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L-*O*-Methylthreonine-Resistant Mutant of *Arabidopsis* Defective in Isoleucine Feedback Regulation[1]

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Threonine dehydratase/deaminase (TD), the first enzyme in the isoleucine biosynthetic pathway, is feedback inhibited by isoleucine. By screening M_2 populations of ethyl methane sulfonate-treated *Arabidopsis thaliana* Columbia wild-type seeds, we isolated five independent mutants that were resistant to L-*O*-methylthreonine, an isoleucine structural analog. Growth in the mutants was 50- to 600-fold more resistant to L-*O*-methylthreonine than in the wild type. The resistance was due to a single, dominant nuclear gene that was denoted *omr1* and was mapped to chromosome 3 in GM11b, the mutant line exhibiting the highest level of resistance. Biochemical characteristics (specific activities, K_m, V_{max}, and pH optimum) of TD in extracts from the wild type and GM11b were similar except for the inhibition constant of isoleucine, which was 50-fold higher in GM11b than in the wild type. Levels of free isoleucine were 20-fold higher in extracts from GM11b than in extracts from wild type. Therefore, isoleucine feedback insensitivity in GM11b is due to a mutant form of the TD enzyme encoded by *omr1*. The mutant allele *omr1* of the line GM11b could provide a new selectable marker for plant genetic transformation.

TD, the first enzyme in the biosynthetic pathway of Ile, catalyzes the formation of 2-oxobutyrate from Thr in a two-step reaction. The first step is a dehydration of Thr followed by rehydration and liberation of ammonia (Bryan, 1990). All the reactions downstream from TD are catalyzed by enzymes that are shared by the two main branches of the biosynthetic pathway that lead to the production of the branched-chain amino acids Ile, Leu, and Val. In bacteria and yeast, resistance to the Ile structural analog thialle was associated with a loss of feedback sensitivity of TD to Ile (Umbarger, 1971; Kielland-Brandt et al., 1979). In *Rosa* cells, resistance to the Ile structural analog OMT was also associated with a TD that had reduced sensitivity to feedback inhibition by Ile (Strauss et al., 1985). Being in tissue culture and having high ploidy level, it was not possible to determine the genetic basis of feedback insensitivity to Ile in the *Rosa* variant, the only plant mutant with an Ile-insensitive TD.

We are isolating and characterizing mutants of the branched-chain amino acid biosynthetic pathway at the whole plant level that are insensitive to feedback inhibition. The availability of such mutants in a nearly isogenic background allows investigation of the regulation of the levels of these amino acids. Amino acid overproduction by feedback-insensitive mutants might be a source for plants of higher nutritional value. With this objective in mind, in this study we have isolated and characterized mutants that are resistant to the Ile structural analog OMT in the nearly isogenic background of *Arabidopsis thaliana*.

MATERIALS AND METHODS

Seed Mutagenesis

About 100,000 seeds of *Arabidopsis thaliana* (L.) Heynh Columbia wild type were allowed to imbibe (v/v) in an aqueous solution of 0.2% EMS for 18 h at room temperature. The mutagenized seed (M_1) was washed several times with distilled water, planted in subpopulations, and allowed to self-fertilize to produce M_2 seed. The M_2 seed were harvested from each subpopulation separately, surface sterilized, and planted on agar-solidified medium containing 0.5 mM OMT. In this medium wild-type seedlings were completely bleached and died immediately after germination, whereas resistant mutants were able to germinate and produce normal shoots and roots. Five mutants, one from each M_2 subpopulation, were selected to ensure the independent origin of each mutational event. Each of the five mutants was carefully transferred to soil, allowed to self-fertilize, and its progeny was tested for resistance to OMT. Homozygous lines from each of the five mutants were isolated after testing the resistance of the progeny of single plants to OMT for two more generations.

Genetic Stocks

A. thaliana seed was used for the selection of OMT-resistant mutants. Mapping crosses were done using the following multirecessive marker stock lines:

(a) W100, homozygous for nine markers: *an* (angustifolia) and *ap1* (apetalous) on chromosome 1; *py* (pyrimidine-requiring) and *er* (erecta) on chromosome 2; *hy2* (long hypocotyls) and *gl1* (glabra) on chromosome 3; *cer2* (eceriferum) and *bp* (brevipedicellus) on chromosome 4; and *tt3*

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Abbreviations: ALS, acetolactate synthase; EMS, ethyl methane sulfonate; OMT, L-*O*-methylthreonine; TD, threonine dehydratase/deaminase.

(transparent testa) on chromosome 5. W100 was also heterozygous for male sterility, *ms*, a tenth marker on chromosome 5, which was not scored in our mapping crosses.

(b) MSU21, homozygous for the recessive markers *ap1* and *clv1* (clavata) on chromosome 1 and *er* on chromosome 2.

(c) MSU12, homozygous for the recessive markers *as* (asymmetric leaves), *er1*, *cer8* (eceriferum), and *cp2* (compacta) on chromosome 2.

(d) MSU22, homozygous for the recessive markers *hy2*, *gl1*, and *tt5* (transparent testa) on chromosome 3 and *er* on chromosome 2.

(e) MSU15, homozygous for the recessive markers *cer2*, *bp*, and *ap2* on chromosome 4 and *er1* on chromosome 3.

(f) MSU23, homozygous for the recessive markers *cer3* (eceriferum), *tz* (thiazole-requiring), and *gl3* (glabrous) on chromosome 5 and *er1* on chromosome 2.

Growth Conditions

Growth resistance tests were performed according to Mourad et al. (1994). To test for growth resistance to OMT, seeds were surface sterilized in 30% (v/v) commercial bleach followed by six rinses in sterile, distilled water and then planted in Petri plates containing minimal medium (Haughn and Somerville, 1986) with 0.5% Suc, 0.6% Difco agar, and a filter-sterilized aqueous solution of OMT was added after autoclaving the medium (for concentrations, see "Results").

In experiments where competition between OMT and Ile, Leu, Thr, and Val in growth inhibition was tested, the medium containing 0.2 mM of the analog was supplemented with 0 to 2 mM Ile, Leu, Thr, or Val.

Extraction of TD

Extraction of the enzyme was done using a modification of the procedure of Dougall (1970) as follows. About 7 g fresh weight of plant material were homogenized in 10 to 15 mL of extraction buffer (20 mM K_2HPO_4, 2 mM Na_2EDTA, pH 8.0, 2 mM DTT, and 1 mM L-Ile) at room temperature because the enzyme is cold labile (Sharma and Mazumder, 1970). The homogenate was then centrifuged at 27,000*g* for 15 min or at 7,200*g* for 30 min. An equal volume of saturated ammonium sulfate was added to the supernatant, which was then centrifuged as before. The pellet was resuspended in 1 mL of extraction buffer except without Ile to prevent the carryover of Ile to the assay mixture. The resuspended pellet was desalted by passing through a Sephadex G-50 column equilibrated in buffer at pH 9.0 (1 M K_2HPO_4, 1 mM Na_2EDTA, pH 9.0, and 1 mM DTT). The column eluate was the enzyme extract.

TD extraction from single F_2 plants was performed by harvesting a few leaves from the plant and scaling down the same extraction procedure as detailed above.

TD Assay

Enzyme activity was assayed in 0.5 mL according to Strauss et al. (1985) as follows: 300 μL of 1.5× assay mixture (1.5× 0.15 M Tris-HCl, pH 9.0, 60 mM Thr, 0.3 M K_2HPO_4, 0.3 mM Na_2EDTA, pH 9.0, 0.3 mM DTT), 100 μL of treatment (5× final concentration of Ile or OMT), and 100 μL of enzyme extract. The reaction mixture was incubated at 30°C for 30 min. To terminate the reaction, 0.2 mL of 50% (w/v) TCA was added. The ketoacid produced was determined according to Friedmann and Haugen (1943) by adding 0.2 mL of 0.1% (w/v) 2,4-dinitrophenylhydrazine in 2 N HCl and incubating for 20 min at room temperature. KOH (0.9 mL of 2.5 N) was then added and mixed, the tubes were incubated for 15 min at room temperature, and the A_{515} was determined.

The pH optimum was determined by using different buffers in the assay mixtures to cover a wide range of pH values: Mes for pH 5.5, 6.0, and 6.5; phosphate for pH 6.5, 7.0, 7.5, and 8.0; Tris-HCl for pH 8.0, 8.5, 9.0, and 9.5.

TD activity of single plant extracts was assayed as above except that it was scaled down to a total volume of 50 μL as follows: 30 μL of 1.5× assay mixture, 10 μL of 2.5 mM Ile (5× the final concentration), and 10 μL of undiluted enzyme extract. The assay was continued exactly as above except that all reagents were added at one-tenth volume.

Extraction and assay of acetolactate synthase was done as previously described (Mourad et al., 1993). Protein determinations for both assays, TD and ALS, were done using the Bio-Rad Protein Assay (Bradford, 1976).

Free Amino Acid Analysis

About 1 g of 3-week-old plants was ground in 3 mL of 0.2 M sodium citrate. The homogenate was centrifuged and the supernatant was filtered through a 0.45-μm Nalgene filter. The filtrate was then mixed with a stock solution of norleucine. Amino acid content was quantitatively determined by HPLC using the Interaction-Sodium Loaded Ion Exchange column (25-μL injection volume) with post-column derivation using *O*-pthaldehyde and subsequent fluorometric detection. For each genotype two different extracts were analyzed. Each extract was analyzed in triplicate.

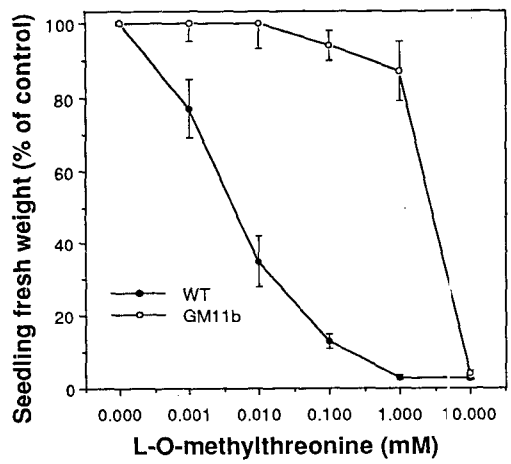

Figure 1. Growth resistance of the mutant line GM11b to OMT compared to the wild type (WT).

Table I. *Approximate concentration of OMT that inhibits 50% of the growth (I_{50}) of Arabidopsis wild-type and OMT-resistant mutants*

Line	M_2 Subpopulation	I_{50} (mM)	-Fold Resistant
Wild type		0.006	
GM1	I	4.0	666.0
GM8a	II	0.9	150.0
GM9f	III	2.0	333.0
GM10d	III	0.4	66.0
GM11b	IV	4.0	666.0
GM12c	IV	2.5	416.0
GM14d	V	1.5	250.0
GM14b	V	2.0	333.0

RESULTS

Isolation of OMT-Resistant Mutants

Five subpopulations, each composed of 20,000 EMS-treated *A. thaliana* wild-type M_2 seeds, were germinated in the presence of 0.5 mM OMT, a concentration that completely inhibited growth of the wild type (Fig. 1). The basis for mutant selection came from the fact that OMT was previously reported to compete with Ile because of structural similarity for incorporation into proteins, thus inhibiting the growth of mammalian cells (Rabinovitz, 1955). Therefore, we hypothesized that whole plant mutants could also be rescued by their ability to overproduce Ile, through the reduced feedback sensitivity of TD to Ile, which in turn could effectively compete with OMT for incorporation into proteins. With this objective in mind we selected a total of eight mutants from five M_2 subpopulations based on their ability to germinate and grow normal shoots and roots in a medium containing 0.5 mM OMT. Based on the concentration of OMT that inhibits 50% of the growth, resistance ranged from 50- to 600-fold above the wild type (Table I). Of the two mutants exhibiting the highest levels of growth resistance (Table I), GM11b (Fig. 1) was chosen for further genetic and biochemical characterization.

Resistance at the Enzyme Level

TD activities in extracts from both genotypes, GM11b and wild type, were similarly inhibited by OMT, albeit at very high concentrations (Fig. 2A). TD activity in extracts from GM11b were 53-fold more resistant to feedback inhibition by Ile than in extracts from the wild type (Fig. 2B). For wild-type TD extracts, the concentration of OMT required for 50% inhibition was about 10,000-fold higher (Fig. 2A) than in the case of plant growth (Fig. 1). Therefore, the toxic effect of OMT on *Arabidopsis* growth is probably not due to TD inhibition.

Table II. *Specific activity in μmol mg^{-1} protein h^{-1} of TD in extracts from Columbia wild type and the mutant line GM11b*

Substrate	Wild Type	GM11b
Thr	1.365 ± 0.005	1.015 ± 0.035
Ser	0.095 ± 0.003	0.120 ± 0.005

Genetic Basis of OMT Resistance

The specific activity of TD, for both Thr and Ser, in extracts from homozygous GM11b was similar to that in wild-type extracts (Table II). This indicated that OMT resistance in GM11b was not due to amplification of TD activity. Also, TD activities in extracts from the F_1 (WT × GM11b) had intermediate levels of resistance to Ile inhibition compared to those from the homozygous-sensitive wild type and the homozygous-resistant GM11b (Fig. 2B). This led us to exclude the possibility of resistance to OMT being either an uptake mutation or due to degradation of the analog by the plant.

Growth resistance to OMT among F_1 progeny as well as in F_2 (3:1) and backcross progeny (1:1) of reciprocal crosses between the wild type and homozygous GM11b revealed that it is controlled by a single dominant nuclear gene (Table III; Fig. 3). The homozygous mutant GM11b was backcrossed five times to wild type in order to eliminate other mutations that might have been induced by the EMS treatment during mutagenesis. We denoted the new mutant allele in GM11b that is responsible for OMT resistance *omr1*. The wild-type sensitive allele would be *OMR1*.

The Map Location of *omr1*

To map *omr1*, GM11b was crossed to the multimarker line, W100, which is homozygous for nine recessive markers and heterozygous for one, male sterility. The F_1 plants were allowed to self-fertilize to produce F_2 seed, which was germinated in the presence of 0.2 mM OMT. OMT-resistant seedlings (75% of the F_2 plants) were transferred to soil and

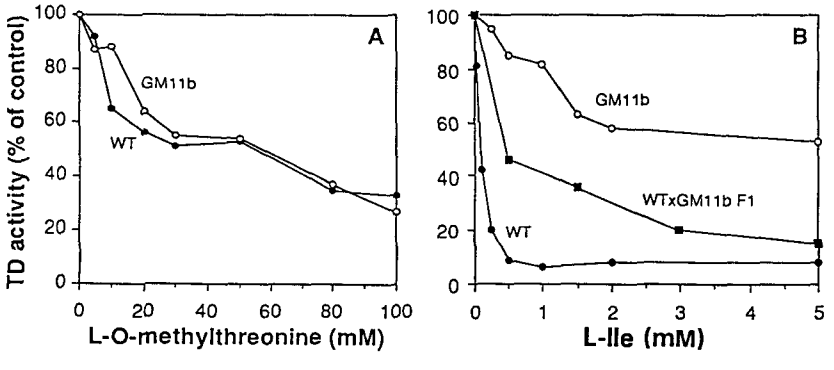

Figure 2. The effect of OMT and Ile on TD activity in extracts from the wild type (WT) and the mutant (GM11b) and their F_1 product (WT × GM11b). A, Inhibition of TD activity by OMT; B, inhibition of TD activity by Ile.

Table III. *Inheritance and genetic analysis of growth resistance to OMT in A. thaliana Columbia wild-type (OMR1/OMR1) and the mutant line GM11b (omr1/omr1)*

WT, Wild type; Res, resistant; Sens, sensitive; BC1, backcross of the F1 to the WT parent.

Cross	Number of Plants			
	Res	Sens	Res:Sens	Chi square
GM11b × WT F1	93.0	0.0	4:0	—
WT × GM11b F1	103.0	0.0	4:0	—
WT × (GM11b × WT F1) BC1	63.0	59.0	1:1	0.14
GM11b × WT F2	78.0	25.0	3.1:1	0.03
WT × GM11b F2	89.0	30.0	2.9:1	0.01

Table IV. *Segregation of nine recessive markers among 266 OMT-resistant F_2 progeny of the cross W100 line × GM11b (omr1/omr1)*

Abbreviations for all the W100 markers, see "Materials and Methods"; ND, not determined.

W100 Marker	Chromosome	Marker Segregation (wild type:recessive)	Marker Linkage to *omr1*
an	1	200:66 (3.03:1)	−
ap1	1	203:63 (3.20:1)	−
py	2	197:69 (2.86:1)	−
er	2	201:65 (3.10:1)	−
hy2	3	256:10 (25.60:1)	+
gl1	3	228:38 (6.00:1)	+
cer2	4	196:70 (2.83:1)	−
bp	4	204:62 (3.29:1)	−
tt3	5	205:61 (3.36:1)	−
ms	5	ND	

scored for segregation for each of the W100 markers except for male sterility (Table IV). Among the resistant F_2 progeny, all of the markers segregated independently except for *hy2* and *gl1* on chromosome 3 (Table IV). Segregation data were also confirmed by scoring for recessive marker segregations among OMT-resistant F_2 plants obtained from crossing GM11b (*omr1/omr1*) to each of five different marker lines (see "Materials and Methods"), each homozygous for up to three recessive markers on the same chromosome.

To calculate the degree of linkage of *omr1* to the markers *hy2* and *gl1*, the F_2 progeny of the two crosses, *hy2/hy2* × *omr1/omr1* and *gl1/gl1* × *omr1/omr1*, was germinated on medium lacking OMT to allow the survival of all the progeny, including the sensitive *OMR1/OMR1*. The F_2 progeny of the cross *hy2/hy2* × *omr1/omr1* was scored for short (*HY2/-*) and long (*hy2/hy2*) hypocotyl, which segregated 3 *HY2*:1 *hy2*. The long-hypocotyl seedlings (*hy2/hy2*) were carefully transferred to one side of a plate containing medium with 0.2 mM OMT, whereas the short-hypocotyl seedlings (*Hy2/-*) were transferred to the other half of the same plate. Most of the *hy2/hy2* were sensitive and died, whereas most of the *Hy2/-* were resistant to OMT and grew normal shoots and roots, confirming the close linkage of *omr1* to the *HY2* locus (Fig. 3B).

In a similar way the F_2 progeny of the cross *gl1/gl1* × *omr1/omr1* was scored for segregation of the *gl1* marker in the absence of OMT (3 *GL1*:1 *gl1*). The nonglabrous (*gl1/gl1*) and the glabrous (*GL1/-*) seedlings were each transferred separately to one half of a plate containing

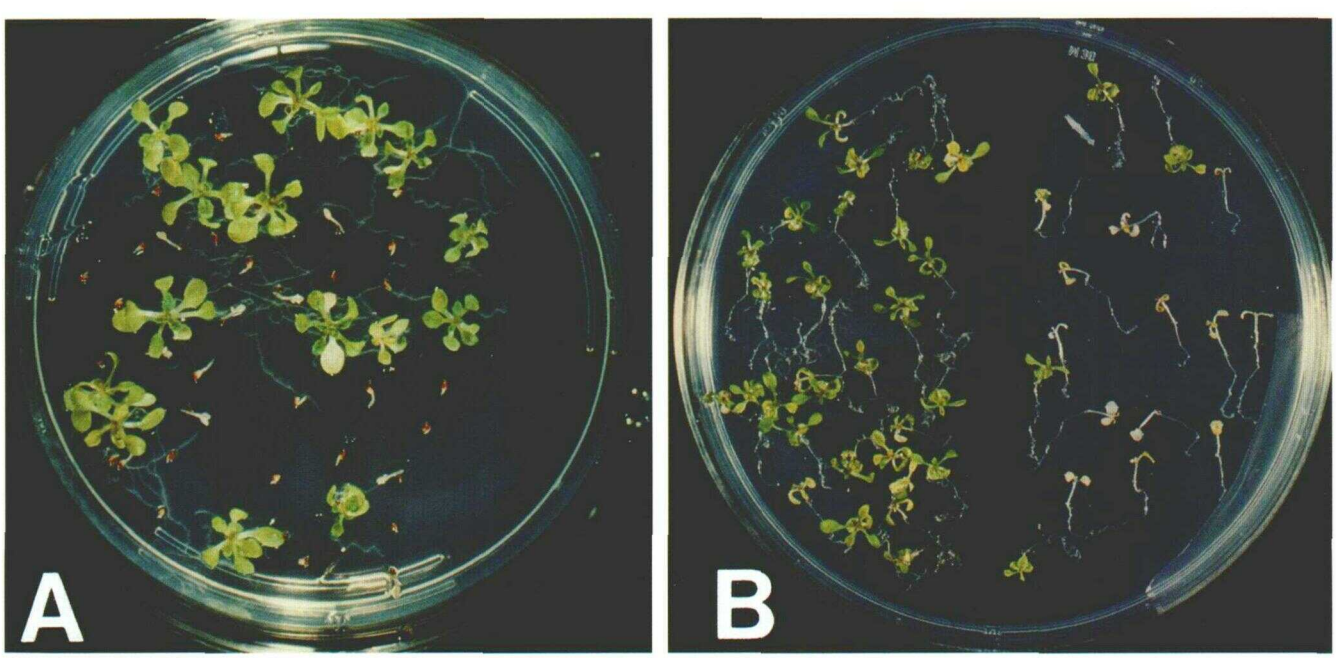

Figure 3. Linkage of *omr1* to the *HY2* locus. A, One of several plates scored for the segregation of growth resistance to 0.2 mM OMT among the backcross progeny (BC1) of the cross WT × (GM11b *omr1/omr1* × WT F_1). B, The F_2 progeny of the cross *hy2/hy2* × *omr1/omr1* (GM11b) was first germinated in minimal medium then transferred to a plate (150 mm) containing minimal medium with 0.2 mM OMT. All of the *HY2/-* (short hypocotyl) were transferred to the right half of the plate, whereas all of the *hy2/hy2* (long hypocotyl) were transferred to the left half of the plate.

medium with 0.2 mM OMT. The OMT-resistant and -sensitive seedlings in each half of the plate were scored. This way it was possible to score for both the parental and recombinant OMT-sensitive classes for both *hy2* and *gl1* markers. This also allowed the precise calculation of recombination frequencies between *omr1* and the two markers *hy2* and *gl1*. *omr1* was found to be 6.53 ± 2.65 centimorgan below *hy2* and 29.51 ± 3.91 centimorgan above *gl1* on chromosome 3 (Table V; Fig. 4).

Co-Segregation of Growth and Enzyme Resistance

If *omr1* controls both growth resistance to OMT at the whole plant level and TD resistance to Ile, then the two resistances should co-segregate. To test this we planted F_2 seed of the cross *OMR1/OMR1* × *omr1/omr1* in medium containing 0.2 mM OMT. The resistant seedlings, the genotype of which would be either *omr1/omr1* or *OMR1/omr1*, were numbered and then transferred to soil and grown under 8 h of light to increase the amount of vegetative growth. Just before flowering, a few leaves from each plant were harvested and homogenized for TD extraction. From each plant, TD activity was assayed in the presence of 0.5 mM Ile, a concentration that can easily distinguish the TD activity from the two genotypes (Fig. 2B), *OMR1/omr1* and *omr1/omr1*. Of the 60 individual F_2 plants tested, TD assays revealed 22 *omr1/omr1*:38 *OMR1/omr1*. The F_3 seed from each of the 60 individual F_2 plants, whose TD activity was tested for the level of Ile resistance, was harvested separately and about 100 F_3 seeds were planted in a plate containing medium with 0.2 mM OMT. Each of the 22 F_2 plants shown by TD activity test to be *omr1/omr1* produced 100% F_3 progeny that was able to grow normally in the presence of 0.2 mM OMT. On the other hand, the F_3 progeny produced from each of the 38 F_2 plants shown by TD activity test to be *OMR1/omr1* segregated 75% resistant:25% sensitive for growth in the presence of 0.2 mM OMT (data not shown). These results suggested that the growth resistance to OMT at the whole plant level and the resistance of TD to Ile are due to the same mutant allele, *omr1*.

Table V. *Recombination frequencies and map distances[a] between omr1 and the markers hy2 and gl1 in chromosome 3*

Marker Pair	Recombination	Map distance
	%[b]	*cM*[c]
omr1:hy2	6.50 ± 2.60	6.53 ± 2.65
omr1:gl1	26.50 ± 2.81	29.51 ± 3.91

[a] Data used to determine the map distances were obtained from 97 F_2 progeny of the cross MSU22 (*hy2/hy2*) × GM11b (*omr1/omr1*) and 353 F_2 progeny of the cross MSU22 (*gl1/gl1*) × GM11b (*omr1/omr1*). [b] Recombination frequencies and SE values were estimated using the product method (Immer, 1930), taken that the markers are in coupling-phase linkage and four recognized classes in F_2 populations, since F_2 populations were scored for markers before transfer to OMT plates, then transferred to 0.2-mM OMT plates to score for resistance. [c] Map distances in centimorgans (cM) and their SE values were calculated using the Kosambi mapping function as mentioned by Koornneef et al. (1983)

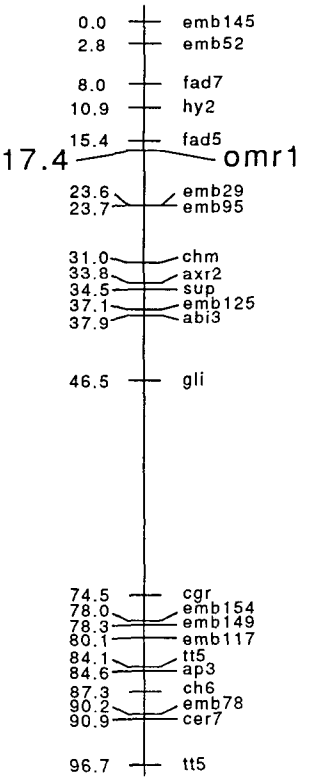

Figure 4. The genetic map of chromosome 3 showing the location of the new mutant allele, *omr1*, OMT resistance. The figures on the left represent the map distance from the first marker mapped (*emb145*) at the tip of the chromosome.

Kinetic Characteristics of TD from Wild Type and GM11b

Substrate saturation curves, and apparent K_m and V_{max} values for Thr, were not significantly different for TD extracted from wild type, *OMR1/OMR1* (K_m = 2.80 mM, V_{max} = 3.13 μmol mg^{-1} protein h^{-1}; Fig. 5, A and B) and from GM11b *omr1/omr1* (K_m = 3.57 mM, V_{max} = 2.22 μmol mg^{-1} protein h^{-1}; Fig. 5, A and B). Similarly, substrate saturation curves and apparent K_m and V_{max} values for Ser were not significantly different for TD extracted from wild type (K_m = 5.00 mM, V_{max} = 0.25 μmol mg^{-1} protein h^{-1}; Fig. 5, C and D) and from GM11b (K_m = 5.00 mM, V_{max} = 0.33 μmol mg^{-1} protein h^{-1}; Fig. 5, C and D). On the other hand, the apparent inhibition constants, K_i, of TD for Ile was 50-fold higher in extracts from GM11b (K_i = 0.50 ± 0.06 mM; Fig. 5F) than from the wild type (K_i = 0.010 ± 0.001 mM; Fig. 5E). The pH optimum of TD activity from wild type and GM11b was similar, about pH 9.0 (Fig. 6).

omr1 Encodes an Ile-Insensitive, Biosynthetic TD

TD activity in extracts from wild-type and GM11b leaves at different developmental stages (first pair, second pair, third pair, and rosette) showed reduction with increased age (data not shown). In the wild type no clear evidence of the emergence of a biodegradative Ile-insensitive form of TD was detected in young leaves (Fig. 7A). At very late stages of development, in senescing leaves showing

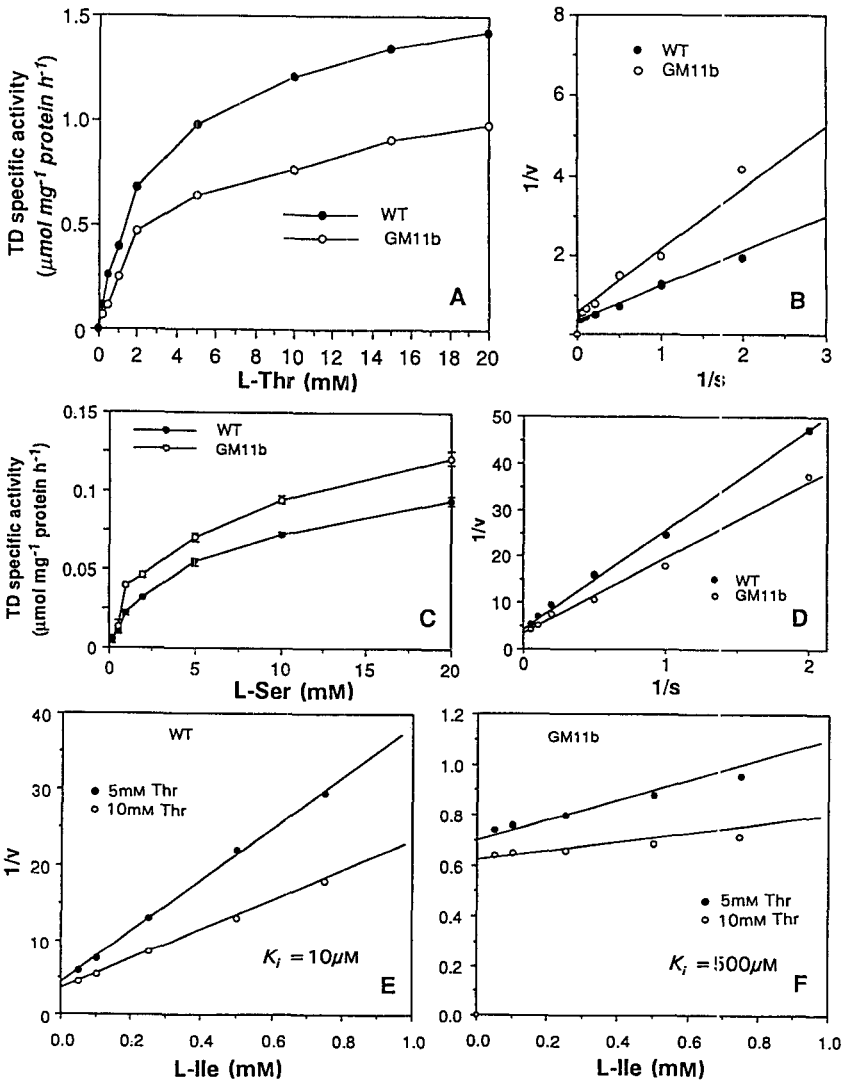

Figure 5. Kinetic characteristics of TD in extracts from the wild type (WT, *OMR1/OMR1*) and the OMT-resistant mutant GM11b (*omr1/omr1*). A, Substrate saturation curves of TD for Thr. B, Lineweaver double-reciprocal plots of TD velocity (v) versus Thr concentration (s). C, Substrate saturation curves of TD for Ser. D, Lineweaver double-reciprocal plot of TD velocity (v) versus Ser concentration. E, Inhibition constant, K_i, of the wild-type (*OMR1/OMR1*) TD for the inhibitor Ile at two Thr concentrations, 5 mM and 10 mM, and deduced from plotting 1/v versus Ile. F, Inhibition constant, K_i, of TD from GM11b (*omr1/omr1*) for the inhibitor Ile at two Thr concentrations, 5 mM and 10 mM.

signs of Chl degradation, a TD form was expressed that displayed low levels of resistance (25–30%, Fig. 7A) to Ile. If this is a biodegradative form of TD, then *Arabidopsis* must have very low levels of it expressed at the senescing stage. Biodegradative TDs expressed at a low level are typical of many plant species (B.K. Singh, personal communication). This is quite different from tomato, which contains a biodegradative form of TD that is almost fully resistant to feedback inhibition by Ile at stages earlier than senescence (Szamosi et al., 1993). *omr1* of GM11b must be a mutant allele that encodes an Ile-insensitive, biosynthetic form of TD, since this form appears at germination and continues to be expressed in the plant until senescence (Fig. 7B).

Amino Acid Analysis

Free amino acid profiles in nonhydrolyzed extracts revealed that GM11b *omr1* had about a 20-fold higher level of free Ile than the wild-type *OMR1* (Table VI). Small differences, up to 2-fold higher, in the levels of all other free amino acids in GM11b than in wild type were statistically insignificant (Table VI).

ALS of GM11b Is Unaffected by *omr1*

ALS is the second enzyme in the biosynthetic pathway of Ile, Leu, and Val, and is known to be slightly feedback inhibited by Val, Leu, and Ile (Miflin et al., 1979). ALS activities in extracts from wild type and GM11b were equally sensitive to feedback inhibition by Ile, Leu, and Val (data not shown). This is a further confirmation that the mutant allele *omr1* encodes an Ile-insensitive TD and has no effect on ALS.

Effect of Leu and Val on TD from Wild Type and GM11b

Val at concentrations up to 10 mM had no inhibitory effect on TD activity from either the wild type or GM11b (Fig. 8A). Leu was inhibitory (Fig. 8B), its effect resembling

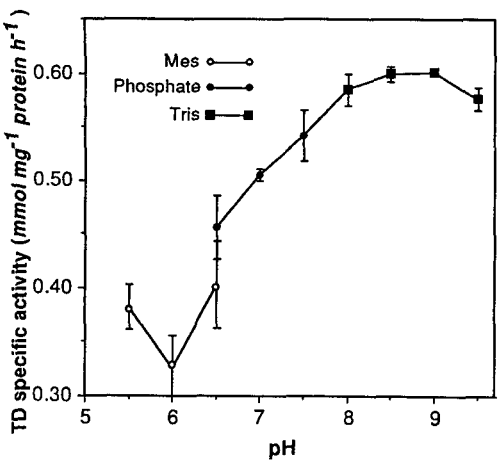

Figure 6. The pH optimum of TD from the wild type (*OMR1/OMR1*). Mes buffer was used to assay TD activity at pH, 5.5, 6.0, and 6.5; phosphate buffer was used at pH 6.5, 7.0, and 7.5; and Tris buffer was used at pH 8.0, 8.5, 9.0, and 9.5.

that found with TD from *Rosa* cells (Strauss et al., 1985). However, TD from both the wild type and GM11b was similarly sensitive to Leu (Fig. 8B).

Ile Reverses Growth Inhibition by OMT

The growth inhibition of wild-type *Arabidopsis* seedlings by 0.2 mM OMT was strongly reversed by added Ile up to 1 mM concentration but not by Leu, Thr, or Val. Ile at 2 mM was, itself, inhibitory (Table VII).

DISCUSSION

Plant TD resembles that of bacteria (Ramakrishnan and Adelberg, 1964; Whiteley and Tahara, 1966) and yeast (de Robichon-Szulmajster and Magee, 1968; Brunner et al., 1969) in that it is a regulatory enzyme in the biosynthetic pathway of Ile. Furthermore, feedback inhibition of TD by Ile was demonstrated in such plants as *Rosa* (Strauss et al., 1985), spinach (Sharma and Mazumder, 1970), tomato (Szamosi et al., 1993), maize (Bryan, 1980; Kirchner and Bryan, 1985), and *Lemna* (Giovanelli et al., 1988). In none of these plant systems was the genetic basis of feedback regulation of Ile biosynthesis elucidated. In bacteria and yeast (Betz et al., 1971; Umbarger, 1971) mutants resistant to thiaIle, a structural analog of Ile, had a TD with reduced sensitivity to feedback inhibition by Ile. The only mutant available in plants with an Ile-insensitive TD was the OMT-resistant line of *Rosa* cells (Strauss et al., 1985). Revealing the genetic basis of the *Rosa* mutant was not possible because of the difficulty of doing genetic analysis on cells growing in culture, and it was further complicated by the high ploidy levels of such cells.

To our knowledge, this report is the first to give details of a whole plant mutant, GM11b of *A. thaliana*, that reveals the genetic basis of feedback regulation of Ile biosynthesis. The mutant line GM11b was selected for growth resistance to OMT, a compound previously reported to be a structural analog of Ile that inhibits growth of mammalian cell cultures by inhibiting incorporation of Ile into proteins (Rabinovitz et al., 1955). OMT was very effective in inhibiting the growth of *A. thaliana* wild-type seedlings. Growth resistance of GM11b to OMT was controlled by a single, nuclear, dominant gene, *omr1*, which was mapped to chromosome 3 by linkage to the markers *hy2* and *gl1*. TD activity in extracts from the true-breeding OMT-resistant line GM11b revealed a 53-fold increase in resistance to Ile inhibition compared to that from the wild type. On the other hand, TD activity in extracts from GM11b and the wild type were similarly affected by OMT, albeit at concentrations 10,000-fold higher than those needed to inhibit plant growth. This observation strongly supported the idea that OMT does not inhibit plant growth by binding to TD. In GM11b, the resistance of TD activity to Ile and the resistance of plant growth to OMT co-segregated, confirming that both levels of resistance were controlled by the same mutant allele, *omr1*.

The kinetic parameters (K_m, V_{max} for both Thr and Ser), specific activities, and pH optimum of TD from wild type

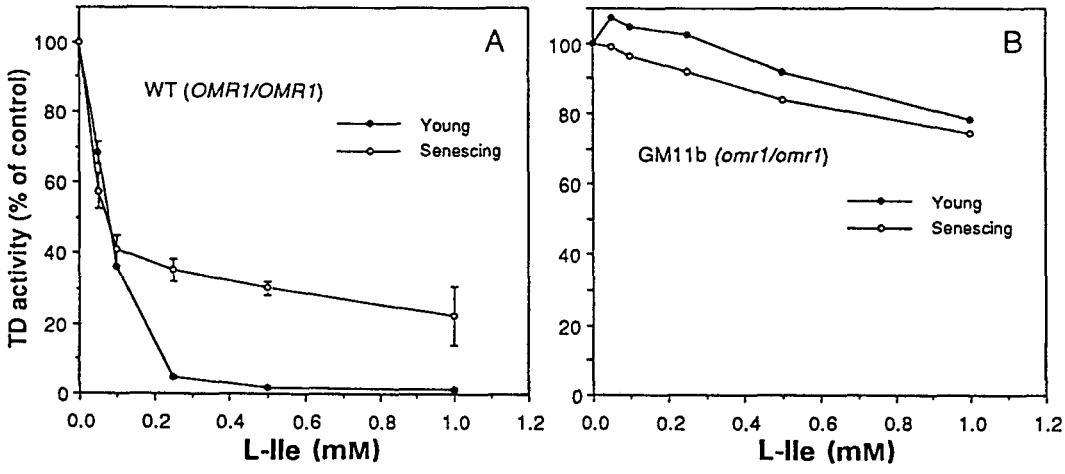

Figure 7. TD activity in young (two-leaf stage) and senescing leaves of *Arabidopsis*. TD activity in extracts from wild type (*OMR1/OMR1*) (A) and GM11b (*omr1/omr1*) (B).

Table VI. *Free amino acid content of wild type OMR1 and GM11b omr1 plants*

Each amino acid is expressed in nmol g^{-1} fresh weight.

Amino Acid	Wild Type (*OMR1*)	GM11b (*omr1*)
Ala	9,465 ± 54	13,439 ± 64
Arg	547 ± 16	1,139 ± 43
Asp	18,343 ± 117	25,369 ± 164
Glu	29,505 ± 198	39,786 ± 551
Gly	16,229 ± 94	35,960 ± 346
His	740 ± 26	1,231 ± 29
Ile	341 ± 18	6,675 ± 75
Leu	386 ± 23	780 ± 4
Lys	777 ± 18	1,333 ± 13
Met	166 ± 3	226 ± 16
Phe	265 ± 3	451 ± 6
Ser	13,263 ± 206	—
Thr	25,175 ± 163	71,661[a] ± 925
Tyr	509 ± 4	603 ± 34
Val	1,207 ± 4	1,893 ± 29

[a] This value represents both Ser and Thr combined because their peaks were not separable in the chromatograms obtained from six different sample injections on the column.

and GM11b were not significantly different. The K_m value of *Arabidopsis* wild-type TD for Thr (2.8 mM) was not much different from that of *Rosa* cells (2.5 mM; Strauss et al., 1985) or spinach (4.5 mM; Sharma and Mazumder, 1970), but it was different from that of tomato (0.25 mM; Szamosi et al., 1993). The pH optimum of *Arabidopsis* TD was about 9.0, resembling that of *Rosa* cells (Strauss et al., 1985) and tomato (Szamosi et al., 1993). On the other hand, the K_i value of TD for Ile was 50-fold higher in extracts from GM11b than that from the wild type. These results confirmed that the mutant allele *omr1* of GM11b must encode a mutant TD molecule with reduced sensitivity to feedback inhibition by Ile. Examining TD activity in extracts from leaves at different developmental stages revealed that *omr1* encodes a biosynthetic TD. ALS activity, the second enzyme in Ile biosynthesis and the first in Val and Leu biosynthesis, of GM11b was as sensitive to inhibition by the three branched-chain amino acids as that of the wild type. This confirmed that *omr1* of GM11b did not affect ALS. TD from *Arabidopsis* wild type and GM11b was equally inhibited by Leu but not at all by Val, and in this was similar to that from *Rosa* cells (Strauss et al., 1985).

The free amino acid profile analysis revealed that Ile was about 20-fold higher in GM11b than in the wild type, a highly significant increase. This is a very high increase in free Ile compared to the 2- to 3-fold increase in Ile observed in the OMT-resistant *Rosa* cells (Strauss et al., 1985). Mutants overproducing Thr were obtained in different plant cell cultures by selecting for resistance to Lys plus Thr (Hibberd et al., 1980; Hibberd and Green, 1982; Cattori-Reynaerts et al., 1983; Diedrick et al., 1990). One dominant mutation in *Nicotiana sylvestris* resistant to a structural analog of Lys, an amino acid of the aspartate family, resulted in a 28-fold increase in free Lys due to a mutant form of dihydrodipiconilate synthase with reduced sensitivity to feedback inhibition by Lys (Negrutiu et al., 1984). Another single dominant mutation in *N. sylvestris* resistant to Lys plus Thr was found to overproduce free Thr in its leaves by 45-fold above the wild-type levels due to a mutant form of aspartate kinase with decreased sensitivity to feedback inhibition by Lys (Frankard et al., 1991). Combining the two dominant mutations of *N. sylvestris* by crossing in one double-mutant genotype resulted in a 15-fold increase in free Lys, but the hybrid was abnormal and completely sterile (Frankard et al., 1992). The 20-fold overproduction of free Ile in GM11b (*omr1/omr1*) of *Arabidopsis* had no effect on the plant's phenotype or fertility.

The inhibition of growth of wild-type *Arabidopsis* brought about by OMT was strongly reversed by Ile, but not Leu, Thr, or Val, in this way resembling *Rosa* cells (Strauss et al., 1985). From these results taken together, we conclude that growth resistance to OMT in the true-breeding line GM11b is by overproducing free Ile through a feedback-insensitive TD encoded by the dominant mutant allele *omr1*. The 20-fold excess of free Ile in GM11b can effectively compete with OMT, its structural analog, for incorporation into proteins. This would allow the mutant GM11b to survive in a medium containing OMT. Therefore, the mode of growth resistance to OMT in *Arabidopsis* (this paper) resembles that reported previously for mammalian cells (Rabinovitz, 1955).

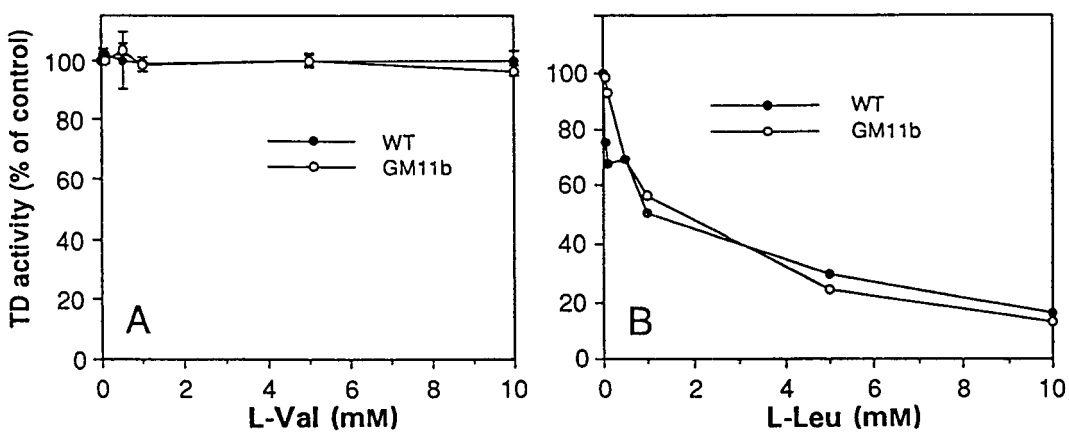

Figure 8. Effect of Val and Leu on TD activity from the wild type (WT) and GM11b. A, Val; B, Leu.

Table VII. *Competition between OMT and branched-chain amino acids in the growth inhibition of wild-type Arabidopsis seedlings*

Treatment	Seedling Fresh Weight
mM	% of control
0.0	100
0.2 OMT	4
0.1 Ile	102
0.2 Ile	100
1.0 Ile	91
2.0 Ile	46
0.2 OMT + 0.1 Ile	39
0.2 OMT + 0.2 Ile	47
0.2 OMT + 1.0 Ile	91
0.2 OMT + 2.0 Ile	56
0.1 Leu	96
1.0 Leu	85
2.0 Leu	53
0.2 OMT + 0.1 Leu	7
0.2 OMT + 1.0 Leu	7
0.2 OMT + 2.0 Leu	7
0.1 Val	83
1.0 Val	6
2.0 Val	6
0.2 OMT + 0.1 Val	12
0.2 OMT + 1.0 Val	10
0.2 OMT + 2.0 Val	4
0.1 Thr	77
1.0 Thr	22
2.0 Thr	14
0.2 OMT + 0.1 Thr	7
0.2 OMT + 1.0 Thr	18
0.2 OMT + 2.0 Thr	12

GM11b is the first genetically characterized mutant at the whole plant level that is highly overproducing Ile by encoding an Ile-insensitive TD. The cloning and molecular characterization of *omr1* and its wild-type allele, *OMR1*, should reveal the feedback binding site of *Arabidopsis* TD. GM11b and the other mutants listed in Table I also represent source material in which the feedback control of TD might be differentially altered. The cloned *omr1* allele could also be used to transform agronomically useful plants with the hope of overproducing free Ile and hence improving their nutritional value. In addition, because *omr1* of GM11b is a dominant allele that expresses high levels of growth resistance to OMT, it could provide an excellent selectable marker for genetic transformation.

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