Plant Physiol. (1994) 104: 881–887

Isolation of an *Arabidopsis thaliana* Mutant, *mto1*, That Overaccumulates Soluble Methionine[1]

Temporal and Spatial Patterns of Soluble Methionine Accumulation

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We isolated *Arabidopsis thaliana* mutants that are resistant to ethionine, a toxic analog of methionine (Met). One of the mutants was analyzed further, and it accumulated 10- to 40-fold more soluble Met than the wild type in the aerial parts during the vegetative growth period. When the mutant plants started to flower, however, the soluble Met content in the rosette region decreased to the wild-type level, whereas that in the inflorescence apex region and in immature fruits was 5- to 8-fold higher than the wild type. These results indicate that the concentration of soluble Met is temporally and spatially regulated and suggest that soluble Met is translocated to sink organs after the onset of reproductive growth. The causal mutation, designated *mto1*, was a single, nuclear, semidominant mutation and mapped to chromosome 3. Accumulation profiles of soluble amino acids suggested that the mutation affects a later step(s) in the Met biosynthesis pathway. Ethylene production of the mutants was only 40% higher than the wild-type plants, indicating that ethylene production is tightly regulated at a step after Met synthesis. This mutant will be useful in studying the translocation of amino acids, as well as regulation of Met biosynthesis and other metabolic pathways related to Met.

Met is used as a precursor of biologically important compounds such as SAM, polyamines, and ethylene. Amino acids of the Asp family include Asn, Lys, Thr, Ile, and Met. The carbon backbone of Met derives from Asp, the sulfur from Cys, and the methyl group from N^5-methyltetrahydrofolic acid (Giovanelli et al., 1980).

Regulation of Met biosynthesis in higher plants has been most extensively studied in *Lemna*. The concentration of soluble Met as well as Met in protein is regulated at a relatively constant level over a wide range of sulfate availability in the culture medium (Datko et al., 1978). Synthesis of cystathionine is suggested to be a regulatory step for L-Met synthesis. When cystathionine is synthesized, the carbon backbone and the sulfur are committed to Met synthesis via homocysteine. The activity of cystathionine γ-synthase, the enzyme that catalyzes transsulfuration of *O*-phosphohomoserine with sulfur of Cys to produce cystathionine, is decreased to 15% of control levels by addition of 2 μM Met in the culture medium (Thompson et al., 1982). Giovanelli et al. (1985) determined that Met feedback regulates its own de novo synthesis at the step of cystathionine synthesis. However, the regulation is not likely done by allosteric feedback inhibition of cystathionine γ-synthase because addition of Met or its metabolites in the assay mixture in vitro had no effect on the enzyme activity (Thompson et al., 1982). These results suggest that the level of cystathionine γ-synthase is regulated by Met. However, the molecular mechanism of the regulation is unknown. Isolation of mutants that are altered in Met biosynthesis will greatly facilitate our understanding of the regulation by Met in plants.

Ethionine is a toxic analog of Met. The toxicity of ethionine is explained by the fact that ethionine is used in place of Met in cellular processes such as protein synthesis and SAM synthesis (Alix, 1982). Mutants that are resistant to ethionine have been reported in several organisms, including prokaryotes (Lawrence et al., 1968; Greene et al., 1970), lower eukaryotes (Spence et al., 1967; Cherest et al., 1973; Sloger and Owens, 1974), and cultured plant cells (Widholm, 1976; Reish et al., 1981; Gonzales et al., 1984; Madison and Thompson, 1988). These ethionine-resistant mutants are reported to overaccumulate soluble Met. The resistance to ethionine is thought to be due to excess soluble Met diluting exogenously applied ethionine and thus reducing the possibility of ethionine being used in the cellular processes. Ethionine-resistant mutants of *Arabidopsis thaliana* have been isolated (Cattoir-Reynaerts et al., 1981), but no further report has been made.

We report here the isolation of *A. thaliana* mutants that overaccumulate soluble Met. The pattern of soluble Met

[1] This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan to M.C., Y.K., and S.N.

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Abbreviations: DAI, day(s) after imbibition; SAM, *S*-adenosylmethionine.

accumulation in the mutant indicates that soluble Met concentrations in plant tissues are temporally and spatially regulated.

MATERIALS AND METHODS

Plant Materials

Arabidopsis thaliana (L.) Heynh. Columbia ecotype (Col-0) was used as a wild-type strain. Ethyl methanesulfonate-mutagenized M_2 seeds of Columbia harboring a *gl1* mutation were purchased from Guhy's Specialty Nursery (Tucson, AZ) and used to isolate ethionine-resistant mutants. The following strains were used for mapping: W100 (*an, ap1, py, er, hy2, gl1, bp, cer2, tt3, ms1*) (Koornneef et al., 1987), MSU22 (*er, hy2, gl1, tt5*), and the *abi3-1* mutant strain (Koornneef et al., 1984). The strains used for mapping are Landsberg ecotype harboring an *erecta* mutation.

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, Japan) embedded in vermiculite and were watered twice a week with MGRL hydroponic medium (Fujiwara et al., 1992).

Selection of Ethionine-Resistant Mutants

Surface-sterilized M_2 seeds were sown aseptically at a density of about 1000 seeds per plate on MGRL-agar medium containing 100 μM DL-ethionine and were incubated at 22°C in the light. MGRL-agar medium is the same as MGRL hydroponic medium (Fujiwara et al., 1992) except that it contains 3% Suc and is solidified with 1.5% agar. Wild-type seeds can germinate, but they will become brown and die before cotyledons are fully expanded. Those seedlings that expanded green cotyledons were recovered and were grown on MGRL-agar medium without ethionine for several days. When the plants were at the four-leaf stage, they were transferred to rockwool bricks embedded in vermiculite. The plants were grown to maturity and self-pollinated in the absence of ethionine.

Linkage Analysis

The ethionine-resistant mutant (male parent) was crossed with tester strains, and F_2 seeds were obtained by self-pollination of the F_1 plants. For linkage analysis with the *abi3-1* mutation, those F_2 seedlings that germinated in the presence of 5 μM ABA were selected (Ooms et al., 1993) and tested for ethionine resistance by transferring the seedlings onto MGRL-agar plates containing 100 μM DL-ethionine. For linkage analysis with other markers, those F_2 seedlings that were resistant to 100 μM DL-ethionine were selected, and segregation of visible markers was scored in the absence of ethionine. Genetic linkage was calculated according to the formulas described by Allard (1956) and Koornneef et al. (1983).

Plant Dissection

The aerial parts were dissected into five parts: rosette region, cauline leaves, inflorescence axis region, fruit region, and apex region. The rosette region included hypocotyl, cotyledons, and foliage leaves. The apex region was about 1 cm of inflorescence from the apex, including flower buds and flowers. The fruit region included immature fruits without the peduncles. The cauline leaves included petioles. The inflorescence axis region was the remainder of the aerial parts including peduncles. Before the plants started to bolt (until 25 DAI), the rosette region included all of the aerial parts.

Assay of Soluble Amino Acids

Plant samples were weighed and immediately frozen in liquid nitrogen and stored at −80°C until assayed. Plant samples were homogenized in a Potter-type homogenizer with 10 mL g^{-1} fresh weight of 80% (v/v) ethanol at 45°C (Duke et al., 1978). Insoluble materials were sedimented by centrifugation for 10 min at 3500*g* at 4°C. Precipitates were washed with one-tenth volume of 80% ethanol at 45°C and resedimented by centrifugation. The supernatants were combined and loaded on a Dowex 50Wx8 column (1.5 mL mg^{-1} fresh weight). The column was washed with 6 volumes of 80% ethanol followed by 6 volumes of deionized water. Amino acids were eluted with 6 volumes of 2 M NH_4OH. The eluate was evaporated to dryness, and the residue was dissolved in 0.2 N HCl. The sample was filtered through an Ultra-free C3GV filter (Japan Millipore, Tokyo, Japan), and soluble amino acids were analyzed by HPLC (model L-6200; Hitachi, Tokyo, Japan) using a 4-mm-diameter × 150-mm column of No. 2619 resin at 37°C. Amino acids were eluted by Li^+ gradient and were detected with *o*-phthalaldehyde. SAM was extracted as described by Peleman et al. (1989) and assayed as above.

Assay of Ethylene Production

A procedure of Guzman and Ecker (1990) was generally followed in the assay of ethylene production. Plant materials (300 mg) were incubated for 24 h at 22°C in an airtight 20-mL glass vial, and ethylene was assayed using a gas chromatograph (model 063, Hitachi). An appropriately diluted standard of 640 μL L^{-1} of ethylene (balance N_2) was used to calculate ethylene concentrations.

RESULTS

Isolation of Ethionine-Resistant Mutants

Approximately 20,000 M_2 seeds of *A. thaliana* were sown on agar plates containing 100 μM DL-ethionine. Twelve plants expanded green cotyledons within 1 week, whereas others were white and did not expand cotyledons. The putative ethionine-resistant plants were transferred to agar medium without ethionine. Nine plants continued to grow and three plants became brown and eventually died. Hereafter, the plants were maintained in the absence of ethionine except when plants were tested for ethionine resistance. All of the nine lines showed ethionine resistance in the M_3 through M_5

generations. These nine lines were back-crossed three times with Col-0 (female parents), and homozygous lines were established in the F_3 generation. One of the lines, designated ET3, was used for further analysis. As shown in Figure 1, wild-type Col-0 plants survived in up to 3 μM of L-ethionine, and ET3 survived 100-fold higher concentrations of L-ethionine.

Soluble Met concentrations in the rosette region of 20 DAI plants were assayed. ET3 mutant plants accumulated about a 40-fold higher concentration of soluble Met than the wild type (see below). Eight other ethionine-resistant lines also accumulated 10- to 40-fold higher concentrations of soluble Met in the rosette region (data not shown). The ET3 mutant plants looked normal except that the fresh weight increase in the rosette region was slightly slower than the wild type during the vegetative growth period and bolting was delayed by 1 to 2 d, resulting in about 40% reduction in fresh weight of the rosette region at the start of bolting (data not shown).

Genetic Analysis

Reciprocal crosses were made between ET3 and Col-0. All of the F_1 plants were resistant to ethionine (eight of eight for both crosses). However, the F_1 plants grew slower on agar plates containing 100 μM L-ethionine than homozygous ET3 plants (data not shown). The F_2 plants segregated resistant and sensitive plants at 3:1 (Table I). The results indicate that the mutant carries a single, nuclear, semidominant mutation. Similar results were observed with the other ethionine-resistant lines (data not shown). Hereafter, we will refer to the mutation carried by ET3 as *mto1–1* for methionine overaccumulation.

ET3 strain was crossed with marker lines, and segregation of the markers and ethionine resistance were analyzed in the F_2 generation. Results obtained with a multiply marked strain, W100, indicated that the *mto1–1* mutation maps to chromosome 3 (data not shown). Linkage analysis was carried out using markers on chromosome 3. As shown in Table II, genetic linkage with the *mto1–1* mutation was observed for *hy2*, *abi3*, and *gl1*. No genetic linkage was observed with *tt5* (data not shown). These results indicate that the *mto1* mutation is located on chromosome 3 between *hy2* and *abi3* (Fig. 2).

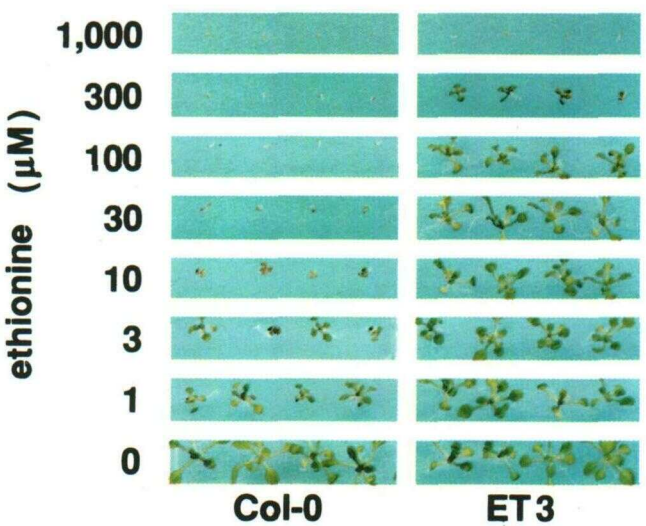

Figure 1. Ethionine resistance of the mutants isolated. Seeds of Col-0 and ET3 mutants were sown aseptically on agar plates containing various concentrations of L-ethionine and were incubated for 14 d.

Table I. *Segregation of ethionine resistance in the F_2 generations of wild-type and mutant plants*

Reciprocal crosses were made between ET3 mutant and wild-type (Col-0) strains, and segregation of ethionine resistance was analyzed in the F_2 generations.

Crosses[a]	Resistant	Sensitive	Total	χ^2 (3:1)
ET3 (f) × Col-0 (m)	247	73	320	0.82[b]
Col-0 (f) × ET3 (m)	221	58	279	2.64[b]

[a] f, Female parent; m, male parent. [b] Not significantly different from 3:1 segregation ($P > 0.05$).

Accumulation of Soluble Amino Acids in *mto1–1* Mutant Plants

Concentrations of soluble amino acids in the rosette region of plants 20 DAI were assayed in wild-type and *mto1–1* mutant plants. Table III shows the concentrations of soluble amino acids that are metabolically related to Met, i.e. products and intermediates of the Asp family and metabolites of Met. As shown in the table, among the intermediates of Met biosynthesis, there was no significant difference in the soluble amino acid concentrations between the wild-type and *mto1–1* mutant plants. On the other hand, concentrations of Met and its metabolites, SAM and *S*-methylmethionine, were much higher in *mto1–1* mutant plants. The concentration of Met sulfoxide was also high in the mutant, but this was most likely due to the oxidation of Met during extraction. The soluble Met content was about 40-fold higher in *mto1–1* mutant plants, and Met constituted as high as 5% (molar basis) of total soluble amino acids (20 protein amino acids) in the mutant plants.

Pro and Arg accumulated in *mto1–1* mutant plants to concentrations that were about 3-fold higher than in the wild type (data not shown). No other amino acids detected in our assays exhibited more than a 2-fold difference in concentration between the mutant and the wild-type plants.

Temporal Patterns of Soluble Met Accumulation

Temporal patterns of accumulation of soluble amino acids in the rosette region of the mutant and wild-type plants were analyzed. As shown in Figure 3A, concentration of soluble Met was 10- to 40-fold higher in *mto1–1* mutant plants until 25 DAI and then decreased to less than twice that of the wild-type plants. On the other hand, the soluble Met concentration in the rosette region of wild-type plants remained fairly constant throughout the life cycle. Both the wild-type and the mutant plants started to bolt between 25 and 30 DAI. Figure 3B shows the total soluble Met contents in the rosette region at various DAI. Total soluble Met contents

Table II. *Mapping of the mto1-1 mutation*

ET3 mutant plants (male parents) were crossed with MSU22 or the *abi3-1* mutant strains, and the segregation of the mutant phenotypes were scored in the F_2 generations.

ET3 × MSU22					
Marker Genes	ETN-r[a]		ETN-s[a]	Total	χ^2 (9:3:4)
	WT[b]	MT[b]			
hy2	559	29	218	806	124.6[c]
gl1	476	112	218	806	12.6[c]

ET3 × *abi3-1* strain					
Marker Gene	ABA-s[d]	ABA-r[d]		Total	χ^2 (12:3:1)
		ETN-r[a]	ETN-s[a]		
abi3	560	69	109	738	120.6[c]

[a] ETN-r and ETN-s, Ethionine-resistant and -sensitive plants, respectively. [b] WT and MT, Plants with wild-type and mutant phenotypes, respectively, of the marker genes. [c] Significantly different from the segregation indicated in the table ($P < 0.005$). [d] ABA-r and ABA-s, ABA-resistant and -sensitive germination, respectively.

increased in both *mto1-1* and wild-type plants during the vegetative stage. After 30 DAI, while total soluble Met contents remained fairly constant in the wild type, more than 90% of soluble Met disappeared from the rosette region in the *mto1-1* mutant plants.

Spatial Patterns of Soluble Met Accumulation

To know the distribution of soluble Met during the reproductive growth stage, aerial parts of plants 40 DAI were dissected, and soluble Met was analyzed. As shown in Figure 4, the soluble Met concentration was high in the apex region and fruit region for both the wild-type and *mto1-1* mutant plants. Compared with the wild type, the mutant plants accumulated 5- to 8-fold more soluble Met in the fruit and apex regions.

Ethylene Production

Ethylene is one of the major phytohormones that is synthesized from Met with SAM and ACC as intermediates. Ethylene production from the rosette region was assayed 21 DAI. At this stage, the content of soluble Met was about 40-fold higher in *mto1-1* mutants than in the wild-type plants (Fig. 3B). As shown in Table IV, ethylene production in *mto1-1* mutant plants was only 40% higher than in the wild type.

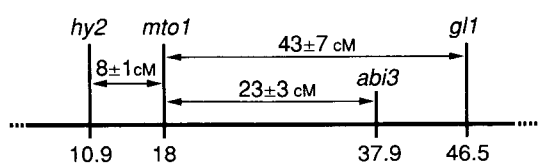

Figure 2. Chromosomal location of the *mto1* mutation. Part of chromosome 3 that covers the map position of the *mto1* mutation is shown, and the estimated map distances ± SD from marker genes are indicated. Map positions of the marker genes were taken from Meinke and Cherry (1992). cM, Centimorgans.

DISCUSSION

In this study we isolated *A. thaliana* mutants that are resistant to ethionine, a toxic analog of Met. Like previous reports of cultured plant cells (Widholm, 1976; Reish et al., 1981; Gonzales et al., 1984; Madison and Thompson, 1988) and microorganisms (Lawrence et al., 1968; Greene et al., 1970), the ethionine-resistant mutants we isolated exhibited elevated levels of soluble Met. Because we selected the mutants from the same batch of a mutagenized M_2 population, the nine mutant lines isolated are not necessarily independent

Table III. *Concentration of soluble amino acids in the rosette region*

Soluble amino acids in the rosette regions of plants 20 DAI were assayed. The averages ± SD of three independent experiments are shown. For those amino acids that are marked with asterisks, the concentrations in Col-0 and ET3 strains were significantly different by *t* test ($P < 0.025$).

Amino Acids	Concentrations		Fold Increase[a]
	Col-0	ET3	
	pmol mg^{-1} fresh wt		
Asp	583.0 ± 147.0	586.0 ± 214.0	1.01
Asn	653.0 ± 249.0	525.0 ± 192.0	0.80
Lys	17.4 ± 2.4	19.3 ± 1.1	1.11
Thr	1185.0 ± 762.0	1048.0 ± 478.0	0.88
Ile	26.4 ± 6.7	33.8 ± 7.4	1.28
Homoserine[b]	1.1 ± 0.6	1.2 ± 0.6	1.05
Cystathionine	6.6 ± 2.4	13.0 ± 4.9	1.97
Met*	14.5 ± 3.4	575.0 ± 83.0	39.7
SAM	0.29 ± 0.09	26.0 ± 19.0	90.5
S-methylmethionine*	4.8 ± 1.8	120.0 ± 29.0	24.8
Met sufoxide*	3.0 ± 0.5	47.3 ± 5.3	15.6

[a] The concentrations in ET3 normalized with those in Col-0. [b] Homoserine was not detectable in the rosette region of plants 20 DAI of both Col-0 and ET3. The concentrations of plants 25 DAI are shown.

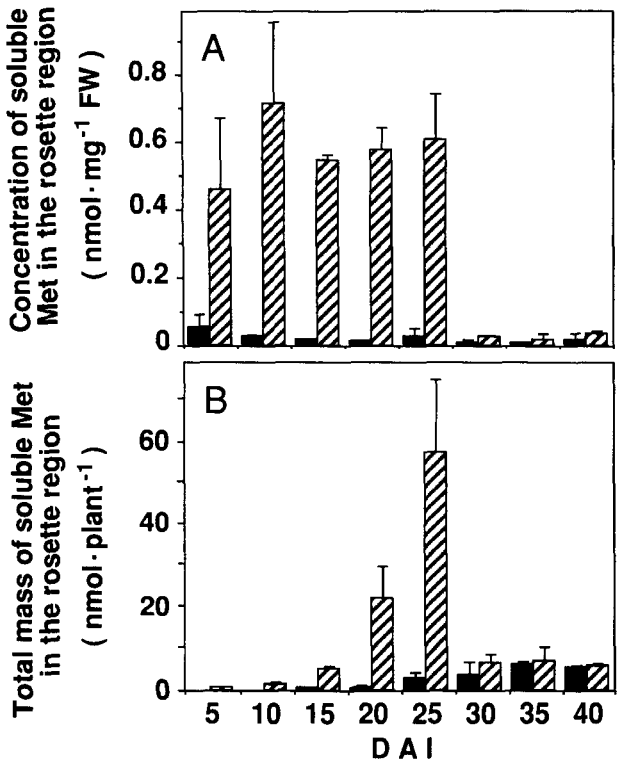

Figure 3. Developmental time course of soluble Met accumulation in the rosette region. Col-0 (■) and ET3 (▨) plants were harvested on the DAI as indicated, and soluble Met in the rosette region was assayed. A, The soluble Met concentration in the rosette region. B, The total soluble Met mass per rosette region. The averages ± SD of three independent experiments are shown. FW, Fresh weight.

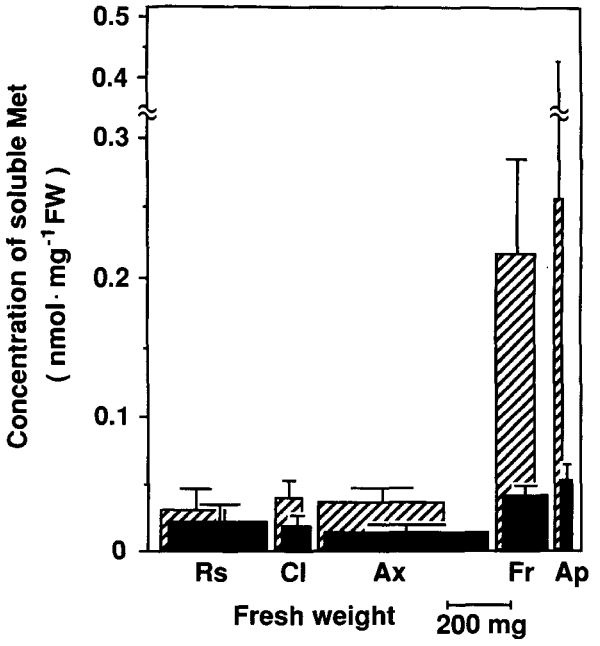

Figure 4. Distribution of soluble Met in aerial parts of plants 40 DAI. Aerial parts of plants 40 DAI were dissected into the rosette region (Rs), cauline leaves (Cl), the inflorescence axis region (Ax), the fruits region (Fr), and the inflorescence apex region (Ap) (see "Materials and Methods"). The experiments were done three times, and the average fresh weights (*x* axis) and the averages ± SD of soluble Met concentration (*y* axis) of Col-0 (■) and ET3 (▨) plants are shown. The area of each block represents the total soluble Met mass. The total soluble Met mass (in nmol/plant) of Col-0 and ET3 plants, respectively, were as follows: 5.1 and 5.6 in the rosette region, 1.4 and 3.5 in cauline leaves, 5.7 and 15.5 in the inflorescence axis region, 5.0 and 26.5 in the fruits region, and 1.9 and 9.7 in the inflorescence apex region. FW, Fresh weight.

of each other. Thus, only one of the mutants, ET3, was analyzed in detail. The fact that *mto1-1* mutant plants accumulated Met in the absence of ethionine indicates that constitutively high levels of Met accumulation resulted in the ethionine-resistant phenotype and that *mto1-1* mutation causes constitutive changes in the metabolic pathway of Met.

Biochemical characterization has been reported for some mutants that accumulate high levels of soluble Met. In the cases of *Saccharomyces cerevisiae* (Cherest et al., 1973; Thomas et al., 1988) and *Escherichia coli* (Greene et al., 1970, 1973; Markham et al., 1984), overaccumulation of soluble Met is caused by a defect in Met *S*-adenosyltransferase activity. SAM is one of the major metabolites of L-Met, and blockage of this reaction leads to overaccumulation of Met. Mutants that overaccumulate soluble Met were also isolated by selecting for Lys plus Thr resistance in a maize cell culture (Hibberd et al., 1980). In this case the accumulation of soluble Met was ascribed to an alteration in feedback inhibition of aspartate kinase activity, the first enzyme in the Asp pathway. In cases of plant cell lines resistant to ethionine, it is not well characterized which step in the pathway is responsible for the elevated levels of Met. Our preliminary results indicated that neither Met *S*-adenosyltransferase nor Asp kinase activity was altered in *mto1-1* mutant plants (data not shown). The final steps for Met biosynthesis in plants are the conversion of cystathionine to Met via homocysteine (Giovanelli et al., 1980). Although the level of homocysteine was below the limit of detection in both the wild-type and *mto1-1* mutant plants, the level of cystathionine was not significantly different between the wild-type and *mto1-1* mutant plants (Table III). This suggests that the *mto1-1* mutation affects either of the two last reactions in Met biosynthesis. Studies of *Lemna* indicated that synthesis of cystathionine is a critical step for the regulation of Met biosynthesis (Thompson et al., 1982; Giovanelli et al., 1985).

Table IV. *Ethylene production by rosette leaves*

Ethylene production by the rosette region of plants 21 DAI was assayed. The averages ± SD of six different samples that were grown in parallel are shown. The amounts of ethylene produced by Col-0 and ET3 strains were significantly different from each other by *t* test ($P < 0.01$).

Strains	Ethylene Production
	nL g⁻¹ fresh wt
Col-0	13.2 ± 3.2
ET3	18.2 ± 1.6

The developmental time course of soluble Met accumulation in *mto1-1* mutant plants showed that the soluble Met content in the rosette region decreased dramatically upon the switch from vegetative growth to reproductive growth (Fig. 3). During the 5-d period between 25 and 30 DAI, more than 90%, or 50 nmol, of soluble Met was lost from the rosette region per plant. Two possibilities can account for this phenomenon: (a) soluble Met that had accumulated in the rosette region was translocated to inflorescence, and (b) Met was degraded in the rosette region, and overproduction was initiated in the inflorescence. Spatial patterns of soluble Met distribution 40 DAI (Fig. 4) showed that the wild-type and *mto1-1* mutant plants accumulated about 35 and 60%, respectively, of soluble Met in the apex region and in fruit region (see legend to Fig. 4), the two major sink organs during the reproductive growth period. It is intriguing to note that the wild-type and *mto1-1* mutant plants showed similar distribution patterns. Moreover, the soluble Met content in the rosette region after the onset of reproductive growth was not significantly different between the wild-type and *mto1-1* mutant plants (Fig. 3B). These observations might suggest that soluble Met accumulated in the rosette region during the vegetative growth period was translocated to the sink organs at the onset of reproductive growth and that the translocation system is intact in *mto1-1* mutant plants. The amount of soluble Met in regions other than the rosette region 40 DAI was about 55 nmol (Fig. 4). This value coincides with the amount of soluble Met that was lost from the rosette region between 25 and 30 DAI, further suggesting that the excess soluble Met was translocated, not degraded.

Taken together, these data suggest that a translocation system is operating to maintain the soluble Met concentration in the rosette region at a certain low level by translocating soluble Met to sink organs. In the absence of sink organs during the vegetative growth period, the *mto1-1* mutation caused increased accumulation of soluble Met in the rosette region. Although we have no information about how the Met is translocated, Riens et al. (1991) and Winter et al. (1992) reported that the soluble amino acid concentration in phloem sap is proportional to cytosolic concentration, suggesting that Met per se is translocated.

Analysis of various metabolites related to Met in *mto1-1* mutant plants will lead to the understanding of the regulation of the Met biosynthesis pathway. As an example, we analyzed the levels of ethylene production. The gaseous phytohormone ethylene is synthesized from SAM via ACC (Yang and Hoffman, 1984). In *mto1-1* mutant plants, the rosette region accumulated about 90-fold more SAM than wild-type plants 20 DAI, whereas ethylene production of the rosette leaves was only 40% higher than the wild type. This result is consistent with previous observations that ACC synthase, the enzyme that catalyzes the formation of ACC from SAM, is the main step of regulation for ethylene synthesis (Kende, 1989).

ACKNOWLEDGMENTS

The authors are grateful to Drs. Mamoru Honma, Junichi Yamaguchi, and Atsuo Kimura and Ms. Tomomi Hotta for their advice and assistance with ethylene assays and to Dr. Hirokazu Tsukaya for his advice regarding plant dissection. We are grateful to Dr. George P. Rédei for providing the Col-0 strain, to Dr. Maarten Koornneef for the W100 and the *abi3-1* strains, and to Dr. Chris R. Somerville for the MSU22 strain. We are also grateful to Ms. Fumiko Hikimoto for her technical assistance and to Ms. Kumi Fujiwara for her general assistance.

Received October 18, 1993; accepted December 17, 1993.

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