Plant Physiol. (1992) 99, 269–275
0032-0889/92/99/0269/07/$01.00/0

Received for publication September 3, 1991
Accepted December 13, 1991

Isolation and Characterization of a Mutant of *Arabidopsis thaliana* Resistant to α-Methyltryptophan[1]

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ABSTRACT

Mutants of *Arabidopsis thaliana* have been selected for resistance to growth inhibition at the seedling stage by α-methyltryptophan (aMT). One mutant, *amt-1* has been characterized in detail. The appearance and growth rate of the mutant in the absence of the inhibitor are similar to wild type, both as plants and callus. However, mutant plant growth is unaffected by 25 micromolar aMT and mutant callus growth by 50 micromolar aMT, concentrations that completely inhibit the growth of wild-type plants and callus, respectively. Tryptophan levels in mutant and wild-type plants are 24.3 ± 2.7 and 4.7 ± 1.2 micrograms per gram fresh weight, respectively, and in the corresponding callus 64.0 ± 2.6 and 31.8 ± 8.4 micrograms per gram fresh weight, respectively. Anthranilate synthase (AS) activity levels in crude extracts from whole plants are 3.09 ± 0.54 nanomoles per milligram protein per hour in *amt-1* and 1.32 ± 0.21 nanomoles per milligram protein per hour in wild-type plants. In crude extracts from callus, anthranilate synthase levels are 11.54 ± 2.05 nanomoles per milligram protein per hour and 7.74 ± 1.58 in *amt-1* and wild type, respectively. Enzyme extracts are inhibited by L-tryptophan; the concentrations required for 50% inhibition (I_{50}) are 3.9 and 1.9 micromolar for *amt-1* and for wild type, respectively. The mutation segregates as a single nuclear allele and shows incomplete dominance. The concomitant increases in both AS activity and its I_{50} for tryptophan suggest that the mutation *amt-1* either resides in one of the AS structural genes or causes increased expression of an AS isoform with an I_{50} greater than the average for the entire extract.

The principal auxin IAA plays a central role in many aspects of plant growth and development. The traditional view holds that IAA is synthesized from tryptophan by one of several possible pathways (14). However, recent work, both genetic and metabolic, in *Arabidopsis* (5) has introduced the possibility that indole is actually the precursor for IAA. As an entry point to a genetic analysis of indole metabolism in a higher plant, we have isolated mutants of *Arabidopsis thaliana* resistant to the tryptophan analog aMT[2].

Most of the previous studies of tryptophan analog-resistant mutants in microorganisms as well as in plants have used 5MT and other ring-substituted analogs as the selective agent. In prokaryotes, eukaryotic microorganisms, and some plants, isolation of mutants resistant to 5MT has led to the recovery of mutants with an altered AS resistant to feedback inhibition by tryptophan (2, 4, 12, 17, 20). This in turn leads to an overaccumulation of tryptophan by as much as 50-fold. In some species (*e.g.* carrot and potato) such mutants are auxin autotrophic in culture, whereas in others (tobacco) this is not the case. However, auxin levels have not been determined in any of these mutants. A second mechanism of resistance to ring-substituted tryptophan analogs has been identified in cell suspension cultures of *Catharanthus roseus*. Sasse *et al.* (13) reported that cultures selected for resistance to 4MT contained higher levels of TDC activity and higher levels of tryptamine and, perhaps surprisingly, of tryptophan. These variants were unstable, and resistance was lost if selection was not maintained.

There have been two brief reports of 5MT-resistant mutants of *Arabidopsis*. In the first (11), mutants were isolated in cell culture. They showed reduced 5MT uptake and were reported to have somewhat elevated levels of tryptophan, although no data were shown. The cultures were not auxin autotrophic and could not be regenerated into plants. These lines are no longer available (M. Jacobs, personal communication). In the second case, a single mutant resistant to 300 μM 5MT was selected at the seedling stage from 35,000 M2 seed of the Lands. genotype (8). The mutant was dominant, and tryptophan levels were not detectably altered (M. Koornneef, personal communication).

As a selective agent, aMT has the advantage of increased solubility, and the site or mode of action of aMT (in which the methyl substituent is on the α-carbon) may differ from the ring-substituted compounds and hence identify different genetic lesions. Whereas the ring-substituted analogs are substrates for both the tryptophan monoxygenase activity from *Pseudomonas savastanoi* and for a cytosolic TDC activity from suspension cultures of *Catharanthus roseus*, aMT is not metabolized by the *C. roseus* TDC, even though it is a competitive inhibitor of the enzyme (13). To our knowledge, aMT has presently been used by only one group of workers for studies of indole metabolism. Smidt and Kosuge (15) isolated two classes of aMT-resistant mutants in *P. savastanoi*. One class had elevated IAA levels, whereas the second class lost the ability to synthesize IAA. Neither class was characterized at the enzymic or genetic level.

In this paper, we describe the isolation of mutants of *A. thaliana* resistant to aMT and characterization of one such mutant.

[1] Supported by U.S. Department of Agriculture grant 87-CR-CR-1-2559.

[2] Abbreviations: aMT, α-methyltryptophan; I_{50}, concentration required for 50% inhibition; 5MT, 5-methyltryptophan; AS, anthranilate synthase; 4-MT, 4-methyltryptophan; TDC, tryptophan decarboxylase; Lands., Landsberg *erecta*; 5MA, 5-methylanthranilic acid; MS, Murashige-Skoog.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seed stock used in these experiments was *Arabidopsis thaliana* (var Columbia and Lands.) and was kindly provided by Dr. Chris Somerville. Seed for aseptic tissue was sterilized as described by Valvekens *et al.* (19). Growth inhibition studies were performed under aseptic conditions on MS salts (10) containing 2% sucrose and vitamins and solidified with 0.7% agar (MS agar). Selective agents were made fresh monthly, filter sterilized, and added to the agar. Plants used for tryptophan determinations were grown individually on MS agar in 95- × 25-mm shell vials for 21 to 28 d. Callus was initiated from sterile seed on callus induction medium (MS agar containing 1 mg/L 2,4-D and 0.05 mg/L kinetin). Callus was passaged on callus maintenance medium (MS agar containing 0.5 mg/L 2,4-D and 0.1 mg/L kinetin). All aseptic tissue was maintained at 25°C under constant illumination (cool-white fluorescent) with a light intensity of 60 μmol/m^2 s. Soil-grown plants were propagated on a 1:1:1 mixture of perlite, vermiculite, and peat irrigated with *Arabidopsis* nutrient salts as described by Somerville and Ogren (16) at 25°C under constant illumination (cool-white fluorescent) with a light intensity of 50 μmol/m^2·s.

Chemicals

L-Tryptophan, aMT, 5MT, anthranilic acid, and chorismic acid were obtained from Sigma. Ammonium sulfate was from ICN. 5MA was a gift from Dr. Rob Last. L-[5-^{3}H]tryptophan was from Research Products International Corp. All solvents were from Fisher and were of HPLC grade.

Mutant Selection

Seed was mutagenized by ethyl methanesulfonate as described previously (16). Mutagenized (M1) seed was germinated, soil grown, and allowed to self to produce M2 seed. To select mutants, M2 seed was sterilized and plated on *Arabidopsis* nutrient salts solidified with 0.7% agar containing an appropriate concentration of aMT. Plates were sealed with Parafilm, incubated at 25°C under constant illumination (100 μEi/m^2·s), and inspected periodically. Putative mutants were picked off, transferred to soil, and allowed to set seed. These seed were then tested for analog resistance.

Growth Measurements

For plant growth measurements, seed were sterilized, vernalized at 4°C for 24 h, then germinated, and grown on MS agar. At various intervals, seedlings were removed and weighed. For the larger seedlings, the weights of individual seedlings were measured and recorded. For the smaller (inhibited) seedlings, groups of 10 to 20 seedlings were pooled and weighed as a group. These latter results are expressed as mean weights per seedling without an SE.

For callus growth measurements, the callus was initiated directly from single seeds on callus induction medium. At various times, callus was weighed singly or in groups as described above.

Tryptophan Measurements

Tryptophan extraction and quantitation by HPLC analysis were as described before (18). Briefly, whole plants or callus were homogenized and extracted first with methanol:chloroform:H_2O (12:5:3) and then reextracted with 80% ethanol. The aqueous phases were combined and dried by rotary evaporation at 36°C. The extract was redissolved in 10 mM sodium acetate (pH 4.8) and analyzed by reverse phase HPLC (Waters μBondapak C_{18}) using 10 mM sodium acetate as the running solvent with detection by fluorescence (254 nm excitation, 360 nm emission). L-[5-^{3}H]Tryptophan was introduced at the beginning of each extraction to monitor the efficiency of recovery.

Enzyme Assays

For AS assays, crude extracts were prepared and assayed essentially as described previously (9). Inhibition experiments were performed using enzyme extracts that had been concentrated by precipitation with 85% ammonium sulfate. The pellet was resuspended in column buffer and then desalted using Sephadex G-25. L-Tryptophan was added to the reactions from an aqueous stock solution diluted in reaction buffer. For whole plant extracts, 7 to 10 g of rosette leaves from 3- to 4-week-old soil-grown plants was used, and for callus 3 to 6 g of callus tissue 3 to 4 weeks postpassaging was used.

Genetic Analysis

Crosses were performed by using pollen from *amt-1* to fertilize emasculated wild-type flowers. F_1 seeds were collected, and individual F_1 plants were grown and allowed to self. The F_2 populations were collected from individual plants. Seeds were sterilized and germinated on MS agar containing aMT. Seedling weights were determined 13 d after the seeds were put onto the medium. Weights of individual seedlings were tabulated and grouped in weight classes in increments of 0.5 mg.

RESULTS

Mutant Isolation

Approximately 35,000 M2 seed were plated at densities ranging from 500 to 2000 seed per 100-mm Petri dish on concentrations of aMT ranging from 75 to 250 μM. The higher concentrations allowed germination, but after 2 weeks the cotyledons were only partly expanded and became chlorotic. Against this background, a small number of seedlings remained pale green and by 15 to 18 d had developed true leaves. From 35,000 seed, 45 possible mutants were removed, allowed to set seed without further selection, and retested in the M3. Nine of these original 45 showed heritable resistance and were retained. One of the more resistant mutants, designated *amt-1*, was used for the detailed characterization described below.

Response of Wild-Type and Mutant Plants to aMT and Other Analogs

Examples of wild-type and mutant plants grown on different concentrations of inhibitor are shown in Figure 1. Growth data are presented in Figure 2. In the absence of inhibitor, the habit and growth rate of mutant plants are essentially the same as the parental Columbia wild type (Fig. 2), although, when large populations are analyzed, increased numbers of bigger plants are observed in wild-type populations compared with the mutant (Fig. 3, A and D). With 12.5 μM aMT, growth of wild-type plants is variable, showing from 0 to 75% inhibition in different experiments, but it consistently shows complete inhibition on 25 μM aMT. By contrast, growth of mutant plants is consistently stimulated by 12.5 and 25 μM aMT and is completely inhibited only by concentrations of aMT in excess of 50 μM. Inhibition could be relieved by the inclusion of 1 to 5 mM L-tryptophan in the medium along with the aMT (data not shown).

We also determined the resistance of wild type and our mutant (*amt-1*) to 5MT and to 5MA, the inhibitor used by Last and Fink (9) to isolate tryptophan auxotrophs of *Arabidopsis*. These results are shown in Table I. The mutant *amt-1* shows an increase in resistance to both aMT and to 5MT in this assay but is as sensitive as wild type to 5MA. In these experiments, we also included the 5MT-resistant mutant, *MK V IV*, isolated and briefly described by Koornneef and van Loenen Martinet (8) for comparative purposes. Our observations confirm the 5MT resistance reported by Koornneef but show that *MK V IV* is no more resistant than its Lands. parent to aMT.

Expression of aMT Resistance in Callus Culture

Callus was induced from wild-type and mutant seed by plating directly on callus induction medium with or without aMT. As shown in Table II, growth of wild-type callus is completely inhibited by 50 μM aMT, whereas mutant callus continues to grow, and mutant growth is, in fact, stimulated by aMT at 50 and 100 μM.

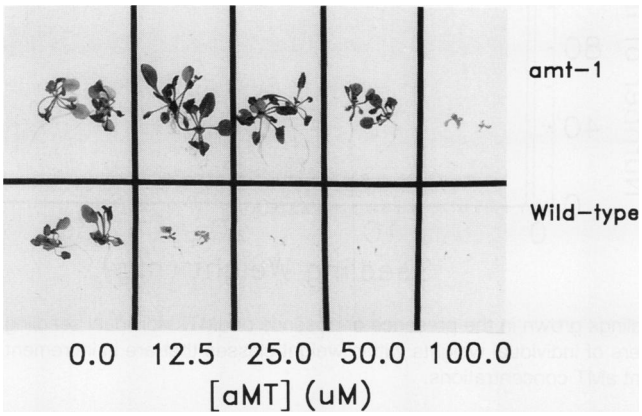

Figure 1. Appearance of *amt-1* and wild-type seedlings after 21 d of growth on media containing from 0 to 100 μM aMT.

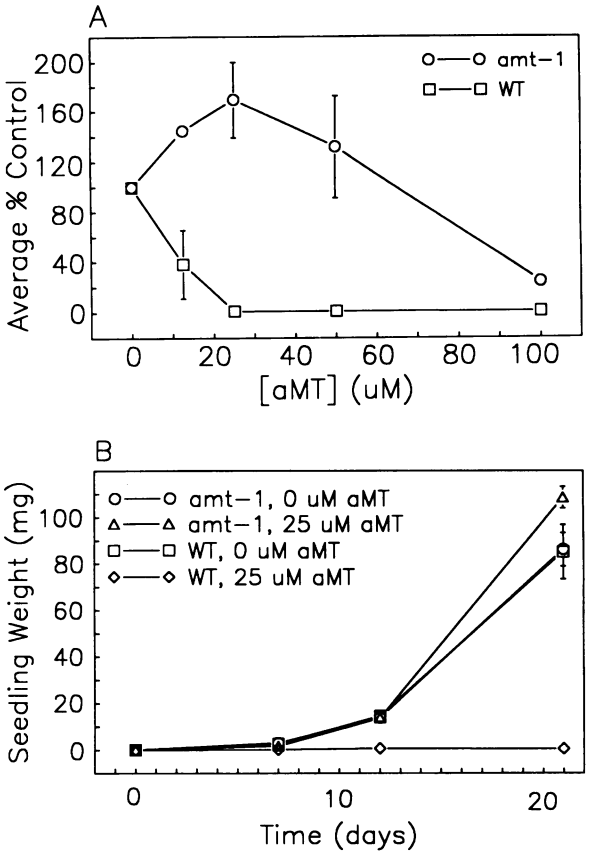

Figure 2. Inhibition of seedling growth by aMT. Wild-type (WT) and mutant seed were germinated and grown on media containing various levels of aMT. Individual plants were weighed after 7, 12, and 21 d. Results are expressed as average percentages of control ± SE (n = 2) at various concentrations of aMT (μM) at day 21 from replicate experiments (A) and as average seedling weights ± SE (n = 8) at various concentrations of aMT (μM) *versus* time (B) from one experiment; where no SE bar is visible, the SE is smaller than the symbol.

Genetic Analysis of *amt-1*

To determine the genetic basis of aMT resistance, M3 and later-selfed populations of seed from the original mutant were examined for segregation of resistance and sensitivity at both the plant and callus levels. When plated on MS agar without inhibitor, mutant and wild-type seedlings show similar size distributions. On agar containing 25 or 50 μM aMT, both wild-type and mutant seed populations showed unimodal distributions of plant size. Although the modes of the mutant and wild-type size distributions are quite different, there is sufficient overlap between these two distributions to prevent a clear distinction between resistant and sensitive seedlings (Fig. 3, A, B, D, and E). In contrast, plating of seed on callus induction medium containing 50 μM aMT provided a clear discrimination between wild-type and mutant populations. Under these conditions, both mutant and wild-type seed germinated with nearly 100% efficiency. However, whereas mutant seed germinated and continued to grow, producing healthy callus on 50 μM, <7% of the wild-type seedlings formed callus on this concentration of aMT. On medium

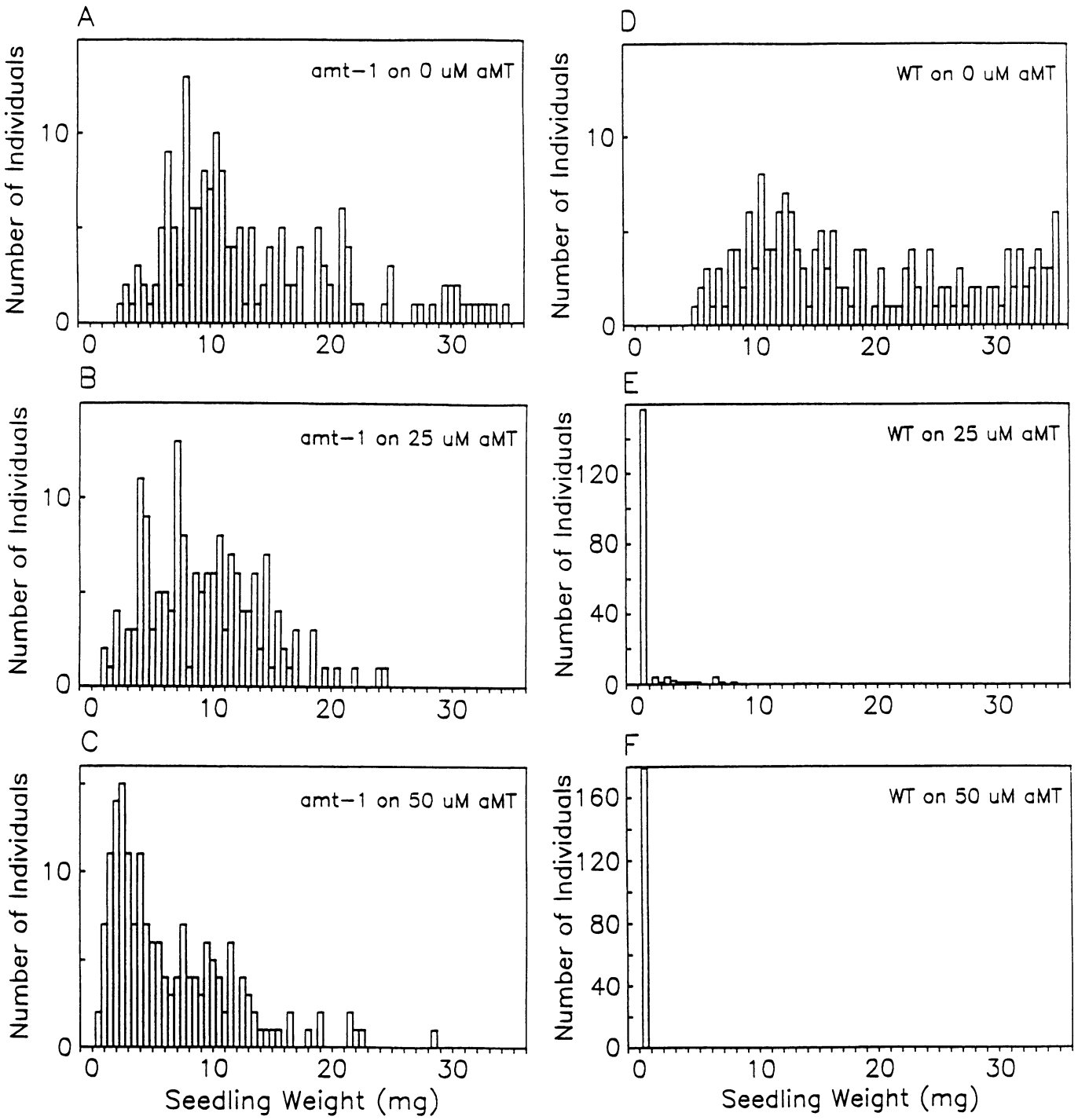

Figure 3. Size distribution in populations of wild-type (WT) and *amt-1* seedlings grown in the presence or absence of aMT. Individual seedlings were weighed 13 d after germination. Results are expressed as the numbers of individual weights within weight classes that are in increments of 0.5 mg. Note that the scales of the ordinates are different for the different aMT concentrations.

Table I. *Resistance of Various Arabidopsis Genotypes to Tryptophan Analogs in Terms of I_{50}*

The concentrations shown are I_{50} determined from fresh weight of 21-d-old seedlings. ND, Not determined.

Genotype	Tryptophan Analog		
	aMT	5MT	5MA
		μM	
Columbia wild type	10.0	17.0	8.0
amt-1	87.0	44.0	7.0
Lands.	14.0	16.0	ND
MK V IV	15.0	21.0	ND

lacking aMT, both mutant and wild-type populations formed callus with 100% efficiency (see Table III). Because the M3 through M7 populations of *amt-1* do not show any segregation of a sensitive phenotype, it appears that the mutant was isolated as a homozygote.

To determine whether one or more loci were involved in the phenotype, mutant plants were crossed with wild type, and the resulting F_1 population was allowed to self. F_2 seed populations were collected from individual F_1 plants, germinated, and grown on MS agar with or without aMT, and the weights of individual plants were determined after 13 d. Two concentrations of aMT were used (25 and 50 μM) to differentiate between fully resistant homozygotes and possibly less resistant heterozygotes. Results are shown in Figures 3 and 4; each histogram represents a population of about 180 individuals. Using the responses of the parental genotypes to define sensitive or resistant phenotypes (Fig. 3), we determined the distribution of sensitive, intermediately resistant, and fully resistant individuals among total F_2 populations. Seedlings that were able to germinate on aMT but died without cotyledon expansion were classified as sensitive. On 25 and 50 μM aMT, these seedlings comprise the 0.0- to 0.5-mg weight class (Fig. 3, B, C, D, and F; Fig. 4, B and C). Those seedlings that were able to grow as well as the mutant parent on aMT were classified as resistant; intermediately resistant seedlings were observed as an increase in the number of seedlings that had significantly reduced weight relative to the parental mutant grown under similar conditions. The F_2 population, grown in the presence of 25 μM aMT, contains less than one-fourth sensitive considering the 0.0- to 0.5-mg seedlings as the sensitive class, and more than three-fourths resistant individuals (see Table IV), and the size distribution in the resistant population is similar to that of the homozygous mutant parent. These results do not fit a normal 1:3 (sensitive:resistant) mendelian ratio for a dominant mutation (Table IV, 25 μM aMT). However, when the F_2 population is grown on a higher concentration of aMT (50 μM), which completely inhibits wild-type growth, one-fourth of the seedlings are sensitive (Table IV, 50 μM aMT), indicating that the mutation in *amt-1* is dominant and that the resistance to aMT is due to a single nuclear locus. Among the nonsensitive F_2 individuals, a new class can be seen (Fig. 4C), which is not as resistant to aMT as the mutant parent (Fig. 3C). The average weight of resistant F_2 seedlings (>0.5 mg) on 50 μM aMT is 3.9 ± 0.35 mg compared with 6.4 ± 0.38 mg for the mutant parent under identical conditions. This F_2 class intermediate in resistance between the two homozygous parents is most likely the heterozygote and indicates that *amt-1* is an incompletely dominant mutation.

Table II. *Inhibition of Callus Growth by aMT*

Wild-type and mutant seed were placed on callus induction media with and without aMT. The callus was weighed after 3 months. Results are the means of five pieces of callus for each data point ± SE.

Callus Source	[aMT]		
	0.0 μM	50.0 μM	100.0 μM
Wild type	34.0 ± 2.0	0.8[a]	0.4[a]
amt-1	41.0 ± 5.1	89.0 ± 13.1	111.0 ± 21.0

[a] Pieces of callus were too small to be weighed separately. The number reported is the average of 20 pieces of callus weighed together and does not have an SE.

Table III. *Segregation of amt-1 Mutation Using Seed Callus Assay*

The numbers shown indicate the fraction of seeds able to form callus on callus induction media with and without aMT. The *amt-1* population used was the M7 generation.

Genotype	[aMT]		
	0.0 μM	25.0 μM	50.0 μM
Wild type	90/90	18/75	6/93
amt-1	87/87	84/84	86/86

Tryptophan Content of Plants and Callus

Free tryptophan levels in both mutant plants and mutant callus are significantly higher than in the corresponding wild-type tissues, the ratio of mutant to wild-type levels being greater in plants than in callus (Table V). In both wild-type and mutant, tryptophan levels are considerably higher in callus tissue than in whole plants.

AS Activities in Plants and Callus

Crude extracts from mutant tissue contained higher levels of AS activity than wild-type extracts, the relative difference being greater for plants than for callus (Table VI). It can also be seen that AS levels are higher in callus than in plants of both genotypes, an observation that parallels the tryptophan levels in these tissues. The sensitivity of enzyme extracts to inhibition by L-tryptophan was also determined. A 0.5-fold increase was observed in the amounts of L-tryptophan required to produce I_{50} using mutant relative to wild-type extracts from whole plants (Fig. 5). Extracts from callus also showed a difference in I_{50} between mutant and wild-type but not as large (data not shown).

DISCUSSION

In this study, we have presented a detailed characterization of an *A. thaliana* mutant resistant to growth inhibition by aMT. Like tryptophan analog-resistant mutants in other spe-

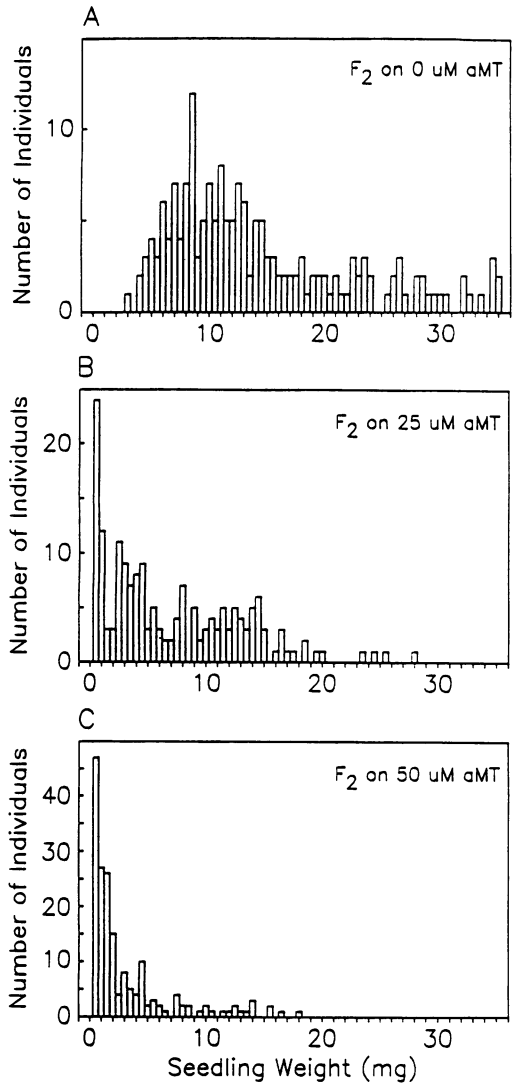

Figure 4. Size distribution of F_2 progeny from *amt-1* × wild-type cross. Pollen from *amt-1* was used to fertilize wild-type flowers. The resulting F_1 progeny were allowed to self, and seeds (F_2) were collected from individual plants. F_2 seedlings were tested for resistance to aMT as described in Figure 3). Results are expressed as in Figure 3. Note that the scales of the ordinates are different for the three graphs.

Table IV. *Genetic Analysis of F_2 Seed Populations*

Results were analyzed using standard mendelian ratios. Sensitive seedlings were those weighing ≤0.5 mg; resistant seedlings were all others.

[aMT]	No. of F_2 Seedlings		χ^2
	Sensitive (expected)	Resistant (expected)	
25.0 μM	24 (43)[a]	149 (130)[a]	$P < 0.01$
50.0 μM	47 (45)[a]	132 (134)[a]	$0.8 > P > 0.5$

[a] Expected ratio for 1:3 sensitive:resistant.

Table V. *Tryptophan Content of Wild-Type and Mutant Plants and Callus*

Tryptophan levels were determined by analyzing whole plant or callus extracts using reverse phase HPLC. The values are the averages from independent extractions.

Genotype	Whole Plants	Callus
	μg/g fresh wt ± SE	
Columbia wild type	4.65 ± 1.18 (n = 5)	31.80 ± 8.40 (n = 2)
amt-1	24.33 ± 2.74 (n = 7)	64.04 ± 2.59 (n = 2)

cies, *amt-1* is dominant, contains elevated levels of tryptophan, and has altered AS activity, although the changes in *amt-1* are smaller than in other plant mutants (2, 4, 12, 20). In some plants, tryptophan analog resistance and elevated levels of endogenous tryptophan led to auxin autotrophy in culture (21). *amt-1* is not auxin autotrophic in culture, and we have not measured auxin levels in the mutant.

It is striking that the rank order of AS activity in the four kinds of extracts examined (mutant and wild-type plants and callus) parallels the levels of tryptophan in these four tissues (*cf.* Tables V and VI). This suggests that tryptophan levels in these tissues may be controlled in part by the level of AS activity. Previous reports of tryptophan analog-resistant mutants in plants have emphasized the altered sensitivity of AS to feedback inhibition by tryptophan. However, a close examination of the literature indicates that some of these mutants showed changes not only in the I_{50} of AS but also in its specific activity. The mutant *amt-1* similarly shows consistent changes in both AS activity and sensitivity to feedback inhibition. These concomitant changes suggest that the phenotype of *amt-1* is not simply due to an increased expression of a normal enzyme but to some qualitative change in the enzyme complex.

In microorganisms, both prokaryotic and eukaryotic, AS usually occurs as a tetramer of two different subunits (for reviews, see refs. 3 and 7). In some of these organisms, the AS enzyme complex also contains catalytic activities for later steps in the tryptophan pathway. The subunit structure of the AS complex in plants has yet to be determined, although some data from maize and pea (6) indicate that the various enzymatic steps in the pathway are due to separate polypeptides. K.K. Niyogi and G.R. Fink (personal communication) identified two AS genes in *Arabidopsis* (sequences homologous to the Trp2 subunit of *Saccharomyces cerevisiae*) that map to two different chromosomes. Earlier biochemical studies of tobacco (1) and potato (2) cell cultures identified two forms of AS that differed in their sensitivity to feedback

Table VI. *AS Activity Levels in Plants and Callus*

Activity levels were determined as described in "Materials and Methods." Data are averages of values from extracts made on different days.

Genotype	Whole Plants	Callus
	nmol/mg protein h ± SE	
Columbia wild type	1.32 ± 0.21 (n = 5)	7.74 ± 1.58 (n = 4)
amt-1	3.09 ± 0.54 (n = 5)	11.54 ± 2.05 (n = 4)

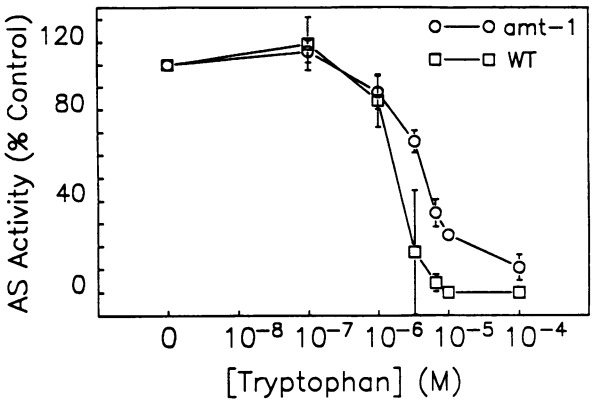

Figure 5. Inhibition of AS by tryptophan. Inhibition assays were performed by adding L-tryptophan (dissolved in reaction buffer) to standard AS enzyme assays (see "Materials and Methods" for AS assay conditions). The values presented are the averages ± SE of triplicate experiments with duplicate reactions for each concentration. WT, Wild type.

inhibition by tryptophan. In tobacco, expression of the more resistant form could not be detected in extracts of leaves but was increased in cultures of 5MT-resistant cell lines (1). The relationship between these AS complexes in the *Solanaceae* and the two genes identified in *Arabidopsis* is unclear. Our results concerning the alterations in expression and feedback sensitivity of AS in mutant and wild-type *Arabidopsis* plants and callus could similarly be due to differential expression of multiple forms of AS. Additional studies, including chromatographic analysis of AS complexes from *Arabidopsis* and mapping of the *amt-1* mutation with respect to the cloned AS genes, will be necessary to clarify this question.

ACKNOWLEDGMENTS

This work was initiated in the laboratory of Dr. Chris Somerville at the Department of Energy Plant Biology Laboratories, Michigan State University. C.D.T. thanks Dr. Chris Somerville for his hospitality and enthusiastic support during his sabbatical at the Department of Energy laboratories.

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