Mutation in the Threonine Synthase Gene Results in an Over-Accumulation of Soluble Methionine in Arabidopsis[1]

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In higher plants, *O*-phosphohomoserine (OPH) represents a branch point between the methionine (Met) and threonine (Thr) biosynthetic pathways. It is believed that the enzymes Thr synthase (TS) and cystathionine γ-synthase (CGS) actively compete for the OPH substrate for Thr and Met biosynthesis, respectively. We have isolated a mutant of Arabidopsis, designated *mto2-1*, that over-accumulates soluble Met 22-fold and contains markedly reduced levels of soluble Thr in young rosettes. The *mto2-1* mutant carries a single base pair mutation within the gene encoding TS, resulting in a leucine-204 to arginine change. Accumulation of TS mRNA and protein was normal in young rosettes of *mto2-1*, whereas functional complementation analysis of an *Escherichia coli thrC* mutation suggested that the ability of *mto2-1* TS to synthesize Thr is impaired. We concluded that the mutation within the TS gene is responsible for the *mto2-1* phenotype, resulting in decreased Thr biosynthesis and a channeling of OPH to Met biosynthesis in young rosettes. Analysis of the *mto2-1* mutant suggested that, in vivo, the feedback regulation of CGS is not sufficient alone for the control of Met biosynthesis in young rosettes and is dependent on TS activity. In addition, developmental analysis of soluble Met and Thr concentrations indicated that the accumulation of these amino acids is regulated in a temporal and spatial manner.

The Asp family of amino acids includes Asn, Lys, Thr, Ile, and Met (Bryan, 1980; Giovanelli et al., 1985, 1988, 1990). Met is a sulfur-containing amino acid that is important not only as a protein component but also due to its role in many cellular processes (Giovanelli et al., 1980; Anderson, 1990). Met serves as a component of methionyl tRNA, which is required for the initiation of protein synthesis, and as a direct precursor of *S*-adenosyl-Met (SAM), the main biological methyl donor in many transmethylation reactions (Chiang et al., 1996). In plant tissues, Met is also metabolized into the phytohormone ethylene via SAM (Matthews, 1999).

Detailed biochemical studies have led to an understanding of the steps involved in the biosynthetic pathway of the Asp family of amino acids in higher plants (Bryan, 1980; Giovanelli et al., 1980, 1985, 1988, 1990; Matthews, 1999). The pathway has two major branch points (Fig. 1). The first is at 3-Asp semialdehyde, which separates Lys biosynthesis, and the second is at *O*-phosphohomo-Ser (OPH), which divides Met biosynthesis from that of Thr and Ile. Cystathionine γ-synthase (CGS; EC 4.2.99.9) converts OPH into cystathionine, which is the first committed step of Met biosynthesis, while Thr synthase (TS; EC 4.2.99.2) converts OPH into Thr. The CGS-catalyzed reaction is suggested to be a key regulatory site of the Met biosynthetic pathway (Ravanel et al., 1998a; Kim and Leustek, 2000; Matthews, 1999). The level of CGS is feedback regulated according to the level of Met (Thompson et al., 1982a; Rognes et al., 1986), and this regulation is suggested to be exerted by controlling the stability of CGS mRNA (Chiba et al., 1999). TS activity, on the other hand, has been shown to be stimulated up to 20-fold by SAM (Madison and Thompson, 1976; Curien et al., 1998). This is in contrast to bacterial TS, in which no such regulation is known. Bacterial CGS utilizes *O*-succinyl homo-Ser as a substrate (Chattopadhyay et al., 1991), and the branch point for Met and Thr biosynthesis exists at homo-Ser, a step before OPH.

Questions remain, however, about the role of CGS in the regulation of Met biosynthesis in higher plants. Thompson et al. (1982b) have shown that down-regulation of CGS to 15% of its control activity in response to exogenous Met was able to support a normal rate of de novo Met biosynthesis in *Lemna paucicostata*. In addition, based on in vitro kinetic data for CGS in Arabidopsis, Ravanel et al. (1998b) suggested that the CGS reaction should normally

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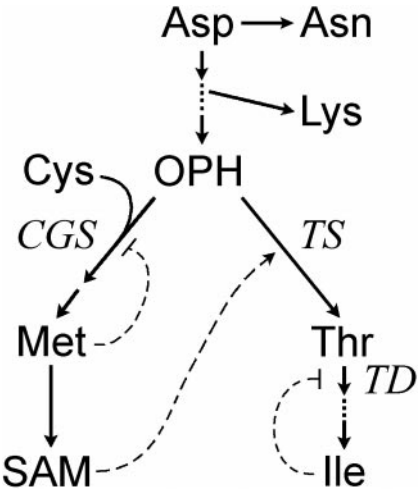

Figure 1. Outline of the Asp family biosynthetic pathway including the branch point at OPH between Met biosynthesis and that of Thr and Ile. The stimulation of TS activity by SAM, negative feedback regulation of CGS according to Met levels, and feedback inhibition of Thr dehydratase (TD) by Ile are indicated as dashed lines.

proceed at approximately 1% to 2% of its maximal rate. Therefore, it has been suggested that in the control of Met biosynthesis in higher plants, the regulation of CGS alone is not sufficient and there may be other factors such as TS activity involved (Thompson et al., 1982a); however, this has yet to be demonstrated in vivo.

To further elucidate the regulatory mechanisms involved in Met biosynthesis and their roles, we adopted a genetic approach of isolating mutants of Arabidopsis with altered soluble Met accumulation. We previously reported the isolation of the *mto1-1* (Met over-accumulation) mutant that accumulated soluble Met up to 40-fold in young rosettes (Inaba et al., 1994). We recently demonstrated that wild-type Arabidopsis has a mechanism to down-regulate the accumulation of CGS mRNA in response to excess Met, and that this mechanism is impaired in the *mto1-1* mutant (Chiba et al., 1999), although it is not known whether Met itself or some other metabolite(s) downstream of Met is acting as a signal molecule. We report here the isolation and characterization of another *mto* mutant, *mto2-1*, and demonstrate that a single base pair mutation exists within the coding sequence of TS. This suggests that a qualitative decrease in TS also results in a phenotype of Met over-accumulation.

RESULTS

Isolation and Genetic Analyses of the *mto2-1* Mutant

Ethionine is a toxic analog of Met (Alix, 1982) and has been successfully used to identify plant mutants that over-accumulate soluble Met (Madison and Thompson, 1988; Inaba et al., 1994). An ethionine-resistant mutant line, MG95, was identified in this study among the T-DNA insertion lines (Feldmann and Marks, 1987), although the mutation was not tagged by T-DNA (see below).

The MG95 mutant (twice backcrossed with wild-type WS) was crossed with wild-type WS (female) and the F_1 plants were tested for ethionine resistance. On agar plates containing 10 μM L-ethionine, wild-type seeds can germinate but die before they expand cotyledons, whereas the MG95 mutant expands green cotyledons and its roots elongate. The F_1 plants could not grow on agar plates containing 10 μM L-ethionine. However, when the L-ethionine concentration was reduced to 3 μM, growth of the F_1 plants was better than wild type (data not shown). The results indicate that the ethionine-resistant mutation carried by MG95 is basically recessive to wild type but shows a weak semi-dominance.

F_3 seeds from individual F_2 plants were pooled (F_2 lines). Eight of the 30 ethionine-resistant F_2 lines did not show kanamycin resistance, indicating that the mutation that confers ethionine resistance is not tagged by T-DNA. In another cross, the wild-type, heterozygous, and MG95 mutant phenotypes segregated at 23:42:27 in the F_2 lines. The result was not significantly different from a segregation ratio of 1:2:1 ($\chi^2 = 0.423$), indicative of a single mutation. A mutant line that did not carry T-DNA was backcrossed a third time with wild-type WS and used for further studies.

MG95 (WS background) was crossed with wild-type Col-0 and segregation of the molecular markers and ethionine-resistance was scored using the F_2 lines that were homozygous for the ethionine resistance mutation. The results indicated that the mutation is located on chromosome 4 between the markers agp66 (75 centimorgans [cM]) and g8300 (81 cM) (data not shown). Since MG95 has elevated levels of soluble Met (see below) and since the mutation locus is different from the previously reported *mto1* mutation located on chromosome 3 (Chiba et al., 1999), the ethionine-resistant mutation carried by MG95 was named *mto2-1*.

Identification of a Single Base Pair Mutation in the TS Gene

The PRHA marker is located between the markers agp66 and g8300 on chromosome 4 (79 cM) and is tightly linked to the gene encoding TS (Bartlem et al., 1999). Due to this close map position between the *mto2-1* mutation and TS gene, the entire coding sequence and a 513-bp upstream region of the *mto2-1* TS gene was sequenced. Comparison with the wild-type WS sequence identified a single base pair mutation in the *mto2-1* TS gene, located 611 bp downstream from the translation start codon. A CTT codon for Leu-204 in the wild-type gene was replaced with CGT for Arg in the *mto2-1* TS gene (Fig. 2A). This amino acid substitution occurred within the consen-

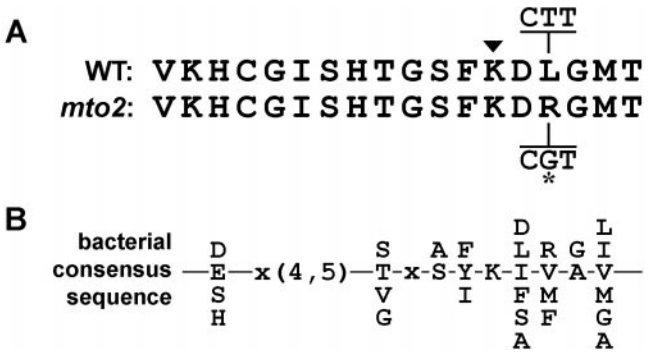

Figure 2. A single base pair mutation in the TS coding sequence of the *mto2-1* mutant alters the enzyme active site. A, Amino acid sequence (190–207) that covers the attachment site for the TS active site-bound PLP coenzyme (Curien et al., 1998) is shown. The wild-type and mutant nucleotide sequence for the affected codon are shown above and below the amino acid sequence, respectively, and the mutation site is marked by an asterisk. The arrowhead indicates the Lys residue that binds PLP. B, The consensus motif for the PLP attachment site determined from bacterial TS sequences (Hofmann et al., 1999; Prosite accession no. PDOC00149). The consensus motif is aligned with the corresponding residues of the Arabidopsis sequences shown in A. x, Any amino acid.

sus sequence for the enzyme active site deduced from bacterial TS sequences (Fig. 2B; Curien et al., 1998; Hofmann et al., 1999; Prosite accession no. PDOC00149). Four independently backcrossed lines of *mto2-1* lines were also confirmed to carry the same single base pair mutation.

The single base pair mutation results in the loss of an *Mfl*I restriction site, and this feature was used to develop a CAPS marker (TSmut) to identify the presence of the mutation by PCR of the TS gene with the TS1F/TS1R primer set (see "Materials and Methods") and digestion of the product with *Mfl*I. Sixteen F_2 lines identified as containing a recombination event between the markers agp66 and nga1139 were screened with the TSmut marker. In all cases, the genotype of the single base pair mutation matched that of the *mto2-1* mutation, as indicated by ethionine resistance, such that lines homozygous or heterozygous for the *mto2-1* mutation were also homozygous or heterozygous for the single base pair mutation, respectively. An additional 39 F_2 lines homozygous for the *mto2-1* mutation were also homozygous for the single base pair mutation. These analyses demonstrate a tight linkage of the single base pair mutation with the *mto2-1* mutation and indicate that they may be the same.

mto2-1 TS Gene Weakly Complements *Escherichia coli thrC* Mutation

The ability of the *mto2-1* TS gene to complement the Thr auxotroph of an *E. coli* strain was analyzed. *E. coli* GIF41 contains a mutation in the *ThrC* gene that codes for TS. Five independent lines each of GIF41 containing either the wild-type Arabidopsis TS gene (GIF41/pKTSwt) or the *mto2-1* TS gene (GIF41/pKTSmto2) were isolated, along with two lines containing only the vector sequence (GIF41/pKK223-3) as controls.

Growth of the lines was examined with and without supplementation of 300 μM L-Thr. The GIF41/pKK223-3 control lines were unable to grow on standard M9 medium and required L-Thr supplementation in the medium (Fig. 3A). In contrast, all GIF41/pKTSwt lines displayed strong growth on M9

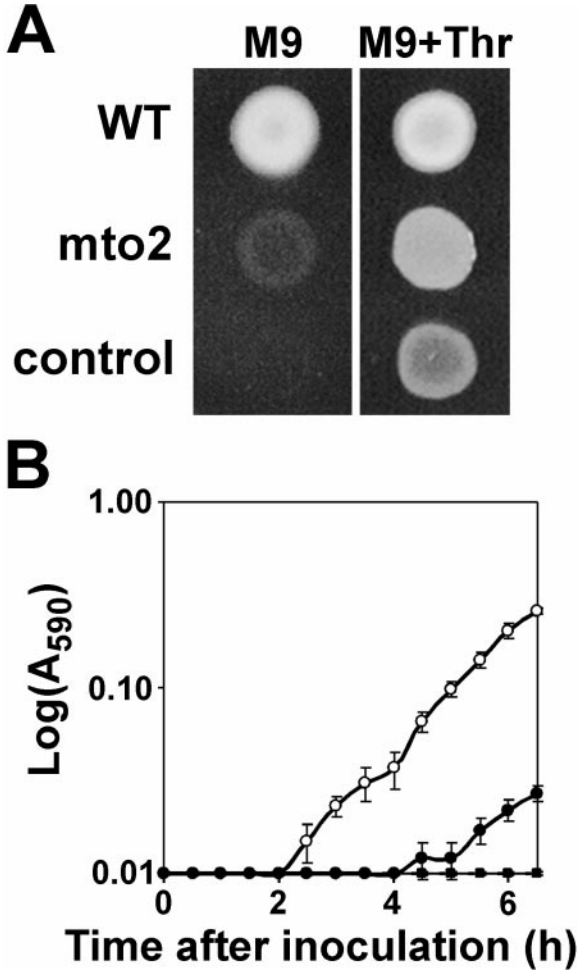

Figure 3. Functional complementation of *E. coli* GIF41 with Arabidopsis wild-type WS and *mto2-1* TS genes. GIF41 is a Thr auxotroph that carries a *thrC* mutation. A, Growth of GIF41 lines on either standard M9 medium (left panel) or M9 medium supplemented with 300 μM L-Thr (right panel). Lines carrying the wild-type Arabidopsis TS gene (WT; GIF41/pKTSwt), the *mto2-1* mutant TS gene (mto2; GIF41/pKTSmto2), or the cloning vector as a control (control; GIF41/pKK223-3) are indicated. Agar plates were spotted with 10 μL of overnight culture and incubated at 37°C overnight. Similar growth was seen for five independent lines of GIF41/pKTSwt and GIF41/pKTSmto2. B, Growth of GIF41/pKTSmto2 lines in liquid M9 medium supplemented with 300 μM L-Thr (○), supplemented with 100 μM IPTG (●), or without supplementation (■). Six-milliliter cultures were inoculated with 200 μL of overnight culture, and incubated in a shaking water bath at 37°C. The averages ± SD of three independent lines are shown.

medium irrespective of L-Thr supplementation, showing that the wild-type Arabidopsis TS gene is able to strongly complement the *thrC* mutation of *E. coli*, as reported by Curien et al. (1996). On the other hand, the growth of lines carrying the *mto2-1* TS gene (GIF41/pKTSmto2) was much slower than that of the GIF41/pKTSwt lines on standard M9 medium in the absence of L-Thr.

The promoter controlling TS gene expression in the vector is inducible by IPTG. Growth of the GIF41/pKTSmto2 lines in liquid M9 medium was enhanced by IPTG (Fig. 3B); however, this was still much slower than that of GIF41/pKTSmto2 lines with L-Thr supplementation (Fig. 3B) or GIF41/pKTSwt lines without IPTG induction (data not shown). This demonstrates that the *mto2-1* TS gene can only weakly complement the *thrC* mutation, and shows that a qualitative difference exists between TS of *mto2-1* and that of the wild type.

TS and CGS Gene Expression

Expression of the genes encoding TS and CGS was analyzed by northern-blot and immunoblot analysis. mRNA and protein accumulation for the TS gene was not appreciably different in the *mto2-1* mutant and wild-type plants at 15 DAI (Fig. 4). In contrast, the accumulation of CGS mRNA and protein in *mto2-1* plants was approximately 40% of that in wild-type plants. This reduction in CGS levels in *mto2-1* does not appear to explain the phenotype of Met over-accumulation, but is likely to be due to feedback regulation of CGS gene expression under conditions of elevated Met (Chiba et al., 1999), rather than being a causative factor.

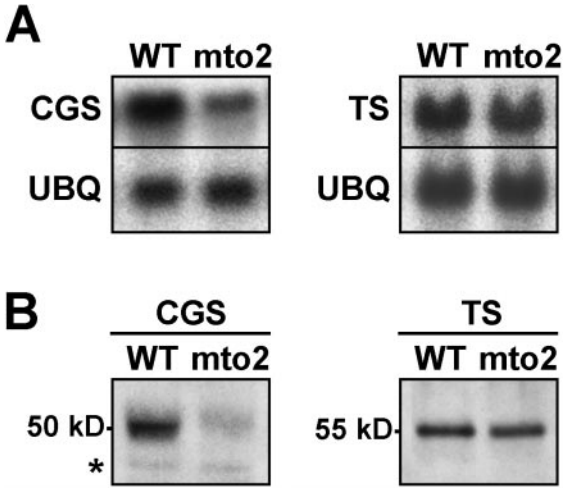

Figure 4. Expression of CGS and TS in 15-DAI seedlings of wild type (WT) and the *mto2-1* (mto2) mutant. A, mRNA accumulation. Total RNA (10 μg) was isolated from 15-DAI rosettes of wild type and *mto2-1* and hybridized with ^{32}P-labeled GCS (left panel) or TS (right panel) cDNA probes as indicated. Membranes were also co-hybridized with a ^{32}P-labeled ubiquitin (UBQ) probe as a loading control (lower panels). A representative result of three independent experiments is shown. B, Protein accumulation. Crude protein extracts (4 μg) from 15-DAI rosettes of wild type and *mto2-1* were separated by SDS-PAGE and subjected to immunoblot analysis using antiserum against CGS (left panel) and TS (right panel) at 1:2,000 dilutions. Bands representing the CGS and TS proteins are marked with their approximate molecular mass. The band marked with an asterisk was also detected with the preimmune serum. A representative result of four independent experiments is shown.

Table I. *Concentration of soluble amino acids in young rosettes*

Soluble amino acids in the rosette region of 15 DAI *mto2-1* and wild-type (WS) plants were assayed. The averages ± SD of at least three independent experiments are shown. For those amino acids marked with asterisks, the concentrations in WS and *mto2-1* plants were significantly different by *t* test ($P < 0.02$).

Amino Acid	Concentrations		Fold Increase[a]
	WS	*mto2-1*	
	pmol mg^{-1} fresh wt		
Asp	476.2 ± 71.2	434.0 ± 22.6	0.91
Lys	11.0 ± 3.2	14.0 ± 4.1	1.27
Thr*	394.1 ± 183.0	25.6 ± 11.2	0.06
Ile	13.4 ± 3.6	11.7 ± 5.3	0.87
Val	72.0 ± 21.6	83.4 ± 48.0	1.15
Leu	16.2 ± 5.2	14.1 ± 6.2	0.87
Met*	7.2 ± 3.3	160.7 ± 116.3	22.30

[a] The average concentrations in *mto2-1* relative to those in WS.

Accumulation of Soluble Amino Acids in *mto2-1* Mutant Plants

Concentrations of soluble amino acids of the Asp family in 15-DAI rosettes of wild-type and *mto2-1* plants were assayed (Table I). Soluble Met concentration in rosettes of the *mto2-1* mutant was significantly increased and about 20-fold higher than that in the wild type at 15 DAI. In addition, soluble Thr concentration was greatly reduced in the *mto2-1* mutant and only about 6% of that in wild-type plants. The concentrations of soluble Asp, Lys, and Ile in *mto2-1* and wild-type rosettes were not significantly different, suggesting that the *mto2-1* mutation does not affect early steps in the pathway or those after Thr formation. No other amino acids detected in our assays exhibited more than a 2-fold difference in concentration between the mutant and wild-type plants.

The concentration of SAM was also increased in *mto2-1* rosettes (45.5 ± 20.4 pmol mg^{-1} fresh weight), approximately 3-fold greater than that in wild type (15.3 ± 0.9 pmol mg^{-1} fresh weight). Inaba et al. (1994) have reported elevated accumulation of SAM in the *mto1-1* mutant, which also over-accumulates Met. SAM levels in the *mto1-1* mutant were re-analyzed in this study, and we need to correct our previous report (Inaba et al., 1994). SAM levels in 15-DAI rosettes of the *mto1-1* mutant (32.1 ± 3.7 pmol mg^{-1} fresh weight) were increased approximately 3-fold compared with that in its Col-0 wild type (11.3 ± 1.2 pmol mg^{-1} fresh weight).

Growth of the *mto2-1* Mutant

The *mto2-1* mutant plants were observed to have reduced growth compared with that of the wild type. The timing of germination (2–3 DAI) and germination rates were similar between the *mto2-1* mutant and the wild type; however, a greater number of *mto2-1* seedlings appeared to stall after germination and did not recover. Cotyledon opening and emergence of the first pair of true leaves was slightly delayed in *mto2-1* seedlings. At 15 DAI, the third leaf set had begun to emerge from wild-type rosettes, whereas the second leaf set was still emerging from *mto2-1* plants. From 20 DAI, the development of *mto2-1* plants appeared to be approximately 5 d behind that of wild-type plants: at 25 and 30 DAI, the *mto2-1* plants resembled those of the wild type at 20 and 25 DAI, respectively. The average fresh weight of the *mto2-1* tissues was between 30% and 50% of that of wild type between 15 and 30 DAI (data not shown).

The difference in growth of *mto2-1* mutant and wild-type plants was most evidently visible in root growth on agar plates. The average length of *mto2-1* roots at 8 DAI was 22% of the average root length for wild-type seedlings (Fig. 5A). On the other hand, the lengths of *mto2-1* and wild-type roots were not appreciably different when cultured on agar plates containing 20 μM L-Thr (Fig. 5B). A slight improvement in *mto2-1* root growth to 50% of the length of wild-type roots was also observed on agar plates supplemented with 20 μM L-Ile (data not shown). L-Ile supplementation is expected to result in an increase in soluble Thr accumulation because it feedback inhibits Thr dehydratase (EC 4.2.1.16; Fig. 1), the enzyme catalyzing Thr deamination for Ile biosynthesis (Bryan, 1990; Singh, 1999). Supplementation of agar plates with either 20 or 100 μM L-Val, L-Leu, L-Lys, or L-Ser did not have an effect on *mto2-1* root growth (data not shown).

Developmental Changes in Accumulation of Met and Thr

Temporal accumulation of soluble Met and Thr in wild-type and *mto2-1* mutant plants was analyzed. As shown in Figure 6A, the concentration of soluble Met was about 20-fold higher in rosettes of *mto2-1* at 15 and 20 DAI compared with that of the wild type. After 20 DAI, the soluble Met concentration in *mto2-1* rosettes decreased markedly and remained not more than 3-fold greater than that in wild-type rosettes up to 40 DAI. On the other hand, the concentration of soluble Met in wild-type rosettes gradually increased over the same period. The marked decrease in soluble Met concentration in young rosettes of *mto2-1* was accompanied by an increase in the soluble Thr concentration. The relative concentration of soluble Thr in *mto2-1* rosettes compared with that in the wild type was very low at 15 DAI (Table I, Fig. 6B), but caught up to about 70% of wild-type level at 20 DAI. The concentration of soluble Thr increased 3- to 4-fold between 20 and 25 DAI, and remained relatively constant in wild-type and *mto2-1* mutant rosettes thereafter (Fig. 6B).

To determine the spatial accumulation patterns of soluble Met and Thr during the reproductive growth stage, aerial parts of 40-DAI plants were dissected and the concentration of soluble Met and Thr analyzed. As shown in Figure 6A, the concentration of soluble Met was 2-fold greater in the cauline leaves, inflorescence apex, and fruits regions of wild-type and *mto2-1* plants compared with that in the rosette and inflorescence axis regions. The concentration of soluble Met was similar between wild type and *mto2-1* in all tissues, although it was slightly higher in fruit.

The concentration of soluble Thr was 1.5- to 2.5-fold higher in *mto2-1* compared with wild type in all tissues examined (Fig. 6B).

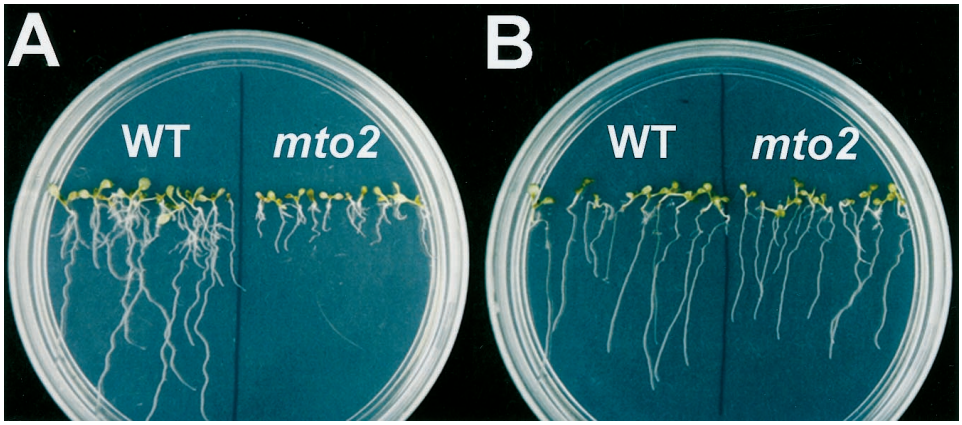

Figure 5. Root growth in response to Thr. Seedlings of wild type (WT) and *mto2-1* (*mto2*) grown on agar medium (A) and agar medium supplemented with 20 μM L-Thr (B) at 8 DAI.

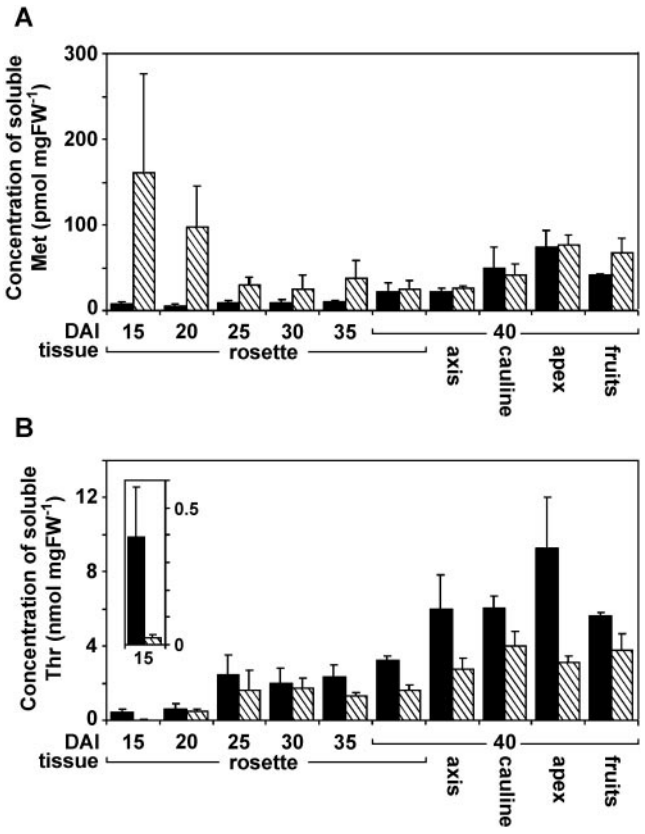

Figure 6. Developmental accumulation of soluble Met and Thr in wild type (■) and *mto2-1* (▨). A, Temporal and spatial changes in soluble Met concentration. B, Temporal and spatial changes in soluble Thr concentration. The inset shows an enlargement of soluble Thr concentration at 15 DAI. For analysis of temporal changes in soluble Met and Thr, rosettes were harvested on the DAI indicated. For spatial analysis of soluble Met and Thr concentration, aerial parts of 40-DAI plants were dissected into the rosette, cauline leaves (cauline), inflorescence axis (axis), inflorescence apex (apex), and fruit regions. The averages ± SD of three independent experiments are shown.

DISCUSSION

We present here the characterization of an Arabidopsis mutant, *mto2-1*, in which soluble Met is over-accumulated and levels of soluble Thr are markedly reduced in young rosettes. The amino acid accumulation profile of *mto2-1* indicated that the branch point in the Met biosynthetic pathway between Met and Thr biosynthesis may be affected. A single base pair mutation within the gene encoding TS in *mto2-1* that altered the amino acid sequence of the enzyme active site was identified and the mutation could not be genetically separated from the *MTO2* locus. Furthermore, functional complementation of an *E. coli thrC* mutation indicated that a qualitative difference exists between wild-type and *mto2-1* TS. These data strongly suggested that the single base pair mutation is responsible for the *mto2-1* mutant phenotype by reducing the mutants' ability to synthesize Thr, and thus allowing soluble Met to over-accumulate in young rosettes due to reduced competition for the common OPH substrate. Similar concentrations of soluble Ile in 15-DAI rosettes of *mto2-1* and wild type indicated that even when soluble Thr concentration is reduced to 6% of normal levels, this is sufficient to support Ile biosynthesis, and that Thr may be preferentially drawn into Ile biosynthesis to fulfill this demand prior to being utilized for protein synthesis. This hypothesis is also supported by the fact that the wild-type concentration of Ile in 15-DAI rosettes was even lower than that of Thr in the *mto2-1* mutant (Table I).

CGS activity is known to be reduced by Met feeding; however, this is not by allosteric inhibition at the enzyme activity level (Thompson et al., 1982a; Rognes et al., 1986) but at the level of gene expression (Chiba et al., 1999). On the other hand, the activity of TS is stimulated up to 20-fold by SAM (Curien et al., 1998). It has previously been proposed that the regulation of CGS levels by Met alone is not sufficient for regulating Met biosynthesis in higher plants, and may also be dependent on other factors such as TS activity (Thompson et al., 1982a). This is supported by comparison of the in vitro kinetic data for these two enzymes (Ravanel et al., 1998a). In 15-DAI *mto2-1* plants, TS activity is expected to be reduced, whereas the lower levels of CGS associated with Met over-accumulation indicates that the regulatory mechanism controlling CGS mRNA accumulation in response to Met over-accumulation (Chiba et al., 1999) is functioning normally. The fact that Met was able to over-accumulate about 20-fold in *mto2-1* when CGS levels were markedly reduced (Fig. 4) suggests that it is also the case in vivo that TS participates in maintaining soluble Met levels in young rosettes and that when TS activity is compromised, the regulation of CGS alone is not sufficient.

Accumulation of mRNA and protein for TS in the *mto2-1* mutant was not appreciably different from the wild type in young rosettes and, together with the functional complementation analysis, suggests that the activity of the *mto2-1* TS is affected rather than expression of the gene itself. The TS enzyme contains an active-site-bound pyridoxal-5′-P (PLP) coenzyme (Curien et al., 1998) for which a consensus binding sequence has been determined for bacterial sequences (Fig. 2B; Hofmann et al., 1999; Prosite accession no. PDOC00149). The amino acid substitution caused by the *mto2-1* mutation (Leu-204 to Arg) occurs within this consensus sequence, affecting the second residue to the C-terminal end of the Lys residue that binds PLP. It is interesting that the wild-type Arabidopsis sequence does not normally conform to this bacterial consensus motif at this residue; however, due to the single base pair mutation, the *mto2-1* amino acid sequence matches the consensus motif at every residue.

Unlike in bacteria, TS from higher plants is markedly stimulated by SAM (Aarnes, 1978; Thoen et al., 1978; Giovanelli et al., 1984; Curien et al., 1998). TS of Arabidopsis exists as a homodimer, whereas TS of *E.*

coli, yeast (Laber et al., 1999), and *Brevibacterium lactofermentum* (Malumbres et al., 1994) have been reported to be monomeric. It is possible that the altered amino acid residue in *mto2-1* TS could influence either SAM stimulation or homodimer formation, which would not affect bacterial TS. However, this is unlikely as sequences at the N-terminal and C-terminal regions of Arabidopsis TS have been implicated in SAM stimulation (Curien et al., 1996) and dimer formation (Laber et al., 1999), respectively, whereas the altered amino acid residue in *mto2-1* resides in the active site toward the center of the coding region. Another possibility is that the substitution affects the TS reaction directly, as suggested by its location within the active site. An explanation for this is difficult with the current consensus motif, which is based on bacterial sequences. At present, the only other sequence covering the TS active site from a higher plant source is an expressed sequence tag from watermelon (*Citrullus lanatus*; GenBank accession no. AI563068), which also encodes for Leu at the site altered in *mto2-1*, as is the case in wild-type Arabidopsis. Further analysis of TS sequences from other higher plant sources is required for further discussion on possible differences in the active site consensus motif for plant and bacterial TS.

Temporal and spatial changes in Met and Thr concentrations were also examined in this study. In 15-DAI rosettes of the *mto2-1* mutant, the relative concentration of soluble Thr was only approximately 6% of that in wild type; however, this increased from 15 DAI and was approximately 70% of wild-type levels between 20 and 25 DAI and 85% of wild-type levels at 30 DAI. The increase in relative Thr concentration accompanied by a marked decrease in soluble Met concentration indicates that the competitive ability of the Thr biosynthetic branch in *mto2-1* is somehow increased over that of Met biosynthesis from 20 DAI. Along with the increase in relative concentration, temporal changes in soluble Thr concentration followed a similar pattern in wild-type and *mto2-1* rosettes, increasing several-fold after 20 DAI. In wild-type rosettes, the increase in soluble Thr concentration occurred without a reduction in soluble Met concentration. It is interesting that the changes in Met and Thr concentrations in wild type and *mto2-1* occur as the plants are entering the reproductive growth stage, possibly reflecting an increased requirement for Thr during this stage. Similar temporal decreases in Met accumulation have also been reported for the *mto1* mutant and wild-type Col-0 (Inaba et al., 1994).

In 40-DAI plants, the concentration of soluble Thr was reduced in all tissues of *mto2-1* compared with that in the wild type. On the other hand, soluble Met concentrations were similar between wild type and *mto2-1*, suggesting that Met biosynthesis may be subject to stricter control in aging plants compared with those at 15 DAI.

Unlike plants and bacteria, mammals are unable to synthesize Met and must obtain this essential amino acid from the diet. Of significant concern is the fact that Met is a limiting amino acid in important legume crops such as soybean, pea, and lupine (Tabe and Higgins, 1998). For this problem to be addressed properly, a clear understanding of the regulation of Met biosynthesis is required. The accumulation patterns for Met and Thr in wild-type and *mto2-1* plants, along with comparisons with the *mto1-1* mutant, suggest that the biosynthesis of Met and Thr is subject to complex developmental regulation that may involve factors in addition to the regulation of CGS and TS activities.

MATERIALS AND METHODS

Plant Materials

The *mto2-1* mutant (isolate name MG95) was isolated in this study from T-DNA insertion mutagenized lines of Arabidopsis (Feldmann and Marks, 1987), obtained from the Arabidopsis Biological Resource Center (Columbus, OH). Arabidopsis ecotype Wassilenskija (WS) was used as a wild-type strain. The strain used for mapping was Arabidopsis ecotype Columbia (Col-0). The Arabidopsis *mto1-1* mutant has been described previously (Inaba et al., 1994; Chiba et al., 1999).

Plant Culture Conditions

Plants were grown at 22°C under continuous white fluorescent light at about 2.4 W m^{-2} s^{-1}. Other than for ethionine-resistant mutant isolation, plants were sown and grown on rockwool bricks (3 × 3 × 4 cm; Nittobo, Tokyo) and watered three times a week with a hydroponic medium (Fujiwara et al., 1992).

For analysis of ethionine-resistant phenotype and root growth, surface-sterilized seeds were sown on agar medium (Inaba et al., 1994). For phenotype analysis, the medium was supplemented with the appropriate concentrations of DL-ethionine. For root growth analysis, the medium was supplemented with 20 or 100 μM of L-Thr, L-Ile, L-Val, L-Leu, L-Lys, or L-Ser where appropriate, and the agar plates were incubated on an approximately 45° angle.

Isolation of Ethionine-Resistant Mutants

Ethionine-resistant mutants were isolated as described previously (Inaba et al., 1994), except that lower concentrations of ethionine were used. T_4 generation seeds from T-DNA insertion lines (24,500 seeds representing 4,900 T-DNA insertion lines) were sown on agar plates containing 10 μM DL-ethionine. Those seedlings that expanded green cotyledons were saved and ethionine resistance was re-analyzed in the next generation using agar plates containing 30 μM DL-ethionine. One line, MG95, was identified. The MG95 line, which carries the *mto2-1* mutation, showed ethionine resistance for two successive generations.

Genetic Analysis

Genetic crosses were carried out by brushing the anthers of a male parent to the stigma of a female parent. For backcrosses, wild-type (WS) plants were used as female parents.

Segregation of ethionine resistance was analyzed as follows. F_3 seeds that were obtained by self-pollination of individual F_2 plants (F_2 lines) were sown on agar plates containing 10 μM of L-ethionine. At this concentration, wild-type seeds can germinate but die before they expand cotyledons. On the other hand, the *mto2-1* mutant expands green cotyledons, and roots elongate under the same conditions.

For genetic mapping, a *mto2-1* mutant backcrossed three times was crossed with wild-type Col-0 strain, and linkage of the *mto2-1* mutation with cleaved amplified polymorphic sequences (CAPS; Konieczny and Ausubel, 1993) and simple sequence length polymorphism (SSLP; Bell and Ecker, 1994) markers was determined. The information on CAPS markers was obtained from The Arabidopsis Information Resource (http://www.arabidopsis.org/). Map positions of markers were obtained from the Recombinant Inbred Map (Lister and Dean, 1993; http://nasc.nott.ac.uk/, a June 26, 1999, version).

Assay of Soluble Amino Acids

The dissection of aerial parts of plants into the rosette, cauline leaves, inflorescence axis, fruit, and apex regions, and the determination of soluble amino acid accumulation was carried out according to the method of Inaba et al. (1994). Prior to the initiation of bolting (20–22 d after imbibition [DAI]), the rosette region included all of the aerial parts.

The analysis of SAM accumulation was carried out following a protocol adapted from Creason et al. (1985). Plant samples (500 mg) were homogenized with 2.5 mL g^{-1} fresh weight of 0.2 M $HClO_4$. Insoluble material was removed by centrifugation at 7,500*g* for 10 min, and then washed twice with 1.25 mL of 0.2 M $HClO_4$. The crude extracts were diluted 4-fold with water and loaded on 1-mL Bond Elut SCX columns (100-mg sorbent mass, Varian, Palo Alto, CA) that had been pretreated with 2 mL of methanol followed by 2 mL of water. The columns were washed with (in order) 3 mL of 0.5 M HCl, 2 mL of water, 2 mL of 80% (v/v) ethanol, and 0.5 mL of solution A (Creason et al., 1985). Following the wash steps, SAM was eluted with 2 mL of solution A and filtered through an Ultra-Free C3GV filter (Japan Millipore, Tokyo). Aliquots (500 μL) of the eluates were analyzed by HPLC (model L-6200, Hitachi, Tokyo) using a 4-mm-diameter $\times$ 150-mm column of no. 2619 resin according to the method of Creason et al. (1985). A_{254} was monitored.

Northern Analysis

Total RNA was prepared by phenol extraction (ISOGEN-LS, Nippon Gene, Tokyo) and 10 μg was separated in 1.2% (w/v) agarose-formaldehyde gels prior to transfer to nylon membranes (Gene Screen Plus, NEN Life Science Products, Boston). Membranes were hybridized with a TS cDNA probe (GenBank accession no. N37209), a CGS cDNA probe (GenBank accession no. X94756), or *sam-1* and *sam-2* cDNA probes (Peleman et al., 1989). As a loading control, the membranes were also hybridized with a ubiquitin probe (UBQ) for the *UBQ5* gene of Arabidopsis (Rogers and Ausubel, 1997). The ^{32}P-labeled probes were prepared by random primer labeling (Feinberg and Vogelstein, 1984) using a DNA labeling system (Multiprime, Amersham-Pharmacia Biotech, Uppsala), and northern hybridization was carried out according to the manufacturer's instructions.

Immunoblot Analysis

Crude plant protein extracts were prepared as previously described (Ishikawa et al., 1991). Quantification was carried out according to the method of Bradford (1976) using a protein assay reagent (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin (TaKaRa, Kyoto) as a standard. Protein samples (4 μg) were separated on 7.5% (w/v) SDS-PAGE gels (Laemmli, 1970) prior to immunoblot analysis using an enhanced chemiluminescence western-blot analysis system and detection kit (Amersham-Pharmacia Biotech) according to the manufacturer's instructions. Antisera against TS (Curien et al., 1996) and CGS (Kim and Leustek, 2000) were used at a dilution of 1:2,000. Detection was carried out using a luminoimager (EPIPRO 7000, Bio-Rad Laboratories).

DNA Sequencing and Analysis

Genomic DNA was sequenced using a terminator cycle sequencing ready reaction kit (Prism BigDye, PE-Applied Biosystems, Tokyo) and a DNA sequencer (model 377, PE-Applied Biosystems) according to the method recommended by the manufacturer. A series of nine primers based on the published 2,233-bp TS genomic sequence (GenBank accession no. AB027151; Bartlem et al., 1999) were used to amplify genomic DNA by PCR and to sequence both strands of the TS gene from *mto2-1*. Forward primers were TSfor1 (5′-TGTTCACATGTTGCTTTCAG), TS1F (5′-CTTTCGTCTTGTCTCTTCAATG), TS2F (5′-GTGATCTCTTCGATTCGCGT), and TS3F (5′-CACAGAGGAGGAGCTGATGG). Reverse primers were TS1R (5′-GATTCAAACTTTGCGATAGAAC), TS2R (5′-GCATCCATCAGCTCCTCCTC), TSmqR (5′-GAGGCGCAGTAAGCAGATAG), TS3R (5′-GGCCATGTGCTTTTACCAAC), and TSbk1 (5′-GTACAGGAGATGACGACGGG).

Functional Complementation Analysis

The sequence encoding the mature TS protein was cloned into the pKK223-3 expression vector (Amersham-Pharmacia Biotech) by PCR as previously described (Curien et al., 1998), except that cetyl-trimethyl-ammonium bromide-extracted DNA (Murray and Thompson, 1980) from WS and two independently backcrossed lines of *mto2-1* were used as the insert source, and the ligation product was transformed into *Escherichia coli* DH5α (Sambrook et al., 1989). Recombinant plasmids were re-

transformed into *E. coli* GIF41 (*thrC1001 thi-1 relA spoT1*; Malumbres et al., 1994), a Thr auxotroph mutant. The integrity of the ligation sites was reconfirmed by restriction digestion, and the presence or absence of the single base pair mutation was confirmed by PCR and *Mfl*I digestion. Vectors containing the wild-type Arabidopsis and *mto2-1* mutant TS genes were referred to as pKTSwt and pKTSmto2, respectively.

For functional complementation analysis, GIF41/pKTSwt and GIF41/pKTSmto2 lines were initially cultured overnight in liquid M9 medium (Davis et al., 1980) supplemented with 50 μg mL^{-1} ampicillin and 300 μM L-Thr in a 37°C shaking water bath. Cultures were washed and resuspended in M9 medium minus Thr by centrifugation. Ten microliters of each culture was spotted onto M9 medium solidified with 1.4% (w/v) agar and supplemented with 50 μg mL^{-1} ampicillin and 300 μM L-Thr when necessary. Plates were incubated overnight at 37°C. For growth analysis in liquid culture, washed overnight cultures were used to inoculate 5 mL of liquid M9 medium supplemented with 50 μg mL^{-1} ampicillin and 300 μM L-Thr or 100 μM isopropyl β-D-thiogalactopyranoside (IPTG). Liquid cultures were incubated in a 37°C shaking water bath, and growth was measured as an increase in A_{590}.

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