Functional Analysis of Methylthioribose Kinase Genes in Plants[1]

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Through a biochemical and a genetic approach, we have identified several plant genes encoding methylthioribose (MTR) kinase, an enzyme involved in recycling of methionine through the methylthioadenosine (MTA) cycle. OsMTK1, an MTR kinase from rice (*Oryza sativa*), is 48.6 kD in size and shows cooperative kinetics with a V_{max} of 4.9 pmol/min and a $K_{0.5}$ of 16.8 μM. MTR kinase genes are the first genes to be identified from the MTA cycle in plants. Insertional mutagenesis of the unique *AtMTK* gene in Arabidopsis (*Arabidopsis thaliana*) resulted in an inability of plants to grow on MTA as a supplemental sulfur source. *MTK* knock-out plants were not impaired in growth under standard conditions, indicating that the MTA cycle is a nonessential metabolic pathway in Arabidopsis when sulfur levels are replete. In rice, *OsMTK* genes were strongly upregulated in shoots and roots when plants were exposed to sulfur starvation. Gene expression was largely unaffected by lack of nitrogen or iron in the nutrient solution, indicating that *OsMTK* regulation was linked specifically to sulfur metabolism.

Met is a sulfur-containing amino acid that can be activated by ATP to *S*-adenosyl-Met (AdoMet). AdoMet serves as a substrate in many biochemical reactions. When AdoMet is utilized for the synthesis of ethylene, certain polyamines, and siderophores, methylthioadenosine (MTA) is produced as a by-product. MTA can be recycled to Met, thus allowing high rates of ethylene, polyamine, or siderophore synthesis without net consumption of Met. This Met salvage pathway has been characterized and was described in plants at the biochemical level in the 1970s and 1980s (Wang et al., 1982; Yang and Hoffman, 1984; Miyazaki and Yang, 1987). It occurs not only in plants but also in bacteria and in animals and is termed the MTA cycle. Recent progress in detailing enzyme characteristics and in identifying their corresponding genes has come almost exclusively from work on prokaryotes (Sufrin et al., 1995; Cornell et al., 1996; Sekowska et al., 2001; Sekowska and Danchin, 2002).

In bacteria and in plants, MTA is depurinated to 5-methylthioribose (MTR) through the enzymatic activity of MTA nucleosidase. MTR kinase catalyzes the subsequent phosphorylation of the C-1 hydroxyl group of the Rib moiety of MTR to yield 5-methylthio-Rib-1-P. In animals, MTA phosphorylase carries out both functions in a single ATP-independent step (Schlenk, 1983). Hence, MTR kinase exists in prokaryotes and plant species but not in animals. 5-Methylthio-Rib-1-P subsequently undergoes enzymatic isomerization, dehydration, and oxidative decarboxylation to 2-keto-4-methylthiobutyrate, the immediate precursor of Met (Miyazaki and Yang, 1987).

Bacterial MTR kinases have been partially purified from *Enterobacter aerogenes* (Ferro et al., 1978) and *Klebsiella pneumoniae* (Gianotti et al., 1990), and their biochemical function has been studied in *K. pneumoniae* (Cornell et al., 1996) and *Bacillus subtilis* (Sekowska et al., 2001). In plants, MTR kinase has been partially purified from seeds of *Lupinus luteus* (Guranowski, 1983). Genes encoding MTA cycle enzymes, including MTR kinase, have so far only been described in microbes but not in plants. Through biochemical and molecular genetic studies, we identified and studied three MTR kinase genes from plants: two from Oryzae (*OsMTK1* and *OsMTK2*) and one from Arabidopsis (*Arabidopsis thaliana*; *AtMTK*). The ability to manipulate these genes has in turn allowed us to study the significance of the MTA cycle in plant growth and development.

RESULTS

MTR Kinases Are Encoded by Unique Genes in Most Plants

With the amino acid sequence of MTR kinase from *K. pneumoniae* as query (Fig. 1; accession no. AF212863) BLAST searches were performed in the translated rice (*Oryza sativa*) genomic database. Two sequences were found that showed 34%/60% and 33%/60% identity/similarity to Klebsiella MTR kinase. The proteins were termed OsMTK1 and OsMTK2. *OsMTK1* and *OsMTK2* genes are located in tandem on chromosome 4 and encompass nucleotides 103,839 to 100,698 (*OsMTK1*) and nucleotides 109,551 to 106,919 (*OsMTK2*) of the rice genomic BAC sequence OSJNBa0043A12. Through

[1] This work was supported by the Deutsche Forschungsgemeinschaft (grant no. SA 495/7–1 to M.S.). K.A.C. received support from U.S. Department of Agriculture (grant no. 02–00047).

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.053587.

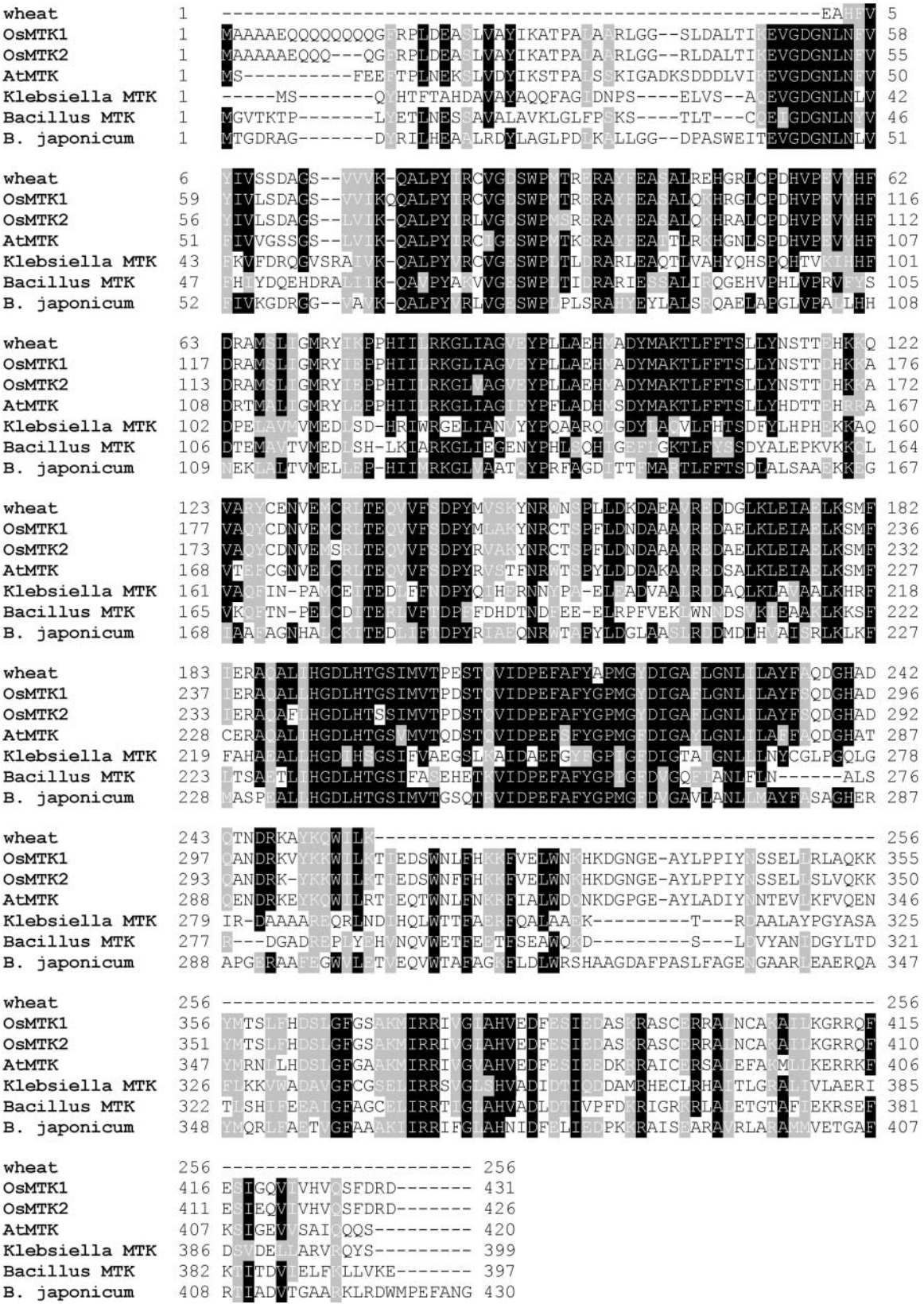

Figure 1. Sequence alignments of MTR kinase proteins from bacteria and plants. MTR kinase homologs from rice (accession nos. AY593959 for OsMTK1 and CAE02820 for OsMTK2), wheat (accession nos. CK171718 and BQ804437), tobacco (accession no. CK298305), Arabidopsis (accession no. NP_564555), *K. pneumoniae* (accession no. AF212863), *B. subtilis* (accession no. O31663), and *Bradyrhizobium japonicum* (accession no. NP_771538) were aligned. Identical amino acids are shaded black. Amino acids conserved in at least 70% of the sequences are shaded gray.

comparison with rice expressed sequence tag (EST) sequences found in the National Center for Biotechnology Information BLAST EST database, the genes were annotated and an open reading frame was identified for each gene consisting of 1,293 nucleotides encoding 431 amino acids for OsMTK1 (accession no. AY593959) and of 1,278 nucleotides encoding 426 amino acids in the case of OsMTK2 (accession no. CAE02820). The deduced polypeptides had a molecular mass of 48,600 D and 48,200 D, respectively. The two OsMTK proteins showed 95% overall identity to each other (Fig. 1). Further sequence comparison revealed that intron sequences of *OsMTK1* and *OsMTK2* were more than 60% identical.

An Arabidopsis MTR kinase homolog was identified on chromosome 1, where it is encoded by the gene At1g49820. The deduced protein had 71% identical and 86% similar amino acids to OsMTK1 (Fig. 1). Arabidopsis has only one copy of the MTR kinase gene, which we termed *AtMTK*. In EST databases, open reading frames with high similarity to rice MTK were identified from orange (*Citrus sinensis*), tobacco (*Nicotiana benthamiana*), tomato, potato (*Solanum tuberosum*), soybean (*Glycine max*), *Vitis vinifera*, *Medicago truncatula*, barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), maize (*Zea mays*), onion (*Allium cepa*), *Sorghum bicolor*, and *Saccharum officinarum*, but no homologs were found in animals or yeast. This result is consistent with the fact that, in animals, MTA recycling does not involve hydrolysis of MTA to MTR by MTA nucleosidase and subsequent phosphorylation of MTR by MTR kinase as in plants and in bacteria. Instead, MTA is phosphorylated and cleaved to methylthio-Rib-1-P and adenine in one step by the enzyme 5′-deoxy-5′-methylthioadenosine phosphorylase (Schlenk, 1983; Thomas and Surdin-Kerjan, 1997). Our homology search did not reveal a yeast MTK homolog and thus confirmed previously published biochemical data indicating that yeast conforms to the animal-type Met recycling pathway with phosphorylytic cleavage of MTA (Marchitto and Ferro, 1985).

For many plant species, more than one MTK EST was found. However, these EST sequences were redundant, encoding identical amino acid sequences for each species. With the exception of two soybean partial cDNAs (97.2% identical) and six ESTs from hexaploid wheat (93% or more identical), all ESTs from one species were identical at the nucleotide level, strongly supporting the idea that in most plants a single-copy gene encodes MTR kinase. The sequence differences found at the nucleic acid level in ESTs from soybean and wheat might represent genotypic variance. Rice is an exception to that general observation in that it possesses two MTK genes. The two genes from rice are highly conserved not only with respect to the encoded proteins, as described, but also with respect to intron/exon structure (data not shown). Furthermore, they share 60% overall identity in intron sequences (data not shown). Taken together with the fact that *OsMTK1* and *OsMTK2* are located in tandem on the genome, we suggest that they have evolved only recently through gene duplication. Most ESTs identified from rice were identical to the corresponding OsMTK1 sequence, indicating that OsMTK1 was more abundantly expressed than OsMTK2. In the remaining EST sequences, it was not possible to decide if they derived from OsMTK1 or OsMTK2, as these were more than 95% identical at the nucleic acid level.

Database searches performed in the BLAST conserved-domain database (Marchler-Bauer et al., 2003) revealed that the OsMTK protein contained the putative conserved domain COG4857. Proteins with this domain are predicted to be kinases. In exon 4, a domain of unknown function (DUF 227) rich in His and Asp was identified, possibly indicating a metal-binding function. The software EMBL-EBI InterProscan (Mulder et al., 2003) classified OsMTK proteins as members of the MTR kinase family. Since no obvious targeting signals were detected with the programs TargetP (Emanuelsson et al., 2000) and PSORT (Nakai and Kanehisa, 1991), we hypothesize that MTK proteins are localized in the cytoplasm.

OsMTK1 Encodes an Enzyme with MTR Kinase Activity

Recombinant OsMTK1 protein from rice was overexpressed in *Escherichia coli* and purified by GSH-affinity chromatography to study enzyme activity. The specific activity was determined to be 19.4 nmol min^{-1} mg of $enzyme^{-1}$. This activity is much lower (approximately 3- to 30-fold less) than that found for *B. subtilis* and *K. pneumoniae* MTR kinases. Several factors could account for this lower specific activity: it may represent innate activity characteristics specific to OsMTK1, or be due to effects arising from the fusion protein engineering scheme that in turn affected enzyme activity. A plot of substrate versus velocity yielded a sigmoidal-shaped rather than a parabolic curve that suggested cooperative enzyme kinetics (Fig. 2). Kinetic constants were calculated from nonlinear regression analysis of substrate-velocity data using the equation: $V_o = V_{max}/[1 + (S/K_{0.5})^n]$. Curve fitting was accomplished by sums of squares minimization of residual differences (least-squares fitting; Bowen and Jorman, 1995). The estimated $K_{0.5}$ for the substrate MTR at ATP = 4 mM was 16.8 μM (Fig. 2A). The $K_{0.5}$ [MTR] of OsMTK1 is thus quite similar to the K_m value of 12.2 μM reported previously for the *K. pneumoniae* MTK enzyme (Cornell et al., 1996). The estimated $K_{0.5}$ of OsMTK1 for the substrate ATP at MTR = 100 μM was 184 μM (Fig. 2B). The maximal enzyme velocity was 4.9 pmol/min. Evidence of cooperative enzyme behavior was indicated by calculated Hill coefficients of 2.4 for MTR and 4.9 for ATP. This result is not unexpected since previous reports indicate that both bacterial and plant MTR kinases associate as dimers in their native state (Guranowski, 1983; Cornell et al., 1996; Ku et al., 2004).

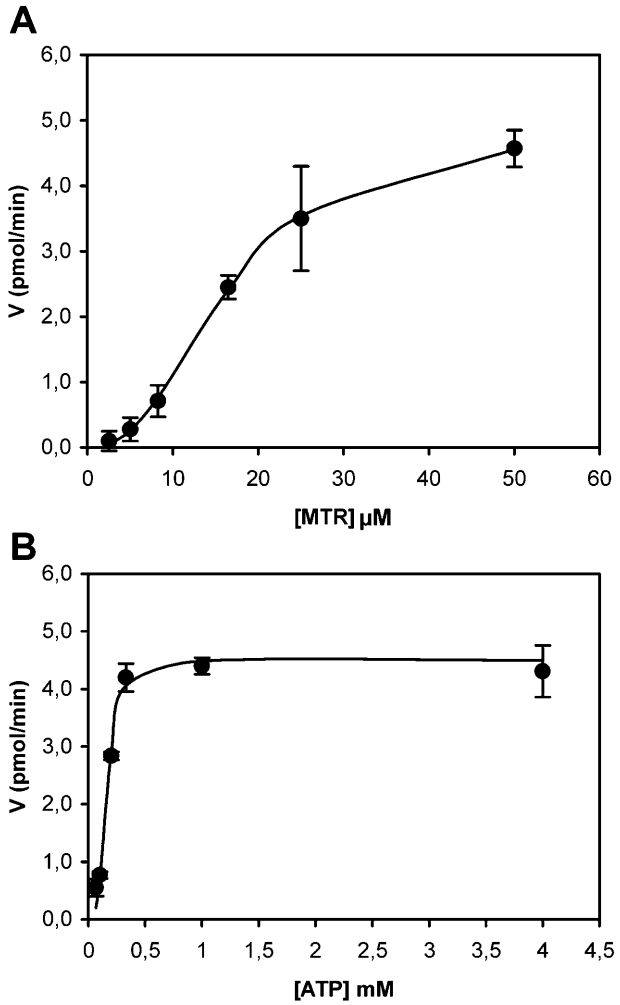

Figure 2. Substrate versus velocity plot of recombinant OsMTK1 activity. The estimated $K_{0.5}$ for the substrates (A) MTR at ATP = 4 mM was 16.8 μM (sum of square = 0.009) and (B) ATP at MTR = 100 μM was 184 μM (sum of square = 0.008). The maximal velocity was 4.9 pmol/min. Evidence of cooperative enzyme behavior is indicated by calculated Hill coefficients of 2.4 for MTR and 4.9 for ATP. Data points are averages of three determinations.

OsMTK Expression Is Not Induced in Submerged Deepwater Rice

AdoMet is a precursor of ethylene biosynthesis. AdoMet is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS) producing MTA as a by-product. Thus, the MTA cycle is directly linked to ethylene synthesis in plants. In deepwater rice, submergence triggers ethylene formation in the youngest internode (Stünzi and Kende, 1989) through increased ACS activity. ACS activity is elevated mainly in the growing region, i.e. in the intercalary meristem and in the elongation zone of the internode (Cohen and Kende, 1987). We used the deepwater rice system to study expression of *OsMTK* after promotion of ethylene synthesis by submergence treatment (Fig. 3). In nonsubmerged control plants, *OsMTK* expression in the youngest internode was higher in differentiated

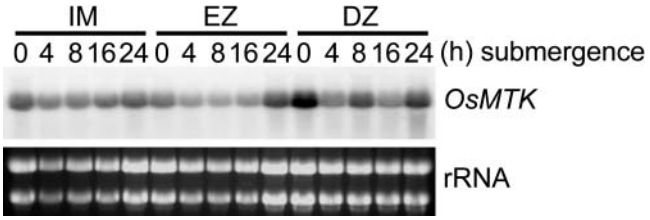

Figure 3. Regulation of *OsMTK* expression in deepwater rice. RNA was isolated from the intercalary meristem, and the elongation and the differentiation zones of submerged deepwater rice plants. Ethidium bromide-stained ribosomal RNA is shown as a control for loading. The observed changes in *OsMTK* expression were confirmed in a second independent experiment (data not shown).

tissue than in the intercalary meristem or in the elongation zone (Fig. 3, 0 h). When rice plants were partially submerged, *OsMTK* transcript levels in the differentiation zone strongly declined within 4 h, while transcript abundance remained largely unaltered in the growing region of the internode (Fig. 3). This is particularly obvious after 1 d of submergence, when ethylene production was induced yet *OsMTK* expression was not. A spatial or temporal correlation between ethylene biosynthetic rate and *OsMTK* gene expression was thus not observed.

OsMTK Is Up-Regulated under Sulfur-Limiting Conditions

MTA is a sulfur-containing compound. The thio group contained in MTA is salvaged through the MTA cycle. Many genes involved in sulfur metabolism have been shown to be up-regulated when sulfur becomes limiting. To investigate if regulation of *OsMTK* genes was linked to sulfur metabolism, we subjected rice plants to sulfur shortage. As controls, we included treatment of plants without nitrogen or without iron in the nutrient solution. Phenotypic analysis indicated that 3 weeks of starvation caused reduced shoot growth and chlorosis in plants grown without nitrogen (Fig. 4A). No phenotypic changes were observed in shoots after starvation with Fe^{2+} or when plants were starved for sulfur as compared to plants supplied with a complete nutrient solution (Fig. 4A).

Following starvation treatment, *OsMTK* mRNA levels were measured in shoots and roots from plants subjected to one of the four treatments (complete, sulfur deficient, nitrogen deficient, iron deficient). A low level of expression was observed in both roots and shoots, with slightly higher mRNA levels in shoots when plants were supplied with sufficient mineral nutrients (Fig. 4B). Regulation through an altered mineral supply was observed in iron-, sulfur-, and nitrogen-deficient medium, albeit to very different degrees. Omission of iron or nitrogen from the nutrient solution resulted in slight down-regulation of *OsMTK* expression. Very high gene induction was observed in sulfur-deficient plants. Specific up-regulation of

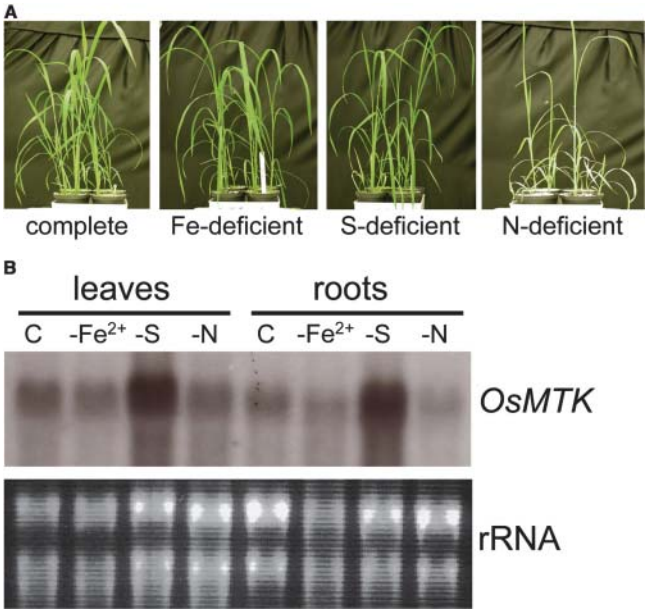

Figure 4. *OsMTR* is specifically induced by sulfur deficiency. A, Five-week-old rice plants which were grown for the last 2 weeks on complete medium, on medium lacking iron, on medium lacking a sulfur source, or on medium lacking a nitrogen source. B, Northern-blot analysis of roots and leaves harvested from the plants shown in A. C indicates growth of plants on complete medium; $-Fe^{2+}$, −S, and −N indicate growth of plants on medium lacking iron, sulfur, or nitrogen.

OsMTK expression in response to sulfur deficiency provided a regulatory link between the MTA cycle and general sulfur metabolism. Analysis of sulfur-containing amino acids in shoots and roots of the same plants that were used for northern-blot analysis indicated that Met levels were lower in plants grown in Fe^{2+}-deficient, sulfur-deficient, and nitrogen-deficient media as compared to plants grown with a complete mineral nutrient supply, indicating that Met is not the direct signal that triggers *OsMTK* gene regulation in rice (data not shown). In yeast, AdoMet has been shown to be the crucial metabolite for regulation of the sulfur network (Thomas and Surdin-Kerjan, 1997), and recent reports support an essential role for AdoMet in the posttranscriptional regulation of plant Met biosynthetic enzymes as well (Chiba et al., 2003).

Insertional Mutagenesis Results in Knock Out of *AtMTK* in Arabidopsis

Knockout mutants of Arabidopsis were used as a genetic tool to study *MTR* kinase gene function. Arabidopsis has one assigned *MTR* kinase gene (Fig. 1), for which we identified two T-DNA insertion mutants in the Sequence-Indexed Library of Insertion Mutations in the Arabidopsis Genome collection at the Salk Institute (La Jolla, CA) designated line 532,926 and line 633,954. These were obtained and insertion of the T-DNA in *AtMTK* was verified through PCR analysis. Line 532,926 harbored an insertion in the promoter or 5′ untranslated region of *AtMTK*, whereas line 633,954 harbored an insertion between exons 5 and 6 (Fig. 5A). Homozygous plants from each line were selected to study transcription of *AtMTK* in the T-DNA insertion lines. Transcript levels were compared in wild-type and mutant plants using reverse transcription (RT)-PCR. RT-PCR with primers AtMTK-R3 and AtMTK-F3 (Table I) were expected to amplify a fragment of 1,314 bp from full-length *AtMTK* cDNA (Fig. 5B). Amplification from wild-type cDNA produced a 1.3-kb fragment (Fig. 4C). The same size fragment was amplified from line 532,926, whereas no product was obtained for line 633,954 (Fig. 5C). This finding suggested that T-DNA insertion in the 5′ untranslated or promoter region of *AtMTK* of line 532,926 did not affect gene transcription. On the other hand, insertion in intron 5 of *AtMTK* prevented synthesis of a full transcript in line 633,954.

To analyze if a truncated mRNA of *AtMTK* was present in line 633,954, RT-PCR with primers AtMTK-R1 and AtMTK-F3 (Table I) was performed. It was expected to produce a DNA fragment 515 bp in size (Fig. 5B). In wild type and in both T-DNA-tagged lines, a product of approximately 500 bp was obtained (Fig. 5C). However, the amount of amplified product was much lower for line 633,954 than in wild type or line 532,926, possibly due to degradation of the truncated mRNA produced in line 633,954. The results indicated that mutant line 633,954 was knocked out for expression of intact *AtMTK* mRNA. We therefore refer to line 633,954 plants as knock-out mutant. In line 532,926 *AtMTK* transcription did not appear to be affected.

AtMTK Knock-Out Plants Cannot Utilize MTA as Sulfur Source

Growth and development of wild-type and knock-out mutant plants were observed under normal growth conditions. Throughout the life cycle, no phenotypic differences were obvious between wild type and mutant (Fig. 6; data not shown), indicating that expression of *AtMTK* was not required for normal plant development under sulfur-replete conditions. Since *MTK* function relates to sulfur metabolism, we studied seedling and plant development under sulfur-limiting conditions. Arabidopsis seeds from wild-type, *AtMTK* knock-out line 633,954, and insertion line 532,926 plants were placed on different media with or without sulfur supplemented as SO_4^{2-}, Met, or MTA at different concentrations. Plants grown on complete medium served as a positive control, whereas plants grown on medium deficient in sulfur were used as a negative control. Morphological differences between nutrient-starved and supplemented plants first became visible after 10 to 12 d (data not shown). Effects on shoot growth were assayed after 3 weeks.

Plants from all three lines showed severe growth retardation when placed for 3 weeks on media without a sulfur source (Figs. 6, A and B, and 7). No differences between mutants and wild type were observed under

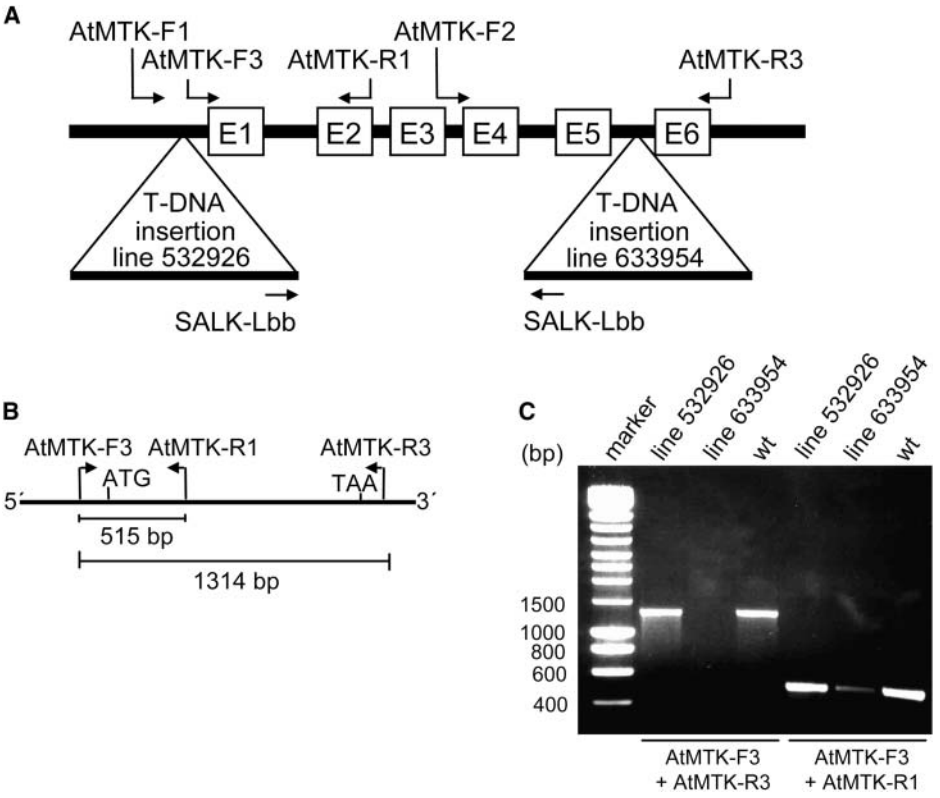

Figure 5. Characterization of Arabidopsis insertional mutant lines 633,954 and 532,926, in which *AtMTK* is tagged. A, Structure of *AtMTK* showing the location of the T-DNA insertions found in lines 532,926 and 633,954 and positions of gene-specific primers used to study the mutants. B, Schematic drawing of *AtMTK* cDNA and of the fragments amplified from mRNA by reverse transcription using the primers indicated. C, Agarose gel showing DNA fragments amplified from reverse-transcribed mRNA using the primers indicated in B. Line 633,954 expressed only truncated *AtMTK* mRNA at lower abundance compared to wild type or line 532,926.

these conditions, which caused arrested growth and induced reddish coloring of shoots that was attributed to stress-induced anthocyanin production (Fig. 6B). Supplementing sulfur-deficient media with SO_4^{2-} resulted in a dose-dependent recovery of shoot growth that was comparable in wild type and both mutant lines (Figs. 6C and 7A). While 5 μM SO_4^{2-} was not sufficient to alleviate growth retardation, addition of 50 μM SO_4^{2-} and 500 μM SO_4^{2-} led to partial and complete recovery of shoot growth, respectively (Figs. 6, A and C, and 7A). Supplementation with Met was sufficient to partially restore growth (Figs. 6, A and D, and 7B). However, even at 500 μM Met, shoot fresh weight was restored to only about two-thirds of that obtained with complete media or with 500 μM SO_4^{2-} as sulfur source (Fig. 7B).

Table I. *Primer sequences*

Primer	5′ → 3′ sequence
AtMTK-F1	GTACCAAAAGCCTATCATATGG
AtMTK-F2	GATGATGATGCTAAGGCTGTG
AtMTK-F3	AAGCAGAGAAGCAAAGAGATG
AtMTK-R1	CTTCTGTGCTCTGTGGTATCG
AtMTK-R3	CAAGAACTTCAGCAGAAGAAG
Salk-Lbb	GCGTTCGAGCAGGGACTC
OsMTK-F1	GAATTCGCCGCGGCGGCGGAGCAG
OsMTK-R1	GCGGCCGCATCAGTCCCGATC
5′pGEX	GGGCTGGCAAGCCACGTTTGGTG
3′pGEX	CCGGGAGCTGCATGTGTCAGAGG

Again, no differences in growth response were observed between mutant and wild-type plants.

In order to see if plants were capable of utilizing MTA as sulfur source, we supplemented sulfur-deficient media with MTA. Wild-type and mutant plants grown on plates containing 5 μM or 50 μM MTA showed no significant increase in fresh weight (Fig. 7C). With 500 μM MTA added to sulfur-deficient media, wild-type plants and plants from insertion line 532,926 showed partial recovery of shoot growth (Figs. 6, A, B, and E, and 7C). By contrast, plants of the knock-out mutant line 633,954 did not grow on 500 μM MTA (Figs. 6, A, B, and E, and 7C). Their shoot fresh weight was comparable to that of plants grown without sulfur. After 3 weeks of growth on 500 μM MTA, wild-type plants and plants from the ineffectual insertion mutant line 532,926 were twice as big as plants of the MTR kinase knock-out mutant. The difference in fresh weight of wild-type and knock-out mutant plants grown on 500 μM MTA was statistically significant at a $P < 0.001$ ($F = 45.24$, degrees of freedom = 1.94) according to ANOVA.

DISCUSSION

In plants, MTA is generated from AdoMet as a by-product of ethylene, polyamine, and siderophore biosynthesis. It is recycled to Met in a biochemical pathway termed the MTA cycle (Miyazaki and Yang,

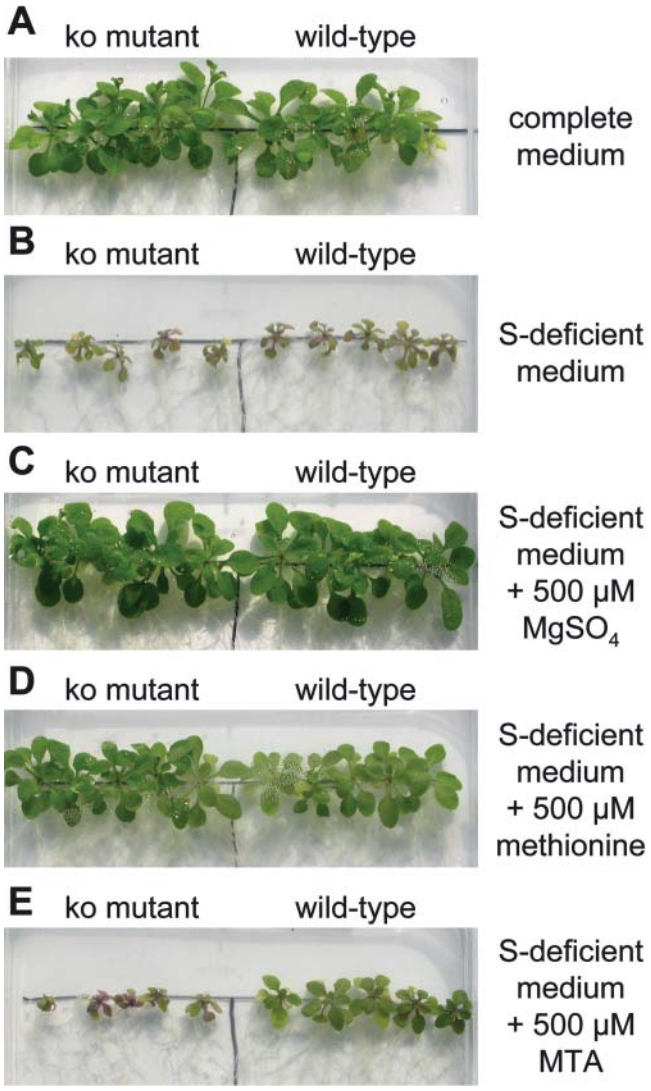

Figure 6. The Arabidopsis *AtMTK* knock-out line 633,954 cannot utilize MTA as sulfur source. Plants from wild type and *AtMTK* knock-out line 633,954 (ko mutant) were grown on 5 different media for 3 weeks: A, full medium; B, sulfur-deficient medium; C, sulfur-deficient medium supplemented with 500 μM $MgSO_4$; D, sulfur-deficient medium supplemented with 500 μM Met; and E, sulfur-deficient medium supplemented with 500 μM MTA.

1987). The MTA cycle exists in bacteria, animals, and plants. Enzymes of the MTA cycle have been identified and characterized in all three. However, the corresponding genes have not been described in plants. This report of MTR kinase genes from Oryza and Arabidopsis is the first to describe regulation and functional characterization of an MTA cycle gene from plants.

Homology at the amino acid level between bacterial and plant sequences was 30% to 35%, which was sufficient to identify putative plant MTK homologs. Recombinant rice OsMTK1 protein was used to study enzyme activity. OsMTK1 displayed kinase activity with an affinity toward MTR as substrate of 16.8 μM, which was comparable to the $K_{0.5}$ of 12.2 μM described previously for the bacterial enzyme (Cornell et al., 1996). The K_m described for partially purified MTR kinase from *L. luteus* was 4.3 μM (Guranowski, 1983), which is approximately one-fourth of that determined for recombinantly expressed rice MTR kinase. In lupin and numerous bacterial species, the enzyme has been shown to require divalent metal ions such as Mg^{2+} or Mn^{2+}, consistent with its activity as an ATP-dependent kinase. Even though a requirement of OsMTK1 for metal ions was not specifically tested, enzyme assays were performed in the presence of Mg^{2+} owing to these earlier findings.

Functional identification of *MTK* as an MTA cycle enzyme-encoding gene was further achieved in Arabidopsis through a molecular genetic approach. Insertional mutagenesis of the unique MTK gene in Arabidopsis eliminated synthesis of full-length *AtMTK* transcripts and resulted in an inability to utilize MTA as sulfur source. Since utilization of sulfate and Met as sulfur supplements were not impaired in the mutant, the mutation can be assigned specifically to the MTA metabolism. The possibility exists that the mutation resulted in an inability of the plant to take up MTA. However, since biochemical analysis identified OsMTK1 as true MTR kinase, it is more likely that knock out of the Arabidopsis homolog impaired turnover of the MTA derivative MTR in the MTA cycle.

Since *AtMTK* is a unique gene, we consider its knock out in Arabidopsis as MTA cycle knock out. Despite elimination of the ability to utilize MTA as a sulfur source, *AtMTK* knock-out plants did not display phenotypic differences to wild-type plants with respect to growth and development under the conditions applied. This indicated that MTA recycling is a nonessential function in Arabidopsis. Without MTA recycling, Met used for ethylene or polyamine synthesis ought to be provided through the primary Met synthetic pathway in the *AtMTK* knock-out mutant.

OsMTK transcript levels in the internode of nontreated intact deepwater rice plants were highest in differentiated tissues. During submergence, mRNA levels in the differentiation zone of the internode were down-regulated, whereas expression was largely unaltered in the growing region. Submergence results in elevated synthesis of ethylene in the growing region but not in the differentiation zone (Cohen and Kende, 1987). Expression levels of *OsMTK* in the meristem and elongation zone thus appeared to be sufficient to support elevated ethylene synthesis following submergence. Hence, strict correlation between ethylene synthetic rate and *OsMTK* mRNA amount was not observed. During tomato fruit ripening, activity of MTR kinase was up-regulated concomitant with the burst in ethylene production (Kushad et al., 1985). However, activity of the MTA-salvaging enzyme MTA nucleosidase was highest in green tomato fruits that did not produce a significant level of ethylene. Thus, in

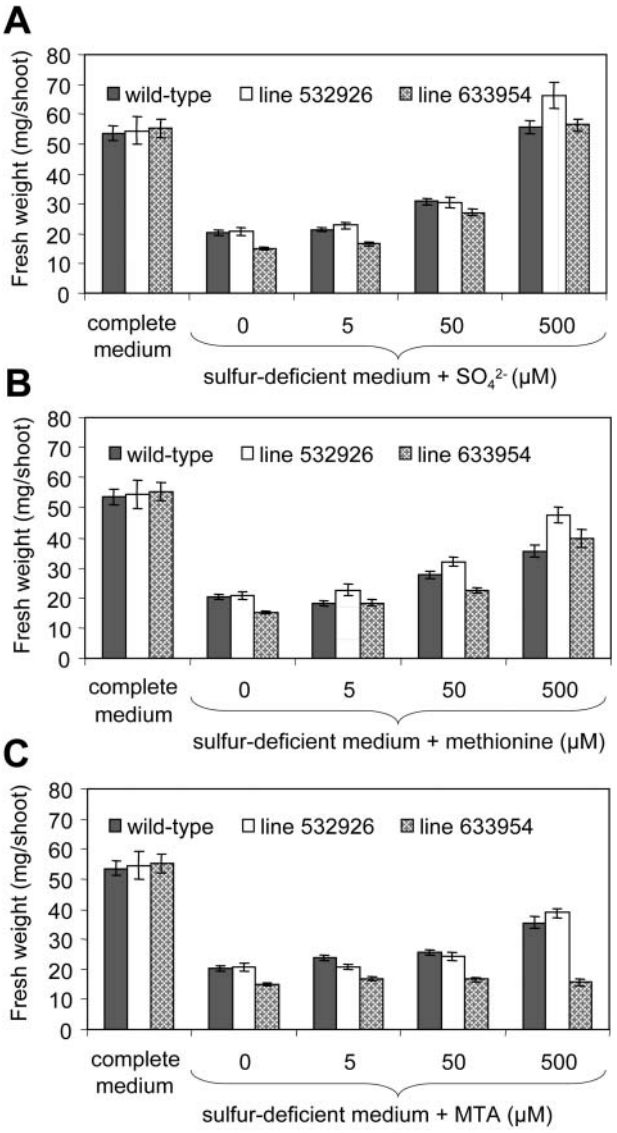

Figure 7. Average shoot fresh weight of wild-type and mutant Arabidopsis plants grown on complete medium or on sulfur-deficient medium supplemented with 0 μM, 5 μM, 50 μM, or 500 μM of (A) $MgSO_4$, (B) Met, or (C) MTA. Results are averages (±SE) of 2 to 3 independent experiments with up to 70 plants per treatment.

tomato, activity of MTA cycle enzymes was not tightly linked to ethylene production in the fruit. Highest *OsMTK* mRNA levels in the differentiation zone argued against a correlation between gene expression and cell division rate, which might establish a requirement for polyamines. Neither was there a link to high metabolic activity that exists in growing regions much more than in differentiated cells. Thus, the significance of high *OsMTK* transcript levels in differentiated tissue remains elusive. In summary, expression pattern and regulation of *OsMTK* genes in the well-characterized youngest deepwater rice internode were not tightly linked to known functions of MTA recycling.

Omission of sulfur or iron from the nutrient solution for 2 weeks following treatment with complete medium for 3 weeks did not affect plant growth, whereas lack of nitrogen resulted in yellow leaves and stunted growth after 2 weeks. Irrespective of the physiological responses, *OsMTK* transcripts were up-regulated only when plants were subjected to sulfur deficiency. Nitrogen is a macroelement required for many of the essential compounds found in cells including amino acids. Despite the fact that nitrogen deficiency severely affected plant growth, expression of *OsMTK* genes, which are involved in regenerating Met, was not up-regulated under these conditions, indicating that *OsMTK* gene regulation is most tightly linked to sulfur metabolism.

Some graminaceous plants are known to produce phytosiderophores under iron-limiting conditions (Shojima et al., 1990). Phytosiderophores are secreted by roots to facilitate uptake of complexed iron from the soil (Ma et al., 1995). Synthesis of phytosiderophores requires AdoMet and generates MTA as a by-product. Growth of wheat without iron resulted in elevated levels of phytosiderophores (Ma et al., 1995). In barley roots, iron deficiency induced expression of *IDI1*, which was postulated to encode an E2 enzyme that catalyzes formation of 2-keto-methylthiobutyric acid in the MTA cycle (Yamaguchi et al., 2000). In rice, iron deficiency did not cause *OsMTK* induction in shoots or roots, where regulation through iron homeostasis might primarily be expected. The possibility exists that rice uses strategies other than synthesis of phytosiderophores to cope with iron deficiency. However, it was shown recently that rice nicotinamine synthase genes are expressed in both leaves and roots and are up-regulated in iron-deficient roots (Inoue et al., 2003). Nicotinamine is the biosynthetic precursor of phytosiderophores. It is also possible that *OsMTK* mRNA levels were largely unaffected by iron deficiency because they were not sufficiently limiting under the conditions applied.

Identification of genes encoding the MTR kinase enzyme of the MTA cycle opened up the avenue to study the role of MTA recycling for plant growth and development. Regulation of MTR kinase genes clearly showed a link with sulfur metabolism. Future work will be aimed at studying the effect of MTA cycle knock out in Arabidopsis on sulfur-containing amino acid pools, as well as on the ability of the plants to synthesize ethylene or polyamines, both of which depend on Met as a substrate. It will also be of interest to understand how expression of different MTA cycle genes and their corresponding encoded enzyme activities are coordinated. Lastly, it will be exciting to learn how regulation of the MTA cycle is integrated in the general sulfur metabolic pathway. Identifying and characterizing genes of the MTA cycle is the first step toward these goals.

MATERIALS AND METHODS

Plant Material

Seeds of deepwater rice (*Oryza sativa* L. cv Pin Gaew 56) were originally provided by the International Rice Research Institute (Los Bañjos, The Philippines). Rice plants were grown as described (Sauter, 1997).

Wild-type seeds of the Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 were obtained through GABI-KAT (Max-Planck Institut, Köln, Germany). Two independent Arabidopsis T-DNA insertion knock-out mutant lines were obtained from the Salk Institute. Line 532,926 carried an insertion in the 5′ untranslated region of the putative AtMTK gene 120 nucleotides upstream of the start codon, whereas line 633,954 had an insertion in the last intron of the gene (Fig. 4). Arabidopsis T_2 plants homozygous for the T-DNA insertion were identified by PCR and used for all subsequent experiments. To characterize Arabidopsis T-DNA insertion lines, a rapid genomic DNA isolation method was used (Weigel and Glazebrook, 2002). One μL of DNA was used in a 25-μL PCR reaction. For line 532,926 primers AtMTK-F1 and AtMTK-R1 and for line 633,954 primers AtMTK-F2 and AtMTK-R3 were used to amplify the wild-type allele (Fig. 4). Primers AtMTK-R1 and Salk-Lbb were used to amplify DNA from the T-DNA-tagged line 532,926 and primers AtMTK-F2 and Salk-Lbb were used for line 633,954 to detect the allele carrying an insertion (Fig. 4; Table I). Arabidopsis Columbia-0 wild-type plants were used as a control.

Unless stated otherwise, Arabidopsis seeds were sown on 1:1 sand:humus that was frozen at −80°C for a few hours to avoid insect contamination. Plants were watered regularly with tap water. Seeds were stratified at 4°C in the dark for 4 d and then transferred to a growth chamber under long-day conditions with 16 h of light (100 μE m^{-2} s^{-1}) and 8 h of darkness at 22°C and 18°C, respectively.

Starvation Experiments

Rice seeds were pregerminated as described (Sauter, 1997) and planted into pots filled with perlite. Plants were watered with deionized water for 1 week, and subsequently with 0.1% (v/v) Wuxal Top N 12-4-6 (12% nitrogen, 4% P_2O_5, 6% K_2O plus trace elements; Aglukon, Düsseldorf, Germany) in deionized water for an additional 10 d. Subsequently, pots were washed with 400 mL of deionized water, and 4 plants each were grown for 2 more weeks in 1 of 4 different media. Control plants were grown in complete medium that consisted of 1:4 diluted modified Murashige and Skoog medium (Murashige and Skoog, 1962), which contained macro salts, micro salts, and $FeSO_4$/Na_2EDTA but lacked vitamins and myoinositol. This medium had a final sulfate concentration of 432.5 μM, resulting from sulfate contained in macro salts (375 μM), micro salts (32.5 μM), and in Fe/Na-EDTA (25 μM). Iron-deficient medium consisted of complete medium lacking Fe/Na-EDTA. To generate a sulfur-deficient medium, sulfate salts were replaced with equimolar amounts of chloride salts in macro and micro salts, such that the sole sulfur source left in the medium was from $FeSO_4$. Medium deficient in nitrogen lacked KNO_3 and NH_4NO_3 in a medium otherwise identical to complete medium. All media were used in a 1:4 dilution. After 2 weeks of starvation treatment, roots were cut, washed in tap water, and frozen in liquid nitrogen. Leaves were collected and frozen in liquid nitrogen for RNA extraction.

Arabidopsis seeds were surface sterilized for 15 min in 1 mL of 0.5% (w/v) sodium hypochlorite. After brief centrifugation, seeds were resuspended in sterile autoclaved water. This washing step was repeated five times. Seeds were laid out using a brush under sterile conditions on square plates containing media of one of eleven different compositions solidified by 0.8% low EEO agar (Sigma-Aldrich, Taufkirchen, Germany). Complete medium contained 1.5% (w/v) Suc and 0.5× Murashige-Skoog salts (Murashige and Skoog, 1962) with 865 μM sulfate. This sulfate concentration resulted from sulfate contained in macro salts (750 μM), micro salts (65 μM), and from the preparation of Fe/Na-EDTA from $FeSO_4$ (50 μM). Sulfur-deficient medium differed from complete medium in that sulfate salts of macro and micro salts were replaced by equimolar amounts of chloride salts and by omission of thiamine. The only sulfate source left in this medium originated from $FeSO_4$. All other media were based on the sulfur-deficient media supplemented with $MgSO_4$, Met, or 5′-methylthioadenosine each at concentrations of 5 μM, 50 μM, or 500 μM. Seeds were stratified, transferred to a growth chamber, and kept at conditions described above. Shoot fresh weights were measured after 3 weeks of growth under conditions as described above for Arabidopsis plants.

Growth Experiments

Partial submergence of deepwater rice plants was carried out for 0 h, 4 h, 8 h, 16 h, or 24 h as described by Lorbiecke and Sauter (1998). The intercalary meristem from 0 to 5 mm above the second node, the elongation zone from 5 to 10 mm, and differentiated tissue below the highest node were harvested, frozen in liquid nitrogen, and stored at −80°C until RNA extraction. For northern analysis, 20 μg total of RNA were loaded per lane.

RNA Extraction, Northern Analysis, and RT-PCR

Total RNA was extracted from rice tissues and used for northern-blot analysis (Sauter, 1997) or RT-PCR. Hybridizations were carried out as described (Sauter, 1997). Since *OsMTK1* and *OsMTK2* are nearly identical at the nucleotide level, the probe derived from the coding region of *OsMTK1* (nucleotides 393–1,305 of the published cDNA, accession AY593959) detected both *OsMTK1* and *OsMTK2* transcripts.

Total RNA was isolated from leaves of T-DNA-tagged Arabidopsis lines 532,926 and 633,954 and from wild-type plants of ecotype Columbia 0 (obtained from the Max Planck Institute). Four micrograms of total RNA were used per RT-PCR assay (5′ RACE kit; Invitrogen, Karlsruhe, Germany). For each line, two independent RT reactions were carried out with primer AtMTK-R3 to monitor the presence of a full-length mRNA or with primer AtMTK-R1 to detect the N-terminal 497 bp of the coding region. For PCR amplification, primers AtMTK-F3 and AtMTK-R3 or primers AtMTK-F3 and AtMTK-R1 were used (Table I; Fig. 4).

Sequencing

The DNA sequencing method used was based on the termination of chain extension developed by Sanger et al. (1977). The samples were sequenced in the Sequence Laboratories Göttingen GmbH (Göttingen, Germany) with the 5′ and 3′ pGEX sequencing primers (Table I; Amersham Biosciences, Uppsala).

Database Searches and Sequence Analysis

DNA and protein homologs of MTR kinase sequences were searched for in the available protein and nucleic acid databases with the BLAST algorithm (Altschul et al., 1997). Database searches were performed on the BLAST servers of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) and the Genomenet Bioinformatics Center based at Kyoto University, Japan (http://blast.genome.ad.jp). Additional information on genomic sequences, ESTs, and available mutants was obtained through The Arabidopsis Information Resource (http://www.arabidopsis.org), The Institute for Genomic Research (http://www.tigr.org/tdb/e2k1/osa1), and the Nottingham Arabidopsis Stock Centre (http://www.nasc.nott.ac.uk) Web sites. Alignments of DNA and protein sequences were made using the BioEdit 5.0.9 software (Hall, 1999).

Overexpression of OsMTR Kinase Protein

A rice EST containing an open reading frame homologous to known bacterial MTR kinase genes was obtained from the Arizona Genomics Institute (OsJNEc 15D06, accession no. CB658835). The putative OsMTK1 cDNA was cloned into the pGEX-6P-1 expression vector (Amersham Biosciences) with primers OsMTK-F1 and OsMTK-R1 (Table I). OsMTK-F1 generated an *Eco*RI restriction site by mutating three nucleotides upstream of the start codon and the start codon itself (ATG, Met) to GAATTC. This resulted in replacement of the start Met by Phe. A *Not*I restriction site was generated two nucleotides downstream of the stop codon by using primer OsMTK-R1 (Table I). This construct was sequenced to verify correct cloning and introduced into the *Escherichia coli* host BL21. Overexpression, purification, and cleavage of GST-OsMTK1 were carried out according to the GST Gene Fusion System Manual (Amersham Biosciences). Overexpression was induced with IPTG. Several pGEX recombinants were identified, all of which expressed a fusion protein with a mass of 70 kD. GST-OsMTK1 was purified by Glutathione Sepharose 4B affinity chromatography (Amersham Biosciences). Purified fusion protein was cleaved with PreScission Protease (Amersham Bioscien-

ces), and recombinant OsMTK1 protein was again purified by passage over Glutathione Sepharose 4B. Recombinant OsMTK1 protein contained 8 additional amino acids (Gly-Pro-Leu-Gly-Ser-Pro-Glu-Phe) at the amino-terminal end and had a molecular mass of 50 kD.

Preparation of ^{14}C-MTR

^{14}C-MTR was synthesized from [^{14}C-methyl]AdoMet (Moravek, Brea, CA) in two steps. First a 1-mL volume containing 50 μCi [^{14}C-methyl]AdoMet, pH 3, was hydrolyzed to ^{14}C-MTA in a boiling water bath for 1 h (Schlenk, 1983). The reaction pH was raised to 6.5 with 50 mM imidazole, and 0.1 mg recombinant *E. coli* MTA/SAH nucleosidase was added to hydrolyze the ^{14}C-MTA to ^{14}C-MTR and adenine. The enzymatic cleavage continued for 24 h at 37°C. Thin-layer chromatography of 10 μL of the reaction mix on cellulose 254 plates (solvent: 9 parts ethylacetate/1 part water) indicated complete hydrolysis of the MTA. Following precipitation of the enzyme by 10% TCA, ^{14}C-MTR was further purified by chromatography on a 10-mL Dowex 50WX8 resin column (Bio-Rad, Hercules, CA) eluted with dH_2O. Eight milliliters of ^{14}C-MTR was vacuum concentrated to 1 mL with 0.8 mM MTR final concentration, neutralized with imidazole (50 mM final concentration), and the specific activity estimated at 77.4 cpm/pmol MTR by liquid scintillation counting.

MTR Kinase Assay

MTR kinase activity was assessed using a modification of the previously reported procedure (Cornell et al., 1996). In brief, standard kinase reactions were carried out in a total volume of 20 μL and contained 4 mM ATP, 20 mM $MgCl_2$, 2 mM dithiothreitol, 100 mM sodium phosphate at pH 8.5, at pH 8.5, 100 μM ^{14}C-MTR, 400 ng bovine serum albumin, and 30 to 300 ng enzyme. Background activity was determined by replacing the MTR kinase with an equivalent amount of bovine serum albumin. Assays were initiated by the addition of enzyme and allowed to proceed for 15 min at 37°C. Reactions were terminated by the addition of 180 μL of ice-cold ethanol, and centrifuged at 13,000*g* for 10 min at 4°C to remove precipitated protein. Samples of 50 μL of supernatant each were applied to 4 cm^2 pieces of Whatman DE52 anion-exchange paper (Clifton, NJ) to capture ^{14}C-MTR-1-P and allowed to air dry. Uncharged ^{14}C-MTR was removed by 3 $\times$ 1 L washes of dH_2O in a shallow tray. Washed filters were dried for 1 h at 55°C then transferred to 7-mL scintillation vials containing Cytoscint (MP Biomedicals, Irvine, CA) scintillation cocktail. Bound radioactivity was determined using a Beckman liquid scintillation counter (Beckman Instruments, Fullerton, CA). All reactions were performed in triplicate. The amount of enzyme was varied in an assay to limit substrate conversion to 10% or less. Kinetic constants were determined using varying concentrations of ^{14}C-MTR in the reaction mix. Kinetic constants were determined by nonlinear regression analysis of substrate-velocity data using the equation $V_o = V_{max}/[1 + (S/K_{0.5})^n]$ and least-squares fitting (Bowen and Jorman, 1995).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AY593959.

ACKNOWLEDGMENTS

We would like to thank Timo Staffel for excellent technical assistance. Receipt of two Arabidopsis T-DNA insertion mutants from the Salk Institute is gratefully acknowledged.

Received September 17, 2004; returned for revision October 21, 2004; accepted October 21, 2004.

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