Expression and Functional Analysis of the CorA-MRS2-ALR-Type Magnesium Transporter Family in Rice

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Maintenance of an appropriate magnesium ion (Mg2+) concentration is essential for plant growth. In Arabidopsis thaliana, the CorA-MRS2-ALR-type proteins, named MRS2/MGT family proteins, are reportedly localized in various membranes and they function in Mg transport. However, knowledge of this family in other plant species is extremely limited. Furthermore, differential diversification among dicot and monocot plants suggested by phylogenetic analysis indicates that the role of the Arabidopsis MRS2/MGT family proteins is not the same in monocot plants. For a further understanding of this family in higher plants, functional analysis and gene expression profiling of rice MRS2/MGT family members were performed. A phylogenetic tree based on the isolated mRNA sequences of nine members of the OsMRS2 family confirmed that the MRS2/MGT family consists of five clades (A–E). A complementation assay in the yeast CM66 strain showed that four of the nine members possessed the Mg2+ transport ability. Transient green fluorescent protein (GFP) expression in the isolated rice protoplast indicated that OsMRS2-5 and OsMRS2-6, belonging to clades D and A, respectively, localized in the chloroplast. Expression levels of these genes were low in the unexpanded yellow–green leaf, but increased considerably with leaf maturation. In addition, diurnal oscillation of expression was observed, particularly in OsMRS2-6 expression in the expanded leaf blade. We conclude that OsMRS2 family members function as Mg transporters and suggest that the genes belonging to clade A encode the chloroplast-localized Mg2+ transporter in plants.

Keywords: Chloroplast • CorA • Magnesium • 28Mg • MRS2/MGT • Oryza sativa.

Abbreviations: ER, endoplasmic reticulum; GFP, green fluorescent protein; Mg, magnesium; RT–PCR, reverse transcription–PCR.

Introduction

In living plant cells, the magnesium ion (Mg2+) is the most abundant divalent cation. Mg is essential for photosynthesis as the central atom of the chlorophyll molecule. In every subcellular organelle, appropriate Mg2+ concentrations are required for the functioning of numerous enzymes in the form of Mg–ATP complexes or cofactors to form a particular structure (Maguire and Cowan 2002, Shaul 2002). In view of the biological significance of Mg2+, its transport across the different membranes is thought to be mediated by particular proteins capable of recognizing Mg2+.

Among several proteins reported to be associated with membrane Mg2+ transport, the bacterial CorA protein and its eukaryotic homologs, Alr1 and Mrs2, are the best-studied Mg2+ transporters mediating Mg2+ influx (Knoop et al. 2005, Niegoski and Eshaghi 2007, Moomaw and Maguire 2008). Homologous proteins of this superfamily can be identified in all organisms, including animals, plants, fungi and prokaryotes (Knoop et al. 2005). The crystal structure and functional analysis of TmCorA from Thermotoga maritima and the recently reported MjCorA from Methanocaldococcus jannaschii revealed a unique gating model for the CorA family protein functioning in a pentameric assembly as a ligand-gated ion channel (Eshaghi et al. 2006, Lunin et al. 2006, Payandeh and Pai 2006, Payandeh et al. 2008, Gusak et al. 2012). The CorA-MRS2-ALR-type proteins have a characteristic glycine–methionine–asparagine (GMN) tripeptide motif at the end of the first of two transmembrane domains, and this motif is thought to be essential for proper Mg2+ transport (Knoop et al. 2005).

In plants, homologs of the CorA type transporter have been identified in Arabidopsis thaliana by two research groups and named as AtMRS2 (Schock et al. 2000) or AtMGT (Li et al. 2001) family genes. The AtMRS2/AtMGT family has nine members and two pseudogenes. The physiological significance of AtMRS2/AtMGT family members has been gradually demonstrated. For example, the vacuole-targeted proteins AtMRS2-1/AtMGT2 and AtMRS2-5/AtMGT3 function in Mg2+ transport...
across the vacuole, thereby serving to maintain cation balance in the vacuole (Conn et al. 2011). AtMRS2-10/AtMGT1 overexpression in tobacco plants increased Mg concentration and conferred tolerance to a low Mg environment as well as toward aluminum (Al) stress (Deng et al. 2006). Similarly, a member of the MRS2/MGT family in rice (Oryza sativa) was recently found to localize on the plasma membrane and participate in the Al stress response (Chen et al. 2012).

Based on the previous studies, we could postulate that MRS2/MGT family proteins function as Mg\(^{2+}\) transporters in subcellular Mg\(^{2+}\) partitioning for maintaining plant growth. However, information on plant MRS2/MGT transporters has been obtained mostly from Arabidopsis studies, with as yet only one study in rice. Moreover, phylogenetic analysis has indicated differential diversification of this MRS2/MGT gene family among dicot and monocot plants (Gebert et al. 2009). Given the potential diversity of the MRS2/MGT family among plant species, it is desirable to characterize the MRS2/MGT family in several plant species for better understanding of its significance. In the present study, the rice MRS2/MGT family was characterized and the expression pattern and Mg\(^{2+}\) transport abilities of its members were investigated.

### Results

#### Identification of MRS2/MGT family genes in rice

Based on the complete genome sequence database of rice, nine genes were identified as OsMRS2/MGT family members (Table 1). These genes were named OsMRS2-1 to -9, as listed in Table 1. The protein sequences (Fig. 1a) were deduced from isolated mRNA sequences. Variation in the splicing pattern was found only in OsMRS2-5 (Fig. 1a; Supplementary Fig. S1). The protein sequence deduced from the smaller variant lacks a highly conserved domain, and thus the larger variant was assumed to be the functional OsMRS2-5 protein (Fig. 1a). Indeed, the protein sequence of OsMRS2-5 deduced from the larger variant produced a better alignment than that from the smaller variant. Accordingly, the full-length coding sequence of the larger variant was used in the alignment, yeast complementation assay and subcellular localization experiment. At the C-terminus of the first transmembrane region, OsMRS2-1, -2, -3, -6, -7 and -9 had the characteristic GMN motif (Fig. 1a). This motif was found to be altered either to AMN or to GIN in OsMRS2-8 (Fig. 1a). A phylogeny of the rice and Arabidopsis MRS2/MGT gene families confirmed that there are five clades to which at least one member of each family belonged (Fig. 1b). In clades B, C, D and E, each OsMRS2 gene did not necessarily have a one-to-one counterpart in Arabidopsis MRS2/MGT, whereas in clade A, OsMRS2-6 had the counterpart AtMRS2-11/AtMGT10 (Fig. 1b).

#### Functional complementation assay in yeast

A complementation assay was performed using the yeast mutant CM66, which lacks ALR1 and ALR2 and accordingly cannot grow on standard medium containing <4 mM Mg\(^{2+}\) (Li et al. 2001). When OsMRS2-1, -3, -6 and -9 were individually expressed in CM66, this yeast strain could grow on solid medium containing 1 mM Mg\(^{2+}\) (Fig. 2a). In liquid medium containing 0.1 mM Mg\(^{2+}\), OsMRS2-6 expression could complement growth (Fig. 2b). In addition to OsMRS2-6, OsMRS2-1 or -3 expression improved the growth rate of CM66 in liquid medium containing 1 mM Mg\(^{2+}\) (Fig. 2b). However, complementation ability was not observed with OsMRS2-2, -4, -5, -7 and -8 (Fig. 2a, b). In particular, expression of either OsMRS2-2, -7 or -8 even lowered the growth rate of yeast below that of CM66 (Fig. 2a, b). One possibility was that these OsMRS2 proteins caused interference with the function of yeast organelle membranes to which these proteins were localized. Alternatively, efflux transport of Mg\(^{2+}\) as a consequence of either potential dual efflux ability (Li et al. 2008) or reverse allocation of the proteins on the membrane could also be the reason. In addition, mistargeting of the OsMRS2s to the organelle membranes other than the plasma membrane in yeast potentially occurred, given that the full-length cDNA was used for the complementation assay without any modification to target the plasma membrane. Improvement in the Mg\(^{2+}\) uptake ability of the CM66 strain expressing OsMRS2-6 was confirmed by \(^{28}\)Mg\(^{2+}\) accumulation (Fig. 2c).

#### Subcellular localization in the isolated rice leaf protoplasts

To address the potential physiological significance of the OsMRS2 family, we attempted the subcellular localization of all nine members. To this end, we cloned the full-length open reading frames of all OsMRS2 genes and transiently expressed green fluorescent protein (GFP) fusion proteins in the isolated rice leaf protoplasts. Apparent GFP fluorescence was produced in the chloroplastic OsMRS2-5–GFP and OsMRS2-6–GFP expression, and possibly in the endoplasmic reticulum (ER) by OsMRS2-3–GFP (Fig. 3). The chloroplastic localization of OsMRS2-5 and -6 was consistent with the chloroplast-targeting transit peptide found at the N-terminus, in which the +1, +2 positions typically show [A/S/E]–[A/S/L] (Fig. 1a) (Zybailev et al. 2011). In contrast, in the N-terminus of OsMRS2-4 (Fig. 1a), as well as AtMRS2-4/AtMGT6 and AtMRS2-6/AtMGT5 (Li et al. 2001), which are in the same clade as OsMRS2-5, the characteristic mitochondria-targeting transit peptide, displaying an alternating pattern of hydrophobic (in bold) and positively charged (underlined) amino acids, was found: MFNC4AEKAMVIFNAIV. To date, no other fusion constructs have demonstrated any identifiable localization pattern.

#### Expression pattern in rice

Semi-quantitative reverse transcription–PCR (RT–PCR) was performed to detect the expression of all OsMRS2 family members in different tissues at different growth stages (Fig. 4).
OsMRS2 genes were expressed in leaves, roots and flowers, except for OsMRS2-8, which was rarely expressed in leaves, and OsMRS2-5, which was not detected in the flag leaf (Fig. 4). OsMRS2-5 was actively expressed in the flower, a pattern somewhat similar to the AtMRS2-6/AtMGT5 expression pattern (Li et al. 2008, Gebert et al. 2009). Transcript of OsMRS2-6 was accumulated in leaves, as expected from the chloroplast localization of the OsMRS2-6 protein (Fig. 3), but was also detected in roots (Fig. 4). In Arabidopsis, mRNA of AtMRS2-11/AtMGT10, which localized in chloroplasts, was detected even in the root (Drummond et al. 2006). Considering that the AtMRS2-11/AtMGT10 protein was found only in the shoot, AtMRS2-11/AtMGT10 mRNA was proposed to be unstable in the root (Drummond et al. 2006), as might also be the case for OsMRS2-6.

The chloroplast localization pattern observed for OsMRS2-6 (Fig. 4), together with the fact that OsMRS2-6 is phylogenetically related to AtMRS2-11/AtMGT10 (Fig. 1), suggested that OsMRS2-6 is an ortholog of AtMRS2-11/AtMGT10 and acts in the rice chloroplast similarly to AtMRS2-11/AtMGT10 in Arabidopsis. To examine this possibility, we further investigated the expression profile of OsMRS2-6 as well as other OsMRS2 genes. One characteristic feature of AtMRS2-11/AtMGT10 is the diurnal expression change (Drummond et al. 2006). Accordingly, mRNA amounts in young mature leaves were measured every 4 h to determine whether OsMRS2-6 also shows a diurnal expression change. As a result, OsMRS2-6 was found to accumulate particularly in leaf blades, with diurnal oscillation peaking at the beginning of the light period (Fig. 5). This expression pattern of OsMRS2-6 was also found in publicly available microarray data (RiceXpro; Sato et al. 2011). The genes other than OsMRS2-6 were almost constantly expressed throughout the day; the result for OsMRS2-5 is shown as a typical example (Fig. 5).

If the clade A MRS2 proteins participate in photosynthetic activity in the chloroplast, their gene expression may be associated with chloroplast metabolism. To test this idea, the expression change of OsMRS2 genes in response to leaf

Table 1 MRS2/MGT family members of O. sativa and A. thaliana

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<td>Gebert et al. (2009)</td>
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<td>AT5G22830</td>
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OsMRS2-6
AtMRS2-11
MRS2 (yeast)
CorA (bacteria)
candidate for MRS2 family in Oryza sativa
MRS2 family in Arabidopsis thaliana
other organisms

Fig. 1 Phylogenetic tree of O. sativa and A. thaliana MRS2 proteins. (a) Multiple alignment of OsMRS2 family protein sequences was performed using ClustalW. OsMRS2 sequences were confirmed by rice cDNA sequences. Four-level shading of the alignment indicates conserved sequences using the Dayhoff PAM 250 matrix with GeneDoc software (Nicholas et al. 1997). Predicted C-terminal transmembrane domains (TM1 and TM2) and the conserved GMN motif are indicated. There were two splicing variants for OsMRS2-5, and 46 amino acids were missing in the smaller variants (blue line). (b) A Neighbor–Joining tree of protein sequences of O. sativa and A. thaliana constructed with MEGAS software (Tamura et al. 2011). Bootstrap percentages are indicated at branches where they exceed 70.
maturation was investigated. Low OsMRS2-6 expression was found in the immature yellow–green leaf blade, which was covered by the sheath of older leaves; however, active OsMRS2-6 expression was found in the expanding green leaf blade (Fig. 6). A similar expression pattern, but not as apparent as that of OsMRS2-6, was found in OsMRS2-5 (Fig. 6). The other genes were expressed almost constantly during leaf maturation (data not shown).

Discussion

The Mg\(^{2+}\) transport ability of OsMRS2 family proteins was examined using a yeast complementation assay with a yeast strain, CM66, defective in both CorA/MRS2 homologs located

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**Fig. 2** Complementation of the yeast CM66 mutant by OsMRS2 genes. The wild-type yeast strain CM52 and mutant CM66 transformed with an empty vector were used as a positive and negative control, respectively. (a) Complementation assay on solid medium containing 1 mM MgCl\(_2\). (b) Complementation assay in liquid medium containing either 0.1 or 1 mM MgCl\(_2\). The cell density of each culture medium (OD\(_{600}\)) was monitored over 36 h. (c) Measurement of \(^{28}\)Mg in cells transformed with OsMRS2-6. After treatment with \(^{28}\)Mg (40 kBq ml\(^{-1}\)) containing 1.0 mM MgCl\(_2\) in liquid solution for 30 min, the relative rate of \(^{28}\)Mg absorption for CM52 was calculated. The bars represent standard deviations.

**Fig. 3** Subcellular localization of OsMRS2-3, -5 and -6. OsMRS2-3–GFP was localized to the ER, and OsMRS2-5–GFP and OsMRS2-6–GFP were localized to the chloroplast in isolated rice protoplasts. 35S:OsMRS2-3–GFP, 35S:OsMRS2-5–GFP and 35S:OsMRS2-6–GFP constructs were introduced into the protoplasts isolated from the leaf blade, and fluorescence was visualized by confocal laser microscopy. Bars = 50 \(\mu \text{m}\).

**Fig. 4** Spatial expression of OsMRS2 genes in the newest developing leaf, fully expanded mature leaf and root at the juvenile stage; in the shoot and root parts at the tiller developing stage; and in the flag leaf and flower at the maturation stage. All organs were harvested in the middle of the light period. The transcript of actin1 was amplified as the experimental control.
in the plasma membrane, ALR1 and ALR2. The Mg$^{2+}$ transport ability of OsMRS2-1, -3, -6 and -9 could be demonstrated, whereas transformation of OsMRS2-2, -4, -5, -7 and -8 had no beneficial effect on the growth rate of CM66 under limiting Mg in either liquid or solid medium. However, this result should not be immediately interpreted as their inability to transport Mg$^{2+}$. In Arabidopsis, the Mg$^{2+}$ transport ability of nine members of the AtMRS2/AtMGT family was recently demonstrated by targeting the proteins to the mitochondrial membrane of the yeast mrs2 mutant (Gebert et al. 2009). In contrast, various assays previously performed to detect the Mg$^{2+}$ transport ability of the AtMRS2/AtMGT protein family resulted in variable findings depending on the respective complementation assays. For example, AtMRS2-6/AtMGT5 and AtMRS2-2/AtMGT9 showed little Mg$^{2+}$ transport ability in a complementation assay using yeast CM66 (Drummond et al. 2006), while their transport ability was demonstrated using Salmonella MM281 and/or the yeast mrs2 mutant (Li et al. 2008, Chen et al. 2009, Gebert et al. 2009). These findings suggest the possibility that OsMRS2-2, -4, -5, -7 and -8 likewise have Mg$^{2+}$ transport ability that merely remained undetected in the less favorable CM66 environment and that all OsMRS2 family proteins could in fact function as Mg$^{2+}$ transporters in rice, similarly to the function of AtMRS2/AtMGT family proteins in Arabidopsis. Indeed, OsMRS2-2 was recently shown to function as an Mg$^{2+}$ transporter participating in Mg uptake in planta, and was named OsMGT1 (Chen et al. 2012).

The results of the new complementation assays reported here indicate another problem concerning the affinity of the transporter. Among the nine family members, OsMRS2-6 expression was particularly effective in the complementation of the yeast CM66 mutant in liquid medium containing 0.1 mM Mg$^{2+}$ (Fig. 2b). This result indicates that OsMRS2-6 would potentially be a high-affinity Mg$^{2+}$ transporter. Interestingly, AtMRS2-11/AtMGT10, the counterpart of OsMRS2-6 in clade A, also had a high ability to complement the CM66 mutant (Li et al. 2001, Drummond et al. 2006). However, in Arabidopsis, observations based on complementation efficiency varied depending on the assay. For example, AtMRS2-10/AtMGT1 was suggested to be a high-affinity Mg$^{2+}$ transporter based on the complementation of the Salmonella MM281 strain in medium containing 10 µM Mg$^{2+}$ (Li et al. 2001). In contrast, the expression of the protein in the yeast CM66 mutant only slightly increased growth (Drummond et al. 2006). In this regard, Drummond et al. (2006) suggested that the probability of plasma membrane localization in the host organism could affect complementation efficiency. Furthermore, AtMRS2-7/AtMGT7 was suggested to be a low-affinity Mg$^{2+}$ transporter in view of the finding that the expression of this protein could complement the MM281 strain as effectively as the expression of AtMRS2-10/AtMGT1 only when the medium contained Mg$^{2+}$ in concentrations of at least 5 mM (Mao et al. 2008), whereas the localization of this protein in the mitochondrial membrane could rescue the Mg$^{2+}$ uptake limitation of mrs2 mutant mitochondria as effectively as the localization of AtMRS2-10/AtMGT1 under Mg$^{2+}$ concentrations of 1–9 mM (Gebert et al. 2009). Because AtMRS2-7/AtMGT7 complemented mrs2 respiratory deficiency more...
Cor-A family proteins with the GMN motif, has been reported to have Mg²⁺ transport ability in T. maritima (Knoop et al. 2005). In this study, the protein sequences of all the OsMRS2s were determined based on the isolated mRNA sequences, and the alteration of the GMN motif to AMN in OsMRS2-4 and OsMRS2-5 and to GIN in OsMRS2-8 was confirmed (Fig. 1a). A search of the NCBI nucleotide database suggested that the AMN tripeptide is conserved in monocots such as Sorghum bicolor (Sb01g030170), Hordeum vulgare (AK370290) and Phyllostachys edulis (FP1000010). The significance of the GMN motif to Mg²⁺ transport ability has been described, given that this motif is highly conserved (Bui et al. 1999, Knoop et al. 2005) and that Mg²⁺ transport activity of yeast MRS2 protein was abolished when the glycine residue of the GMN motif was changed into alanine (Kolisek et al. 2003). However, the specific role of the glycine residue of the GMN motif in Mg²⁺ transport ability has not been identified, while the participation of asparagine residues in the structure of an asparagine ring at the entrance of the channel was described based on the recently revealed MycCorA crystal structure (Guskov et al. 2012). In addition, TmcCorA in T. maritima which is one of the best-researched CorA family proteins with the GMN motif, has been reported to be a highly selective cobalt transporter with no Mg²⁺ transport regulation capability (Xia et al. 2011). Accordingly, the in vivo function of each CorA family protein may differ in each species, so that any generalization of the CorA-MRS2-ALR-type protein should be avoided as yet (Xia et al. 2011). The Asn314 residue, a part of the GMN motif, is located at the periplasmic entrance and is suggested to restrict the pore for cation transport in TmCorA (Lunin et al. 2006). In the TmCorA crystal structure, the ϕ and ψ angles of Gly312 of the GMN motif were in the ranges from −93° to −78° and from −0.5° to 13.8°, respectively. In a Ramachandran plot, these main chain dihedral angles are within the ‘favored’ region for a α helix, indicating that Gly312 forms part of a transmembrane helix. Considering the ϕ and ψ values, a substitution of the glycine with alanine would not distort the protein structure and function. A recent report has even implied the involvement of residues in addition to the GMN motif in Mg²⁺ selectivity (Guskov et al. 2012). Given this situation, it remains to be clarified whether OsMRS2-4 and OsMRS2-5, which have a naturally occurring AMN variant, have the function of transporting Mg²⁺ or another divalent cation, although all three proteins lacking the GMN motif failed to complement the Cm66 mutant (Fig. 2a, b).

The presence of a GIN tripeptide has been previously reported as a variant of the originally defined GxN motif, and this alteration was proposed to be associated with cation selectivity (Knoop et al. 2005). The Salmonella typhimurium ZntB protein contains a GIN tripeptide in place of the GxN motif and is regarded as a zinc transporter (Worlock and Smith 2002). Thus, OsMRS2-8 may be capable of transporting divalent cations other than Mg²⁺, possibly Zn²⁺.

One of the characteristics of the Arabidopsis MRS2/MGT family is the diversity of subcellular localization: AtMRS2-10/AtMGT1 localizes to the plasma membrane (Li et al. 2001), AtMRS2-11/AtMGT10 to the chloroplast envelope (Froehlich et al. 2003, Drummond et al. 2006), AtMRS2-7/AtMGT7 to the ER (Gebert et al. 2009), AtMRS2-6/AtMGT5 to mitochondria (Li et al. 2008), AtMRS2-1/AtMGT2 and AtMRS2-5/AtMGT3 to the vacuoles (Conn et al. 2011) and AtMRS2-4/AtMGT6 to either chloroplasts or mitochondria (Gebert et al. 2009, Conn et al. 2011). In rice, a similar localization diversity is found; OsMRS2-5 and OsMRS2-6 were shown to be localized to the chloroplast (Fig. 3) and OsMRS2-3 potentially to the ER, and OsMRS2-2/OsMGT1 was found to be a plasma membrane Mg²⁺ transporter (Chen et al. 2012). In addition, OsMRS2-4, belonging to clade D, was found to carry a potential mitochondrial targeting signal at the N-terminus (Fig. 1a). From a phylogenetic point of view, it might now be suggested that clade A may be defined as a chloroplast transporter group, clade B as a plasma membrane and vacuolar membrane transporter group, and clade D as a mitochondria and chloroplast transporter group. Clade E contains only one member whose localization has been shown, and two members in clade C were found to localize to different organelles, with the result that no definition of these two clades has been possible to date. Furthermore, the finding that the export of proteins from the ER may be dependent on a specific motif required for the interaction with a coat protein complex II component (Mikosch et al. 2009) indicates that the ER localization of the artificial GFP-linked protein may not necessarily be evidence of the intrinsic ER localization of that protein. In this regard, OsMRS2-3 has the potential to be targeted in vivo to organelles other than the ER. In clade A, OsMRS2-6 has the one-to-one counterpart AtMRS2-11/AtMGT10 (Fig. 1b). These two genes showed common characteristics, including chloroplast localization (Fig. 3), gene expression even in the root (Fig. 4) (Drummond et al. 2006) and a diurnal change in the expression level (Fig. 5), although the levels peaked at different times of the day (Drummond et al. 2006). The available A. thaliana microarray database indicated that AtMRS2-11/AtMGT10 showed an expression pattern similar to that of the photosystem-related genes, and their expression levels remained high for several hours during the light period. In contrast, the expression level of OsMRS2-6 was shown to be extremely high around dawn (Fig. 5), mirrored by the expression patterns of some genes encoding light-harvesting complex proteins in rice. It could thus be speculated that the expression of these genes is managed as part of the chloroplast apparatus, and that they function to supply Mg²⁺ to the chloroplast stroma during the daytime. In spinach, the Mg²⁺ concentration in stroma was found to be significantly higher in the light than in the dark period (Portis 1981, Ishijima et al. 2003). All their common features strongly suggest an orthologous relationship between OsMRS2-6 and AtMRS2-11/AtMGT2-10. Accordingly, a phylogenetic tree of clade A of the plant MRS2/MGT transporter family was constructed to identify chloroplast Mg²⁺ transporters (Fig. 7). It suggests that
a wide variety of photosynthetic organisms possess OsMRS2-like proteins.

In this study, OsMRS2-6 and -5 were suggested to be chloroplast Mg$^{2+}$ transporters. However, there were several differences between the two members. OsMRS2-5 expression did not show a diurnal change (Fig. 5) and was less associated with leaf maturation (Fig. 6). Furthermore, the expression level of OsMRS2-5 was markedly lower than that of OsMRS2-6 (Fig. 5). Based on these findings, we propose that OsMRS2-6, rather than OsMRS2-5, contributes largely to the Mg$^{2+}$ transport into the chloroplast. Further characterization of these two members is required to clarify the functional significance of each member in photosynthesis and/or metabolism in the chloroplast.

**Materials and Methods**

**Plant culture conditions**

Rice seedlings (O. sativa L. cv. Nipponbare) were grown in half-strength Kimura B nutrient solution (pH 5.6) in a growth chamber set to 30°C and 70% humidity (Tanoi et al. 2011). The day/night cycle was 16/8 h, and the photosynthetic photon flux density was 100 μmol m$^{-2}$ s$^{-1}$ during the daytime. After the 25th day of germination, tiller development began. The plants were then transplanted to culture soil (Bonsol No. 2; Sumitomo Chemical) and cultured in a greenhouse until ears had developed.

**Isolation and sequencing of full-length OsMRS2**

Based on the predicted full-length cDNA sequences of all the OsMRS2 genes obtained by searching the Rice Annotation Project Database (RAP-DB; Ohyanagi et al. 2006) and the Knowledge-based Oryza Molecular biological Encyclopedia (KOME, Kikuchi et al. 2003), primers to amplify the entire coding region of every OsMRS2 gene were constructed (Supplementary Table S1). Following this step, RT–PCR was performed to amplify the entire coding regions of all OsMRS2 genes from mRNA isolated from the rice seedlings. The amplified cDNA fragments were cloned into the pGEM$^{	ext{TM}}$-T Easy vector (Promega) for sequencing.

**Phylogenetic tree construction**

Protein sequences of the AtMRS2/AtMGT family members used for phylogenetic analysis were based on the GenBank database. Other sequences homologous to OsMRS2-6 were obtained by BLAST search of the databases at NCBI (USA) and Kazusa DNA Research Institute (Japan). Multiple sequence alignment was performed with the ClustalW module within MEGA v.5. (gap opening penalty = 10, gap extension penalty = 0.2, protein weight matrix = Gonnet) (Tamura et al. 2011). Bootstrap values were calculated from 10,000 replicates to construct a phylogenetic tree by the Neighbor–Joining method.

**Complementation assay in a yeast mutant**

The Mg$^{2+}$ transport ability of each OsMRS2 protein was examined by a yeast complementation assay. All nine OsMRS2 genes were amplified from full-length cDNA clones using the PCR primers lying downstream from their start codon and just upstream of the stop codon (Supplementary Table S1). Each open reading frame having no stop codon was ligated into the Gateway$^{	ext{®}}$ pENTR/D-TOPO vector (Invitrogen), and...
transferred into the destination vector pYES-DEST52 (Invitrogen) to construct the C-terminal GFP fusion proteins. The yeast CM66 mutant strain, which lacks the plasma membrane-localized Mg\(^{2+}\) transporter genes ALR1 and ALR2 (Li et al. 2001), was transformed with the pYES-DEST52-OsMRS2 plasmid or the empty vector as a negative control. Transformants were identified by growth on ampicillin-containing medium. The growth capability of each transformant on solid medium containing 1 mM Mg\(^{2+}\) and liquid medium containing 0.1 or 1 mM Mg\(^{2+}\) was determined.

For the solid medium experiment, 10\(^2\)–10\(^4\) yeast cells were spotted on the medium and cultured for 2 d. For the liquid medium experiment, growth curves of transformants were determined over 36 h using a plate reader (DS Pharma Biomedical). CM52, the wild-type yeast strain (Li et al. 2001), was transformed with the empty vector, was used as the positive control.

Mg uptake of CM66 transformed with OsMRS2-6 was compared with that of CM52 and CM66 transformed with the empty vector. Radioactive \(^{28}\)Mg as a tracer was prepared following Iwata et al. (1992) at the National Institute of Radiological Sciences (NIRS, Japan). During log-phase growth in a synthetic complete medium lacking uracil and containing galactose (SC-URA) and 10 mM MgCl\(_2\), the supernatant following centrifugation at 500 g for 2 min was discarded and the yeast cells were resuspended in the SC-URA without Mg. The yeast strains were further washed three times by centrifugation and resuspended in SC-URA without Mg to grow for 24 h. They were then cultured in SC-URA liquid medium containing \(^{28}\)Mg (40 kBq ml\(^{-1}\)) and 1.0 mM MgCl\(_2\) as a carrier at 4 or 30°C. After 30 min of treatment, each yeast strain was washed three times with SC-URA containing 10 mM MgCl\(_2\) by centrifugation at 500 g for 2 min. Measurement of \(^{28}\)Mg was performed using a gamma counter (Hitachi-Aloka), and the OD\(_{600}\) was determined to calculate the Mg\(^{2+}\) uptake rate. The Mg\(^{2+}\) amount absorbed at 4°C was subtracted from that absorbed at 30°C to determine the actual Mg\(^{2+}\) uptake rate.

Subcellular localization using transient rice protoplast transformation

The 35 S promoter:OsMRS2-x–GFP construct was produced as follows: full-length cDNA cloned into the Gateway\(^\text{®}\) pENTR/D-TOPO vector (Invitrogen) was transferred into the pUGWS vector (Nakagawa et al. 2007). Rice protoplasts were isolated from shoots of 12-day-old seedlings according to a previously described procedure (Chen et al. 2006), with modification of the enzyme solution: 2.0% cellulase R-10 (Yakult), 1.0% macerozyme R-10 (Yakult), 0.6 M mannitol, 1.0% bovine serum albumin and 0.1% CaCl\(_2\). Each pUGWS vector harboring the OsMRS2-x–GFP genes was introduced into the isolated protoplasts using polyethylene glycol as previously reported (Chen et al. 2006). The GFP signal was detected using an FV500 confocal laser microscope (Olympus).

Semi-quantitative transcription analysis

Three-week-old rice seedlings were sampled and divided into the youngest leaf, mature leaves and the root. At the tiller developmental stage, the shoot and root parts were sampled. Flag leaves and flowers were collected from mature rice plants. RNA extraction was performed using the Gen pureRNA Kit (DOJINDO), and first-strand DNA was produced using SuperScriptIII First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). An RT–PCR product for each OsMRS2 gene was amplified with a thermal cycler (Applied Biosystems) using the primers listed in Supplementary Table S1. The rice actin 1 gene (OsRac1; Os03g0718100) was used as a reference gene.

Quantitative transcription analysis

To investigate diurnal rhythmicity in gene expression, young mature leaf blades and leaf sheaths of 3-week-old seedlings were sampled for RNA isolation every 4 h from the beginning of the light period. Leaf blades were also sampled at 4 h of the light period, when leaves were just emerging, maturing or completely matured, to investigate the expression change related to leaf maturation. For emerging leaf blades, the samples were separated into the upper green part, which was about to expand, and the lower part, which had not yet emerged from the sheath of the older leaf and was yellow–green in color, as illustrated in Fig. 6. RNA was extracted using the RNeasy Plant Mini Kit (Qiagen), and a 400 ng aliquot of RNA was used for cDNA synthesis using SuperScriptIII First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen) according to the manufacturer’s instructions. The gene expression level was quantified by real-time RT–PCR using Fast SYBR Green Master Mix (Invitrogen) on the StepOne\textsuperscript{TM} Real-Time RT-PCR System (Applied Biosystems). The primers used are listed in Supplementary Table S1. Each plasmid dilution from 16 to 10,000 copies per aliquot was amplified to prepare a calibration curve for quantitative determination.

Supplementary data

Supplementary data are available at PCP online.

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References


