cd2+ and Cu2+, in a reproducible and economical way, we employed a small-scale liquid culturing system in 24-well plate set-ups. This allowed the growth and monitoring of individual plantlets of different mutant lines under several nutritional conditions in parallel, and the scoring and statistical evaluation of developmental stages and biomass accumulation. Detrimental effects of higher concentrations of these elements were similar in mutants and the wild type. However, growth retardation phenotypes seen upon hydroponic cultivation under low Mg2+ could be ameliorated when Ca2+ concentrations were concomitantly lowered, supporting indications for an important interplay of these two most abundant divalent cations in the nutrient homeostasis of plants.

Introduction

Despite its fundamental role as a cofactor in many biological processes, surprisingly little is as yet known about the homeostasis of Mg2+ in multicellular eukaryotes. Aside from its crucial role in a vast number of enzymatic reactions including nucleotide metabolism and the turnover of nucleic acids in transcription, splicing or replication, magnesium has an additional prominent role as the central atom in the Chl molecules of photosynthesizing organisms. In plants, a family of proteins that have alternatively been designated as MRS2 (Schock et al. 2000) or MGT proteins (Li et al. 2001) appear to play a major role in magnesium transport across diverse biological membranes. The plant MRS2/MGT proteins are structurally homologous to bacterial CorA proteins and to the yeast and fungal ALR1/ALR2, MRS2/LPE10 and MNR2 counterparts located in the plasmalemma, inner mitochondrial or vacuolar membranes, respectively (Knoop et al. 2005, Pisat et al. 2009). Animal (metazoa) genomes encode only single homologs of MRS2, likewise targeted to the mitochondria (Zsurka et al. 2001), whereas flowering plants encode gene families encoding approximately 10 proteins targeted to different membranes in the plant cell.

All CorA/ALR/MRS2-like proteins are universally characterized by two C-terminal transmembrane (TM) domains, with the first consistently ending with a GMN tripeptide motif that appears to be an irreplaceable part of the entry pore for

Abbreviations: DKO, double knockout; KO, knockout; LCS, liquid culturing system; MRS, mitochondrial RNA splicing; MS, Murashige and Skoog; QTL, quantitative trait locus; SD, Siegenthaler–Depery, SEFLC, shaking Erlenmeyer flask liquid cultivation; TKO, triple knockout; TM, transmembrane; WT, wild type.
the Mg$^{2+}$ ion (Maguire and Cowan 2002, Papp-Wallace and Maguire 2007, Moomaw and Maguire 2008). A major step towards understanding the unique mechanisms of transporting Mg$^{2+}$—the largest of all hydrated but the smallest of all dehydrated biologically relevant divalent cations—are the X-ray crystallographic and additional structural studies of prokaryotic CorA proteins (Eshaghi et al. 2006, Lunin et al. 2006, Payandeh and Pai 2006, Chakrabarti et al. 2010, Dalmas et al. 2010, Guskov et al. 2012, Guskov and Eshaghi 2012, Pfoh et al. 2012). These structural studies have demonstrated that the Mg$^{2+}$-transporting channel is formed as a CorA pentamer with the pore lined by the first of the two TM domains in each monomer.

Several complementation studies have demonstrated that members of the plant MRS2 gene family can complement bacterial or yeast mutants across wide phylogenetic distances (Schock et al. 2000, Li et al. 2001, Drummond et al. 2006, Li et al. 2008, Mao et al. 2008, Chen et al. 2009, Gebert et al. 2009). The individual AtMRS2 proteins function to varying degrees in re-establishing magnesium uptake in mitochondria of the yeast MRS2 mutant (Gebert et al. 2009). Assigning individual roles for each member of the gene family in planta is more demanding given their tissue- and development-specific expression patterns and potential genetic redundancies between at least some members. Homozygous knockouts (KOs) were obtained and characterized for four of the MRS2 genes in Arabidopsis thaliana: AtMRS2-1, AtMRS2-5, AtMRS2-7 and AtMRS2-10 (Gebert et al. 2009). In contrast, no homozygous KO lines could be raised for the other five functional genes AtMRS2-2, AtMRS2-3, AtMRS2-4, AtMRS2-6 and AtMRS2-11, and it appears that such KOs would be embryo lethal (Li et al. 2008, Chen et al. 2009).

By and large, the observations are congruent with the phylogeny of the gene family given that AtMRS2-1, AtMRS2-5 and AtMRS2-10 are members of one MRS2 subclade, which we refer to as ‘clade B’, and which may be able to substitute functionally for each other. No significant macroscopic phenotypes were observed for the KO plant lines of any of these three genes, as also independently found elsewhere in studies of the single KOs of AtMRS2-1 and AtMRS2-5 (Conn et al. 2011) and AtMRS2-10 (Visscher et al. 2010), and most significantly also not for the similarly obtained double KOs (DKOs) AtMRS2-1/5 and AtMRS2-5/10 in our laboratory (Gebert et al. 2009). However, there seemed to be no overall substitutability among clade B genes given that no DKO combination of AtMRS2-1 and AtMRS2-10 was obtained. We report here our success in raising this DKO line as well as a triple knockout (TKO) line for all three clade B genes AtMRS2-1, AtMRS2-5 and AtMRS2-10. The AtMRS2-1/10 DKO and the AtMRS2-1/5/10 TKO lines show virtually identical phenotypes of strong developmental retardation in low-magnesium environments, supporting a secondary role for AtMRS2-5. It is interesting to note that AtMRS2-5 belongs to the dicot-specific subclade B-D2 of faster evolving MRS2 proteins featuring unique alterations in the two terminal α-helical TM domains (Fig. 1).

Here we use a simple and economic ‘high-throughput’, small-scale liquid culturing system in 24-well microtiter plates (LCS-24) which allows easy and quantifiable phenotypic screening of early seedling development under numerous different nutritional environments for up to 4 weeks depending on the Arabidopsis lines. We demonstrate its usability in quick and facilitated scans for physiological and developmental effects of increased, detrimental concentrations of different metal ions (Cd, Co, Cu, Mn and Zn) on the wild type (WT) and the novel clade B KO lines. Moreover, we have improved a routine set-up for hydroponic cultivation of Arabidopsis plants using cellulose acetate filters as a support material, which allows controlled growth of plants throughout their entire developmental cycle. Experiments in this set-up demonstrate how the obvious phenotypic deficits of the new MRS2 clade B multiple KO lines can be significantly ameliorated by a concomitantly lowered supply of calcium.

**Results**

**Five subclades of MRS2 genes supported with extended angiosperm genome sampling**

Phylogenetic analysis of the A. thaliana MRS2 proteins along with their rice (Oryza sativa) homologs had revealed five MRS2 clades that we labeled A–E (Fig. 1) and which have apparently been established prior to the divergence of monocots and eudicots in angiosperm evolution (Gebert et al. 2009). We find this five-clade concept fully supported with the MRS2 homologs that can now be identified in the several additional angiosperm genomes that have become available: the monocots Brachypodium distachyon, Hordeum vulgare, Sorghum bicolor and Zea mays, and the eudicots Arabidopsis lyrata, Glycine max, Medicago truncatula, Populus trichocarpa, Ricinus communis and Vitis vinifera. All five MRS2 clades A–E defined earlier are clearly set apart from each other and contain at least one gene family member of each species (not shown). Single, individual orthologs are exclusively present only in the most ancient clade A of MRS2 proteins in all species, whereas the other four clades B–E are characterized by independent and differential later diversifications creating additional paralogs in some taxa. Clade B containing the genes under investigation here is a particularly prominent example for diversification and possibly independent neo-functionalization (Fig. 1). Three A. thaliana paralogs exist in clade B—AtMRS2-1, AtMRS2-5 and AtMRS2-10—and each has an unequivocal ortholog in the genome of A. lyrata. However, independent gene duplications have created duplicate genes in the MRS2-5-like dicot subclade (B-D1) in Populus and in the MRS2-1/10 subclade (B-D2) both in Glycine and in Populus. An evolutionarily much more ancient split has created two subclades of paralogs (B-M1 and B-M2) among the monocots. Supporting the idea of genetic redundancy between the MRS2 genes in clade B, homozygous plant lines carrying individual T-DNA gene KOs of AtMRS2-1, AtMRS2-5 and AtMRS2-10 were
Fig. 1 Phylogeny of the MRS2 genes: clade B proteins in flowering plants. The phylogenetic tree shown includes MRS2 clade B protein sequence homologs encoded in the completely sequenced genomes of the dicot species Arabidopsis lyrata (Ara lyr), Arabidopsis thaliana (Ara tha), Glycine max (Gly max), Medicago truncatula (Med tru), Populus trichocarpa (Pop tri), Ricinus communis (Ric com) and Vitis vinifera (Vit vin), and the monocot species Brachypodium distachyon (Bra dis), Hordeum vulgare (Hor vul), Oryza sativa (Ory sat), Sorghum bicolor (Sor bic) and Zea mays (Zea may). Two separate monocot (B-M1 and B-M2) and dicot (B-D1 and B-D2) subclades are obvious. Bootstrap support values (1,000 replicates) are shown where they exceed 70. The B-M2 subclade (filled square), which lacks a detectable ortholog in Hordeum, features a characteristic 9–10 amino acid insert between the conserved array of four β-sheets and the following α-helix conserved at the N-termini of 2-TM-GxN proteins, whereas the B-D2 subclade (open circle) shows amino acid exchanges in six positions of the TM domains highly conserved in the other clades. The B-D1 clade contains recently evolved paralogs in Arabidopsis, Glycine and Populus (filled triangles). All five previously identified plant MRS2 gene clades A–E [top left, restricted to Arabidopsis (At) and Oryza (Os) sequences] are characterized by unique intron patterns, strongly supporting them as separate groups (Gebert et al. 2009).
easily obtained but remained without any significant phenotype, also when cultured under varying magnesium supply (Gebert et al. 2009).

**Obtaining AtMRS2-1/10 double and AtMRS2-1/5/10 triple knockout lines**

Similar to the individual gene KOs of the *A. thaliana* MRS2 clade B genes, we were also able to obtain rather straightforwardly DKO lines for gene combinations AtMRS2-1/5 and AtMRS2-5/10, which similarly revealed no obvious phenotypic alterations, however (Gebert et al. 2009). In contrast, raising homozygous AtMRS2-1/10 DKO or AtMRS2-1/5/10 TKO lines proved difficult. We reasoned that a significantly impaired magnesium homeostasis may cause a bottleneck for seed germination and/or very early seedling development. Hence, we attempted to germinate seeds obtained from selfing of plants that were heterozygous for the knockout of the respective remaining AtMRS2 gene under conditions of high supplemental Mg$^{2+}$ concentrations (i.e. final 11.5 mM) in a sterile magenta box set-up. In this way we were indeed able to raise the AtMRS2-1/10 DKO and AtMRS2-1/5/10 TKO lines, which could be verified by PCR as homozygous KOs for all of the respective loci (Supplementary Fig. S1).

The developing seedlings were grown under additional Mg$^{2+}$ supply and later transferred to standard soil. The resulting plant lines were retarded in growth but ultimately proved to be fertile, completing their growth cycle fully and producing viable seeds. Interestingly, once obtained, the seeds of the AtMRS2-1/10 DKO and AtMRS2-1/5/10 TKO lines did not require additional magnesium supply for germination but also germinated under standard growth conditions, although with lower and highly variable germination rates among different batches in the range of 20–80% compared with the WT (not shown). The closer inspection of seeds harvested from different individual AtMRS2-1/10 DKO or AtMRS2-1/5/10 TKO mutant plants revealed variable fractions of unevenly shaped or crumpled seeds.

Phenotypes of the now available AtMRS2-1/10 DKO and the AtMRS2-1/5/10 TKO lines become most apparent in later growth stages upon flower and seed development (Fig. 2). Although somewhat delayed in initial shoot development, the mutant plants largely catch up in growth, ultimately producing fewer shoots, flowers and seeds than the WT, however (Fig. 2A). Most significantly, both the rosette and the stem leaves turn yellow and wilt much earlier than in the WT upon flower and seed development (Fig. 2C, D).

![Fig. 2](https://example.com/fig2.jpg) Phenotypes of the AtMRS2-1/10 DKO and the AtMRS2-1/5/10 TKO mutant growing on standard soil. Overview (A) and detailed views of flowers (B), stem leaves (C) and rosettes (D) of plants grown on standard soil for 6–9 weeks showing yellowing of leaves in mutant lines.
Physiological characterization of the AtMRS2-1/10 DKO and AtMRS2-1/5/10 TKO lines

To characterize the new mutant lines physiologically with respect to nutrient requirements, we used hydroponic cultivation making use of a new set-up based on cellulose acetate as support material (see the Materials and Methods). Early seedling development up to the fully developed rosette leaf stage of mutant lines was virtually indistinguishable from that of the WT upon cultivation with standard SD (Siegenthaler–Depery) medium providing 1 mM Mg^{2+} (Fig. 3D). However, a significantly earlier yellowing and wilting of rosette leaves again becomes apparent upon shoot and flower formation (Fig. 3A, C), slightly ameliorated but essentially analogous to the phenotypes observed when grown on soil (Fig. 2). Interestingly, the process of early rosette leaf senescence in the mutants could be fully compensated by repeated removal of newly formed shoots (Fig. 3E), suggesting either a leaf vs. shoot competition for the necessary Mg^{2+} taken up via the roots or a disturbed Mg^{2+} repartitioning from leaves to shoot.

Phenotypic differences between mutant lines become dramatically more pronounced under low magnesium concentrations (150 μM) in the media. Plant mutant development becomes significantly retarded in comparison with the WT, which only shows very minor effects on vitality under these conditions of lower magnesium supply. Whereas the WT grows vigorously producing abundant flowers and seeds, the mutants show early yellowing of rosette leaves (Fig. 3I), have almost no stem leaves (Fig. 3H) and overall grow poorly (Fig. 3F). Because of even apical stem yellowing and wilting of most inflorescences (Fig. 3G), they produce nearly no seeds under the lowered Mg^{2+} supply. Moreover, while the effects on rosette leaves in the mutants under standard magnesium supply could be compensated by cutting off the developing shoots (Fig. 3E), heavy chlorosis with later wilting remained under low magnesium supply (Fig. 3I). Taken together, we interpret the above observations as a heavily impaired uptake and/or buffering of magnesium in intracellular stores, probably the vacuole.

Interestingly, our previous work had shown that in the case of the AtMRS2-7 single gene KO, a low-magnesium phenotype could be observed most dramatically upon incubation of seeds in a shaking Erlenmeyer flask liquid culture (here referred to as SEFLC) system. Accordingly, we used set-ups of normal [1,500 μM in Murashige and Skoog (MS) medium] and highly limiting (50 μM) Mg^{2+} supply to investigate seed germination and seedling biomass accumulation of the two novel clade B multiple KO lines compared with the WT (Fig. 5). The stringent, unfavorable germination conditions for the submerged seeds in the SEFLC resulted in reduced overall germination rates also for the WT, but again the DKO and TKO lines germinated at rates of 50–60% relative to the WT (not shown). Biomass accumulation per germinated seedling was significantly reduced in the DKO and in the TKO line to approximately 70% of the WT level under standard Mg^{2+} concentrations and to approximately 80% under the highly limiting magnesium concentrations (Fig. 5B). Moreover, the clump of plant material was still greenish even under highly limiting magnesium supply in the case of the WT, whereas no significant greening indicating the proper development of chloroplasts accumulating Chl was detectable for the two KO lines (Fig. 5A).

No significant growth or developmental phenotypes had been observed previously for any of the AtMRS2 clade B single gene KOs or the double gene KOs in combinations AtMRS2-1/5 and AtMRS2-5/10. Given the new insights, we re-investigated the physiology in a large-scale side-by-side experiment using all seven clade B MRS2 KO lines in parallel for hydroponic cultivation under four different Mg/Ca supply set-ups (1,000/1,500, 150/150, 50/1,500 and 150/150 μM) that now proved to be critical for the phenotype of the newly available AtMRS2-1/10 DKO and the TKO line. We continuously monitored shoot and root growth (Supplementary Figs. S2, S3), and finally determined root, rosette and stem weights. Total shoot weight and the root/shoot ratios of biomass accumulation were calculated and the final Chl content for all seven clade B mutant lines and the WT was determined (Supplementary Figs. S4, S5). None of the new AtMRS2-1/10 DKO or the TKO plants survived the stringent growth conditions under 50 μM Mg^{2+} and 1,500 μM Ca^{2+} (Supplementary Figs. 3, 4). Both lines, however, were able to develop under the
Fig. 3 Phenotypes of the AtMRS2-1/10 DKO and the AtMRS2-1/5/10 TKO mutant growing hydroponically. SD medium containing 1,000 μM Mg²⁺ and 1,500 μM Ca²⁺ (A–E) or 150 μM Mg²⁺ and 1,500 μM Ca²⁺ (F–J) leads to drastic differences directly visible in overviews (A vs. F) and detailed views of flowers (B vs. G) and stem leaves (C vs. H) after 6–9 weeks of growth. Even developing rosettes (D vs. I) already show deficiency symptoms after 4 weeks of growth on low magnesium concentrations. Regular clipping of emerging shoots decreases (J) or fully avoids (E) yellowing and wilting of rosette leaves even after 7 weeks of growth. Plants were grown in a hydroponic system (Araponics) on cellulose acetate filters as a novel support material as described in the Materials and Methods.
slightly enhanced supply of 150 μM Mg²⁺ and 1,500 μM Ca²⁺ although accumulating significantly less shoot and root biomass than all other lines and reaching an early shoot growth plateau (Supplementary Fig. S3A). The discrepancies in biomass accumulation were most pronounced in stems and somewhat less so in rosettes and roots (Supplementary Fig. S4A–D), ultimately resulting in an increase in the root/shoot ratio to 150% under these conditions (Supplementary Fig. S4E). As described above, the new AtMRS2-1/10 DKO and the TKO lines catch up under concomitantly lowered calcium concentrations (150/150 μM Mg/Ca), possibly even with a gain in rosette biomass accumulation in comparison with the other five clade B mutant lines or the WT (Supplementary Fig. S4B). Compared with our two new clade B gene multiple KO lines, none of the three single or the other two double KO lines showed any considerable differences of the parameters now scored. A significant drop in Chl content per leaf surface was observed only under the limiting 150 μM Mg/1,500 μM Ca conditions for the new DKO and TKO lines (Supplementary Fig. S5), but we consider this with caution given the overall impact on plant vitality and the wilting rosette leaves at this stage.

**Standardizing the LCS-24 set-up**

To scan conveniently and economically different lines and nutritional conditions simultaneously while focusing on very early seedling development, we used a small-scale liquid culture set-up, here referred to as 'LCS-24', which makes use of 24-well microtiter plates (Fig. 6A–C). Seed germination in 24-well microtiter plates has been performed before in different research contexts (e.g. Gómez-Gómez et al. 1999; Krol et al. 2010). We here aimed for standardization and adaptation of the set-up allowing more rigorous statistical analysis of early plant growth and development. Among the major advantages, the LCS-24 system is very inexpensive, reproducible, easy to set up in standard laboratories, tolerant against contamination of individual seeds and allows for continuous photodocumentation of plant development (Fig. 6B, C). Upon harvesting, the Arabidopsis plantlets may be investigated in detail and/or subjected to further tests, and their fresh content.
weight can be recorded semi-automatically (see the Materials and Methods). We routinely observe development to stage 1.06 characterized by three fully developed leaf pairs (Boyés et al. 2001) after 11 d, resulting in about 15 mg of fresh weight for WT seedlings under standard conditions. If mutant seedlings are retarded in growth (Fig. 6C) and development is not yet limited by the chambers’ size, cultivation may be extended for up to 4 weeks before final evaluation.

Using the LCS-24 system, we found mean seedling weights of the AtMRS2-1/10 DKO and AtMRS2-1/5/10 TKO lines to be significantly decreased compared with the WT under both low and highly limiting magnesium concentrations: approximately 6 vs. 9 mg per seedling at 150 μM Mg2⁺ and approximately 1 vs. 3 mg per seedling at 50 μM Mg2⁺ (Fig. 6D). Hence, the new LCS-24 miniature set-up confirms the magnesium deficiency phenotype observed in the hydroponic and in the SEFLC set-up very well. No significant effect on biomass accumulation was seen for the novel mutant lines in comparison with the WT under higher magnesium concentrations of 5 or 10 mM in the media (Fig. 6D).

**Influence of other ions in detrimental concentrations: Cd, Co, Cu, Mn and Zn**

Since the LCS-24 is particularly well suited to test numerous nutritional environments in parallel, we also employed it for preliminary scans on the influence of other metal ions at detrimental concentrations. Given that none of the previous experimentation had shown any phenotypic difference between the AtMRS2-1/10 DKO and the AtMRS2-1/5/10 TKO, we focused entirely on the TKO line for these experiments on ion toxicity. We found that concentrations of 75 μM cadmium, 100 μM cobalt or 75 μM copper were inhibiting proper Arabidopsis seedling development in the presence of low concentrations (i.e. 150 μM) of Mg2⁺. Any of those three additions had dramatic effects on Arabidopsis plant growth and biomass accumulation, with 75 μM Cd2⁺ reducing growth by approximately 40% and 100 μM Co2⁺ or 75 μM Cu2⁺ reducing growth by approximately 70%, respectively (Fig. 7C). Analogous growth retardations were observed, however, in the WT and the AtMRS2-1/5/10 TKO mutant alike, indicating that a knockout of the three magnesium transporters neither increases nor decreases tolerance against the three elements at higher concentrations.

A major advantage of the 24-well set-up is that it allows for individual assignment of early developmental stages of seedlings which could provide a more differentiated picture (Fig. 7A). Under low magnesium supply alone, the WT and TKO show a similar distribution over developmental stages, although the reduced germination rate becomes apparent with four compared with two non-germinated seeds (stage 0.1). In the presence of Cd2⁺ or Co2⁺, however, significantly more WT than TKO seedlings have developed into the respective most advanced developmental stages (1.03 or 1.02, respectively). This looks different for the 75 μM Cu2⁺ condition, however. Although significantly limited in overall biomass accumulation, more seedlings have progressed into the two-leaf pair stage in the mutant. Apparently, the essential microelement copper in higher concentrations harms biomass accumulation similarly to the toxic elements cadmium...
or cobalt, but affects the developmental progression of seedlings in our miniature set-up significantly less. Examples of the most highly developed plants under each condition show that in addition to different leaf numbers, the length of the root is much influenced by the various metal ions, but again there is no obvious difference between the WT and the mutant line.

Preliminary experimentation showed that higher concentrations of the essential micronutrients manganese (Supplementary Fig. S6) or zinc (Supplementary Fig. S7) were needed to cause detrimental effects on Arabidopsis seedling development. We additionally experimented with increased cultivation times in the LCS-24 set-up, extended to 22 d. Such an extension of incubation time can lead to much higher weights of up to 60 mg per WT plantlet under favorable conditions, but plant growth and development is then clearly limited by the well size. Under growth-inhibiting conditions, however, seedling development may be followed for much longer, and final growth stages of up to 1.08 can be reached, demonstrating the difference between still ongoing development at strongly reduced overall growth and no biomass accumulation at all. However, no significantly different response was seen when the new TKO line was compared with the WT under either of two conditions of moderately or strongly increased concentrations of manganese or zinc (Supplementary Figs. 56, 57), respectively, other than the overall growth retardation of the TKO line in the low $\text{Mg}^{2+}$ background used in these experiments.

**Discussion**

With the benefit of hindsight, it is now interesting to note that neither of the two initially characterized founding members of the *A. thaliana* magnesium transporter gene family, AtMRS2-1/MGT2 (Schock et al. 2000) or AtMGT1/MRS2-10 (Li et al. 2001) that led to the different naming of the gene families as either MRS2 or MGT genes is highly essential for plant development (Fig. 8). Individual knockouts of these two genes and even in the respective combinations with AtMRS2-5, the third gene of the MRS2/MGT subclade B to which they all belong (Fig. 1), do not cause obvious phenotypes in *A. thaliana* even under magnesium deficiency conditions. In contrast, a simultaneous knockout of the genes AtMRS2-1 and AtMRS2-10 proved much more difficult to obtain.

We here report that we were finally able to raise such an AtMRS2-1/10 DKO line and even an AtMRS2-1/5/10 TKO line for all three clade B genes (Fig. 8). Both the AtMRS2-1/10 DKO and the AtMRS2-1/5/10 TKO show significant defects in growth, vitality and development, but both are fertile and finally produce viable seeds. The developmental retardation phenotypes of both lines become more notable under low magnesium supplies in different set-ups of hydroponic or liquid cultivation, but can interestingly be ameliorated by concomitantly reduced calcium supply in the growth media. Alternatively, the phenotypes of early senescence in rosette leaves upon shoot formation in the two multi-KO lines can be significantly reduced by serially cutting off developing shoots. These observations indicate that the genes involved play a major role in homeostasis and transport of magnesium between different plant tissues. A vacuole membrane (tonoplast) localization is very likely for both AtMRS2-1 and AtMRS2-5 (Carter et al. 2004, Whiteman et al. 2008, Conn et al. 2011), although AtMRS2-5 had previously also been identified in the plasma membrane (Alexandersson et al. 2004). Given that the simultaneous knockout of AtMRS2-1 and AtMRS2-5 did not cause significant phenotypes, it is likely that yet another functional redundancy exists in the tonoplast. One entirely different magnesium transport system across the tonoplast, the MHX1 $\text{Mg}^{2+}/\text{H}^+$ exchanger (Shaul et al. 1999), however, is very likely to be responsible for loading the acidic vacuole with magnesium instead of the likely roles of AtMRS2-1 and AtMRS2-5 in regulating its unloading across the tonoplast along the concentration gradient of magnesium (Fig. 8).
Fig. 7 The effects of increased cadmium, cobalt and copper concentrations screened in the LCS-24 set-up. After 11 d of growth in a low (150 μM) magnesium environment with additional elevated ion concentrations of Cd²⁺, Co²⁺ and Cu²⁺, seedling classification according to developmental stage (according to Boyes et al. 2001) can be performed (A). Individual plantlets can be subject to photodocumentation (B) or subsequent analyses or measurements (such as, for example, root length) after determining their fresh weights (C). Bars in B are 1 cm, the number of plants used for average in (C) is indicated below each bar; error bars show the standard error and asterisks indicate significant differences ($P < 0.05$) from the wild type under a given condition.
CNGC10 appears to contribute to cellular Ca$^{2+}$ transport to the plasmalemma in Arabidopsis. Parallel complementation with only AtMRS2-10/MGT1 recently switched to targeting membrane (Li et al. 2001). AtMRS2-10 instead appears to be targeted to the outer cell membrane (Li et al. 2001).

Very recent studies on the routing of membrane proteins to the tonoplast have interestingly revealed that only minor sequence changes in membrane protein paralogs may lead to an insertion into the plasmalemma as the `default' target in the absence of other targeting signals (Wolfenstetter et al. 2012). We hence conclude that (i) identification of a protein in different membrane compartments at the same time (such as AtMRS2-5) may not necessarily have to imply methodological artifacts but may imply alternative co-sorting to both membrane types and (ii) that the here investigated clade B of membrane proteins (Fig. 1) may be referred to as vacuolar proteins, with only AtMRS2-10/MGT1 recently switched to targeting to the plasmalemma in Arabidopsis. Parallel complementation approaches of the now available AtMRS2-1/10 DKO and the AtMRS2-1/5/10 TKO line with any of the three clade B MRS2 genes, alternatively driven from the strong 3SSS rather than from their native promoters, may provide further interesting clues in the future.

Despite the absence of significant overall developmental phenotypes (Gebert et al. 2009) for the individual AtMRS2-1 and AtMRS2-5 gene KO lines, more sophisticated measurements using SEM/EDS (scanning electron microscopy and energy-dispersive X-ray spectroscopy) suggested individual roles in magnesium storage in leaf mesophyll cell vacuoles that become obvious under serpentine (i.e. high Mg/Ca ratio) conditions (Conn et al. 2011). An unequivocal correlation in the homeostasis of the two macroelements has recently also been observed in a large-scale ionomics study sampling 96 different A. thaliana wild accessions (Baxter et al. 2012). Another large-scale investigation has recently demonstrated that high concentrations of calcium in the substrate inhibit magnesium accumulation in the leaves of Brassica rapa, but not the other way round (Rios et al. 2012).

Notably, population re-sequencing of A. lyrata plants grown on serpentine vs. normal soils had indicated significant allele differences for two MRS2 genes (Turner et al. 2010). However, rather than the clade B genes, these surprisingly turned out to affect the two functional clade E genes AtMRS2-7/MGT7 (Gebert et al. 2009) and MRS2-2/MGT9, a gene for which no homozygous KO lines could be raised and which is involved in pollen development (Chen et al. 2009). Similarly, a scan for QTLs (quantitative trait loci) associated with seed mineral concentrations identified none of the three clade B genes but AtMRS2-2, AtMRS2-3 and AtMRS2-11 instead as candidate loci (Waters and Grusak 2008). A scan for QTLs linked to natural variation of seed cation contents identified MHX as a candidate for seed magnesium content whereas the MRS2 clade E genes on chromosome 5 were identified as QTL candidates associated with manganese content (Vreugdenhil et al. 2004).

Although abundantly expressed already in early seedling development, AtMRS2-5 seems to have a secondary role in any case, as it does not aggravate any of the observed phenotypes when knocked out in combination with the other clade B genes. The much more striking phenotype of the AtMRS2-1/10 DKO line is more difficult to explain. One possible scenario may be that the two genes provide alternative routes for adequate magnesium supply in the cytosol of which either the one or the other has to be intact for very early seed development (Fig. 8). Seed germination and very early seedling development may rely either on magnesium stored in the vacuole of embryo cells or alternatively on magnesium taken up through the emerging seedling radicle where AtMRS2-10 is strongly expressed (Gebert et al. 2009). In contrast to their differential expression during development, no evidence has been found for significant transcriptional regulation of the plant MRS2 genes in response to changes in magnesium supply (Gebert et al. 2009, Hermans et al. 2010a, Hermans et al. 2010b) aside from moderate cell-specific changes of clade B gene transcript levels in serpentine environments (Conn et al. 2011).
**Materials and Methods**

**Plant lines and genotyping**

*Arabidopsis thaliana* ecotype Col-0 and T-DNA insertion lines originally obtained from the Nottingham Arabidopsis Stock Centre (NASC) were used in all experiments. The AtMRS2-1/10 DKO and AtMRS2-1/5/10 TKO lines ultimately resulted from crossing of lines homozygous for one or two of the respective loci (Gebert 2009; Supplementary Table S1) but heterozygous for the remaining one. Screening of the selfed progeny for homozygous individuals was done by genotyping PCR as reported previously.

**General seed and plant handling**

Seeds were surface sterilized (Swinburne et al. 1992) prior to sowing. All seeds were initially stratified for 3 d at 4°C in the dark before they were transferred to a growth chamber (growth day 0) with day temperature limited to 22°C. Light intensity was approximately 45 μmol m⁻² s⁻¹ with a 16/8 h light cycle. Humidity was not actively controlled in plant growth rooms. Temperature control via air conditioning generally operated against heat dissipated by the illumination under long-day conditions and kept overall humidity normal. No obvious impact on plant cultivation during different seasons was recognizable.

**Media**

Hydroponic cultivation was performed in an SD medium background of 5 mM KNO₃, 1 mM KH₂PO₄, 1 mM NH₄Cl, 1-fold concentrated microelements and a 1-fold concentration of Fe-EDTA. Microelements were supplemented from a 1,000-fold stock solution containing 46 mM H₂BO₃, 10 mM MnSO₄, 0.77 mM ZnSO₄, 0.32 mM CuSO₄ and 0.58 mM MoO₃, and Fe-EDTA was supplemented from a 1,000-fold stock solution of 100 mM Na₂EDTA-2H₂O, 370 mM KOH and 100 mM FeSO₄. Adjustments of Mg²⁺ and Ca²⁺ concentrations were done by appropriate supplementation with MgSO₄ and Ca(NO₃)₂ (SD standard concentrations are 1 and 1.5 mM, respectively). Upon variation of MgSO₄, sulfate concentrations were kept constant at 1 mM by compensatory addition of Na₂SO₄. Cultivation in Erlenmeyer flasks and 24-well microtiter plates used standard MS media with Gamborg B5 vitamins (Duchefa M0231) buffered by 0.05% (w/v) MES adjusted to pH 5.7 with KOH. When lowering normal concentrations of 1.5 mM MgSO₄ and 3 mM CaCl₂, media were prepared using the recipe from above using modified stock solutions keeping the sulfate concentration constant. Additionally 1% (w/v) sucrose was added before autoclaving to all MS media.

**Hydroponic cultivation**

For routine hydroponic cultivation of *A. thaliana* with seedlings completing their life cycle under controlled mineral nutrient conditions, we experimented with different media (MS, general hydroponics, SD) and seed carrier materials (agarose plugs, glass and stone wool, cigarette filter materials) to improve economic handling, reliability, reproducibility and standardization, and to decrease the risk of contamination. We ultimately found that commercially available cellulose acetate cigarette filters (e.g. ‘Gizeh slim’) in our hands proved to be a well-suited seed carrier material allowing easy handling also at higher throughputs by ideally fitting into the Araponics (http://www.araponics.com/) seedling holder system or alternatively into 0.5 ml bottom-less plastic test tubes. Gizeh slim filters were prepared by piercing the center with a 3 mm wide pin or toothpick to allow reliable growth of the seedling root and subsequent washing at 37°C for 3 h each in 70% ethanol and distilled water, respectively, before placing them in the Araponics seed holders. Individual Arabidopsis seeds were placed on top of the central hole pierced in the carrier filters placed in SD medium. After stratification, set-ups were first incubated under transparent covering hoods for up to 1 week to provide adequate humidity for early seedling development. To prevent growth of molds and to accommodate plants for the removal of the lids, they were aerated daily. Depending on plant development, Aracon and Aratube containment holders (http://www.arasystem.com) were applied approximately 3 weeks after stratification.

To allow for a continuous monitoring of plant shoot and root growth and ultimately to determine the weight of organs towards the end of the plant life cycle, plants were hydroponically cultivated (see also Supplementary Fig. S2) in home-made carrier systems on containers containing a removable partitioning system to keep individual root systems apart. Fully developed plants were dissected into stem (above rosette), rosette and root sections, and all materials were transferred separately into pre-weighed, cokable plastic containers or, for root material, into tubes with a 1.2 mm hole in the bottom allowing the remaining nutrient solution to be spun off by centrifuging at 5,000×g for 7 min. Determination of fresh and dry weight (after incubation at 60°C for 3 d) was done by computer-assisted weighing (weight recording, zero correction, difference calculation). The length of shoots and roots was measured with ImageJ (http://rsb.info.nih.gov/ij/) on digital images of roots and shoots.

**Shaking Erlenmeyer flask liquid cultivation (SEFLC)**

A fixed number of sterilized seeds were flushed into Erlenmeyer flasks containing 100 ml of autoclaved liquid medium (in triplicate set-ups) and incubated on a rotary shaker at 100 r.p.m. for 11 d. Plantlets were collected from the liquid and surface dried by centrifugation at 4,000×g for 5 min in specially prepared 50 ml Falcon tubes with water outlet holes. Fresh weights were calculated as the difference between the empty tube weight and the weight after centrifugation. The number of germinated plants was determined by counting the numbers of hypocotyls in plant clumps.
Liquid cultivation set-up in the 24-well microtiter plates (LCS-24)

Aliquots of 2 ml of autoclaved media were dispensed into individual cavities of a 24-well microtiter plate. Single sterilized seeds were transferred using a toothpick into each well. To minimize potential experimental differences between incubated plates, different plant lines investigated were included on the same plate with several independent plate replicates. Plates were closed and sealed using parafilm, stratified and subsequently incubated for 11 d (standard) or longer in the case of significant growth retardation of mutants. Assignment of developmental stages was done as described previously (Boyes et al. 2001), followed by careful transfer of individual seedlings to pre-weighted 0.5 ml reaction tubes with a 1.2 mm hole in the bottom. Tubes with plantlets were centrifuged at 4,000×g for 5 min sitting in 2.0 ml reaction tubes to collect superfluous medium adhering to the plant surfaces. Individual plant fresh weights were recorded by computer-assisted weighing (weight recording, zero correction and difference calculation).

Chl measurements

The Chl concentration was determined from Arabidopsis leaf samples of fully developed plants after 43 d of growth prior to harvesting for determination of root and shoot weights. Leaf discs (0.24 cm² surface area) were punched out from the rosette leaf blades, avoiding the major leaf veins in three replicates per individual plant. The extraction of Chl was carried out using the N,N-dimethylformamide (DMF) extraction method (Moran and Porath 1980) and concentration was determined with a NanoDrop 2000c (Thermo Fischer Scientific) at 664 nm. Single erratic measurements occasionally suggesting much higher Chl concentrations than in the two parallel measurements of the same plant sample were excluded from the analyses.

Phylogenetic analysis

Protein sequence alignment and phylogenetic analysis was done using MEGA (Kumar et al. 2008). Phylogenetic tree construction of the full MRS2 protein alignment was done via the Neighbor–Joining (NJ) method using Poisson-corrected distances and the pair-wise gap deletion. Tree node support was estimated with 1,000 bootstrapping replicates.

Data acquisition and statistical analysis

Besides self-written utilities to automate the plantlet weighing process, data were analyzed and visualized with the authors own scripts written in R (R Core Development Team 2012, www.R-project.org) mainly using the ggplot2 (Wickham 2009), plyr (Wickham 2011) and RColorBrewer (www.ColorBrewer.org) packages. Weight measurements were tested for normal distribution (Shapiro–Wilks test) and pairwise equality of variances (F-test). Finally, the significances of weight differences were tested using two-tailed Mann–Whitney U-test with P < 0.05.

Supplementary data

Supplementary data are available at PCP online.

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References


