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# Molecular Basis of α-Methyltryptophan Resistance in *amt-1*, a Mutant of *Arabidopsis thaliana* with Altered Tryptophan Metabolism[1]

Joel A. Kreps[2], Tilak Ponappa, Weiqin Dong, and Christopher D. Town*
Department of Biology, Case Western Reserve University, Cleveland, Ohio 44106

**A mutant of *Arabidopsis thaliana, amt-1*, was previously selected for resistance to growth inhibition by the tryptophan analog α-methyltryptophan. This mutant had elevated tryptophan levels and exhibited higher anthranilate synthase (AS) activity that showed increased resistance to feedback inhibition by tryptophan. In this study, extracts of the mutant callus exhibited higher AS activity than wild-type callus when assayed with either glutamine or ammonium sulfate as amino donor, thus suggesting that elevated AS activity in the mutant was due to an alteration in the α subunit of the enzyme. The mutant also showed cross-resistance to 5-methylanthranilate and 6-methylanthranilate and mapped to chromosome V at or close to *ASA1* (a gene encoding the AS α subunit). *ASA1* mRNA and protein levels were similar in mutant and wild-type leaf extracts. Levels of *ASA1* mRNA and protein were also similar in callus cultures of mutant and wild type, although the levels in callus were higher than in leaf tissue. Sequencing of the *ASA1* gene from *amt-1* revealed a G to A transition relative to the wild-type gene that would result in the substitution of an asparagine residue in place of aspartic acid at position 341 in the predicted amino acid sequence of the ASA1 protein. The mutant allele in strain *amt-1* has been renamed *trp5–1*.**

AS is a key enzyme in the biosynthetic pathway for Trp, the plant hormone IAA, and numerous secondary metabolites. AS converts chorismate to anthranilate in the first committed step in the biosynthesis of Trp. Since chorismate is also an intermediate in the biosynthesis of Phe, Tyr, and several secondary products, it forms an important branch point from which different classes of aromatic compounds can be synthesized (reviewed by Poulsen and Verpoorte, 1991). In microbes, AS usually consists of two nonidentical subunits, referred to as the α subunit (component I) and the β subunit (component II). Component I can convert chorismate to anthranilate in the presence of high levels of ammonia (ammonia-dependent AS activity), whereas component II is responsible for the use of Gln as the amino donor (Hütter et al., 1986). Both subunits are required for the Gln-dependent reaction.

Recent studies have provided a better understanding of the genetics and biochemistry of AS in higher plants. Two genes, *ASA1* and *ASA2*, from *Arabidopsis thaliana* were isolated that complemented mutations in the AS α subunit in both yeast and bacteria (Niyogi and Fink, 1992). Genes for the Arabidopsis AS β subunit were subsequently isolated by complementation in yeast and *Escherichia coli* (Gudelsky et al., 1993; Niyogi et al., 1993). Plant AS has recently been purified from *Catharanthus roseus* (Poulsen et al., 1993) and *Ruta graveolens* (Bohlmann et al., 1995). From these studies it appears that plant AS is similar in organization to the microbial enzyme. Plant AS also is similar to the microbial AS in its sensitivity to feedback inhibition by Trp and the ability to use Gln or ammonia as an amino donor for the synthesis of anthranilate (Poulsen et al., 1993).

As a means to investigate regulation of the Trp pathway, toxic analogs of Trp have been used in metabolic studies of plant cell cultures and as a tool to select mutants. Many of these studies have been conducted with the growth inhibitor 5-methyltryptophan. In a number of species including *Datura innoxia*, *C. roseus*, and *Solanum tuberosum*, variant cell lines resistant to inhibitory concentrations of 5-methyltryptophan were found to have AS that was less sensitive to feedback inhibition by Trp (Carlson and Widholm, 1978; Scott et al., 1979; Ranch et al., 1983). Widholm (1977) described 5-methyltryptophan-resistant carrot cell lines and a potato cell line that were auxin autotrophic.

In addition to the commonly used 5-methyltryptophan, which is a ring-substituted analog of Trp, another potentially useful compound, which may have a different mode of action and is more soluble, is αMT. To study regulation of AS, we isolated an Arabidopsis mutant (*amt-1*) that is resistant to αMT (Kreps and Town, 1992). We previously demonstrated that this mutant has greater than 5-fold higher levels of Trp and exhibits higher levels of AS activity than wild-type plants. Similarly, callus initiated from *amt-1* has higher AS activity in comparison with wild-type callus. AS from the mutant was also found to have greater resistance to feedback inhibition by Trp. In this paper, we further characterize *amt-1* at the genetic, biochemical, and molecular levels. Mapping was carried out to determine the genomic location of the mutation. At the biochemical level, assays of ammonia-dependent AS activity were car-

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[2] Present address: Department of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, MA 01003.

* Corresponding author; e-mail cdt2@po.cwru.edu; fax 1–216–368–4672.

Abbreviations: AS, anthranilate synthase; αMT, α-methyl-DL-Trp; 5MA, 5-methylanthranilic acid; 6MA, 6-methylanthranilic acid; RFLP, restriction fragment length polymorphism.

ried out. Molecular analyses were also performed to determine the level of *ASA1* mRNA and protein expression in mutant and wild-type tissue and to determine the sequence basis of the mutation.

## MATERIALS AND METHODS

### Chemicals

Anthranilic acid (catalog No. A 1506), αMT (catalog No. M 8377), and chorismate (catalog No. C 1259) were obtained from Sigma; 5MA (catalog No. 41,944–3) and 6MA (catalog No. 23,053–7) were from Aldrich. Ammonium sulfate was purchased from ICN. Secondary antibody ($^{35}$S-labeled anti-rabbit antibody raised in donkey) for western blotting was from Amersham (catalog No. SJ.434).

### Plant Material and Growth Conditions

Isolation of *amt-1* from *Arabidopsis thaliana* (Columbia) was described previously (Kreps and Town, 1992). Soil-grown plants were propagated on a mixture of perlite, vermiculite, and peat moss (1:1:1 on a volume basis) and irrigated with nutrient salts as described by Somerville and Ogren (1982). The conditions for callus initiation from seeds and for callus maintenance were as described previously (Kreps and Town, 1992). Growth inhibition was studied by placing surface-sterilized seeds (10 seeds/100- × 15-mm Petri dish) on Murashige-Skoog medium (Murashige and Skoog, 1962) supplemented with 2% Suc, vitamins, and 0.7% TC agar (JRH Biosciences, Lenexa, KS). Following overnight cold treatment (4°C) of the plated seeds in the dark, Petri dishes were oriented vertically and placed at 24°C under constant illumination (35 $\mu$mol m$^{-2}$ s$^{-1}$, cool-white fluorescent) with a Plexiglas screen (yellow 2208) to prevent possible degradation of medium components due to light (Stasinopoulos and Hangarter, 1990). Root lengths were measured 5 and 10 d after transfer to 24°C.

Plant material used for DNA isolation was obtained by transferring surface-sterilized seeds to flasks containing 50 mL of Murashige-Skoog liquid medium with 2% Suc. The flasks were shaken for 14 to 21 d at 24°C under constant illumination (50 $\mu$mol m$^{-2}$ s$^{-1}$). Tissue was harvested, rinsed with distilled water, blotted dry with tissue paper, frozen in liquid nitrogen, and stored at −70°C until extraction.

### Enzyme Assays

AS activity was assayed essentially as described by Last and Fink (1988). For the comparison of ammonia-dependent and Gln-dependent AS activity, extracts were concentrated by precipitation with ammonium sulfate at 50% saturation, resuspended in column buffer (10% glycerol, 0.1 mM DTT, 0.1 mM EDTA, and 0.1 M potassium phosphate, pH 8.0), desalted using Sephadex G-25, and assayed by using ammonium sulfate (50 mM) or L-Gln in the reaction mixture.

### Genetic Analysis

The *amt-1* mutation was mapped by crossing the mutant to MSU14 (*ttg, yi, er*). The $F_2$ population was evaluated based on inhibition of seedling root growth on αMT-containing medium, and *ttg* was simultaneously scored by the absence of stress-induced anthocyanin production (which occurs in wild-type seedlings grown in the presence of αMT). The *yi* phenotype (yellow inflorescence) was scored at the time of bolting. Recombination frequency was determined between *amt-1* and *ttg* by using the program Linkage 1 (Suiter et al., 1983) and was converted to map distances (Kosambi, 1944).

RFLP mapping was performed following digestion of plant genomic DNA with the restriction enzyme *Dra*I and electrophoresis of the digest on 0.8% agarose gels. DNA was transferred to reinforced nitrocellulose (Micron Separations, Westboro, MA) and probed with a genomic clone of *ASA1* (pKN212C, kindly provided by K.K. Niyogi, Whitehead Institute, Cambridge, MA). Hybridizations were done in Church buffer (Church and Gilbert, 1984) for 16 to 24 h at 60°C. Blots were then rinsed at room temperature in a wash solution consisting of 0.1× SSC (1× SSC is 0.15 M NaCl, 15 mM sodium citrate), 0.1% SDS followed by two 30-min incubations in wash solution at 60°C. These conditions have been shown to yield gene-specific hybridization with the probes used (Niyogi and Fink, 1992).

### RNA and DNA Analysis

DNA was isolated based on the method of Rogers and Bendich (1988) for Southern blot analysis or by the method of Keller and Bancroft (1991) for the purpose of sequencing. Total RNA was isolated according to the method of Rochester et al. (1986) from tissue frozen in liquid nitrogen. Tissues used to study expression of *ASA* RNA were rosette leaves of 3- to 4-week-old soil-grown plants and callus harvested 3 to 4 weeks after transfer to new medium. Poly(A)$^+$ RNA was isolated according to the method of Maniatis et al. (1982). RNA gel blot analysis was performed by electrophoresis of denatured samples on formaldehyde agarose gels, transfer to nylon-reinforced nitrocellulose, and probing of the blot with the gene-specific probes for *ASA1* and *ASA2* as described for RFLP mapping (above). The amount of bound probe was determined by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). To correct for differences in loading, blots were also probed with a β-tubulin gene, which under the conditions used hybridizes equally to RNA from all tissues (D.P. Snustad, personal communication).

For sequencing of *ASA1* from *amt-1*, genomic DNA was amplified by PCR and sequenced with a set of intron-based internal primers by cycle sequencing (Sequitherm cycle sequencing kit; Epicentre Technologies, Madison, WI).

### Immunoblot Analysis

Tissue (0.5 g) was ground under liquid nitrogen, extracted into 0.8 mL of AS-grinding buffer (200 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 8 mM $MgCl_2$, 0.2 mM DTT, 60%

glycerol) plus 25 mg of polyvinylpolypyrrolidone (Niyogi et al., 1993) and centrifuged at 12,000*g* for 15 min at 4°C. Protein concentrations of the supernatant were determined by the method of Bradford (1976) with BSA as standard (Bio-Rad protein assay). SDS-PAGE was conducted in 10% acrylamide according to the method of Laemmli (1970) after extracts were mixed with an equal volume of 2× loading buffer and heated to 100°C for 5 min. Following electrophoresis, the proteins were transferred to nitrocellulose by electroblotting (Genie, Idea Scientific, Corvallis, OR) at 24 V for 45 min with transfer buffer (3 g/L Tris base, 14.4 g/L Gly in 20% methanol). To examine the efficiency of protein transfer, blots were stained in Ponceau S (0.1% in 1% acetic acid) for 3 min and then rinsed three times with distilled water. Blots were blocked for 4 h in blocking solution (5% nonfat dry milk in PBS). Primary antibody (kindly provided by R. L. Last and J. Zhao, Boyce Thompson Institute, Ithaca, NY) raised in rabbit against a glutathione-*S*-transferase-ASA1 fusion protein was used in an overnight incubation at room temperature at a 1:2000 dilution in blocking solution. The blots were then washed three times with PBS , containing 0.05% Tween 20. Secondary antibody was diluted 1:1000 in antibody diluent (1% BSA, 0.05% Tween 20 in PBS, pH 7.4) and incubation was carried out for 1 h at room temperature. Blots were then washed three times (PBS containing 0.05% Tween 20), and immunoreactive protein was quantitated with a PhosphorImager.

## RESULTS

### Genetic Analysis

To determine the genomic location of the *amt-1* mutation, a cross was performed between the mutant and the mapping strain MSU14 (*ttg*, *yi*, *er*). Analysis of the $F_2$ population showed that *amt-1* is linked to *ttg* but not to *yi* (Table I), placing *amt-1* at the top of chromosome V. To better localize *amt-1*, we also conducted RFLP mapping to determine the recombination frequency between *amt-1* and *ASA1*, which also maps to chromosome V (Niyogi and Fink, 1992). The genomic clone of *ASA1* (pKN212C) detects a polymorphism between the Columbia and Landsberg *er*/*er* ecotypes (the two parents). Thirty-four $F_3$ families from the cross between *amt-1* and MSU14 were subjected to RFLP mapping. No recombination was detected in the 68 chromatids analyzed, which represents a recombination frequency of 1.47% or less and is equivalent to a distance of 1.5 centimorgans (with confidence intervals of 0–6 centimorgans).

**Table I.** *Genetic mapping of amt-1 to chromosome V*

Crosses were performed with *amt-1* as the male parent and MSU14 (*ttg*, *yi*, *er*) as the female parent. The $F_2$ seeds were tested for resistance to αMT and scored for the visible markers *ttg* and *yi*. Recombination frequency ± SE is presented.

| Loci Tested | Recombination Frequency |
|---|---|
| *amt-1/ttg* | 0.16 ± 0.04 |
| *amt-1/yi* | 0.49 ± 0.09 |

**Table II.** *AS enzyme activity in mutant and wild-type callus extracts assayed in the presence of L-Gln or $(NH_4)_2SO_4$*

| Experiment | *amt-1*, Gln | *amt-1*, $(NH_4)_2SO_4$ | Wild Type, Gln | Wild Type, $(NH_4)_2SO_4$ |
|---|---|---|---|---|
| | *nmol anthranilate mg$^{-1}$ protein h$^{-1}$* | | | |
| I | 10.7 | 3.3 | 8.1 | 2.2 |
| II | 23.7 | 7.6 | 18.7 | 4.9 |
| III | 21.5 | 5.0 | 14.8 | 3.2 |

### Assay of AS Activity

The *amt-1* mutation mapped close to the *ASA1* gene, which encodes the α subunit of AS. Since the α subunit is capable of converting chorismate to anthranilate with ammonia as the amino group donor (Hutter et al., 1986), we measured ammonia-dependent AS activity in extracts from mutant and wild-type tissue. These determinations were performed on callus because callus extracts exhibited higher AS activity than leaf extracts (Kreps and Town, 1992). Although enzyme activity was reduced in both mutant and wild-type extracts when ammonium sulfate rather than Gln was used as the amino donor, mutant extracts consistently exhibited higher AS activity than wild-type extracts (Table II). The ratio of AS activity between mutant and wild type was 1.32 ± 0.08 in the Gln-dependent assay and 1.54 ± 0.02 in the ammonium sulfate assay. The observation of higher AS activity in the mutant in both assays suggested that the elevated enzyme activity may be due to a change in the α subunit (encoded by *ASA1*) of the AS holoenzyme.

### Expression of *ASA1*

Poly(A)$^+$ RNA was isolated, electrophoresed on a denaturing gel, blotted to nitrocellulose, and probed with a fragment of *ASA1* (Fig. 1). Upon correction for differences in loading by re-probing the blot with β-tubulin, similar levels of *ASA1* mRNA were observed in mutant and wild-type leaves (Table III). Extracts of the mutant and wild-type callus also exhibited similar levels of *ASA1* mRNA. However, *ASA1* mRNA is up-regulated in callus when compared with leaf tissue for both *amt-1* and wild type. We also tested the possibility that the mutant and wild type may differ in expression of *ASA2*, an α subunit gene of AS that is similar to *ASA1* but whose mRNA is approximately 10 times less abundant than that of *ASA1* in Arabidopsis (Niyogi and Fink, 1992). Our results revealed no difference in levels of *ASA2* mRNA between mutant and wild-type (plant or callus) tissue (data not shown).

Although RNA gel blot analysis of *ASA1* mRNA indicated no differences between wild type and mutant, we tested the possibility that elevated AS activity in the mutant may be caused by increased levels of ASA1 protein. Immunoblot analysis of protein extracts from *amt-1* and wild-type plants revealed no difference in the amount of ASA1 protein (Fig. 2). Callus extracts had higher levels of ASA1 protein than plant extracts, but there was no apparent difference in the amount of ASA1 protein between mutant and wild-type callus extracts. Callus extracts also exhibited a second band of lower

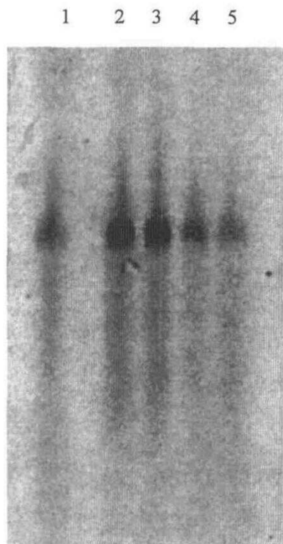


**Figure 1.** RNA gel blot analysis of *ASA1* in *amt-1* and wild-type tissue. Poly(A)$^+$ RNA was isolated from mutant and wild-type tissues, electrophoresed on a formaldehyde agarose gel, blotted onto nitrocellulose, and probed with a gene-specific fragment from *ASA1*. The amount of bound probe was visualized with the help of a PhosphorImager. To correct for loading differences, blots were re-probed with an Arabidopsis β-tubulin gene. Lane 1 corresponds to a loading of 10 μg of total RNA from wild-type plants. Lanes 2–5 were loaded with 2 μg each of poly(A)$^+$ RNA isolated from mutant callus (lane 2), wild-type callus (lane 3), mutant leaf (lane 4), and wild-type leaf (lane 5).

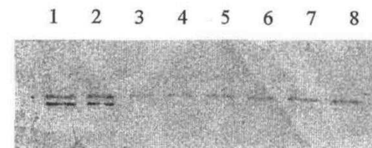


**Figure 2.** ASA1 protein in *amt-1* and wild-type tissue. Extracts were subjected to SDS-PAGE, blotted onto nitrocellulose, and incubated with a 1:2000 dilution of primary antibody (raised in rabbit against a glutathione-*S*-transferase-ASA1 fusion protein). Blots were then incubated with $^{35}$S-labeled (anti-rabbit) secondary antibody and visualized with the help of a PhosphorImager. Lane 1, Mutant callus (5 μg of protein); lane 2, wild-type callus (5 μg of protein); lanes 3 and 5, mutant leaf (5 μg of protein); lanes 4 and 6, wild-type leaf (5 μg of protein); lane 7, mutant leaf (10 μg of protein); lane 8, wild-type leaf (10 μg of protein).

molecular weight. A similar protein band of lower molecular weight than the ASA1 protein is also visible in immunoblots of Arabidopsis leaf extracts (Zhao and Last, 1995).

## Molecular Basis for the *amt-1* Mutation

Our data (summarized below) collectively suggested that the *amt-1* mutation resides in the structural gene for *ASA1*: (a) the mutation was closely linked to *ASA1*; (b) mutant extracts exhibited elevated activity of AS, and mutant AS had reduced sensitivity to feedback inhibition by Trp (Kreps and Town, 1992); and (c) the levels of *ASA1* mRNA and protein were similar in mutant and wild-type extracts. We therefore sequenced the coding regions of *ASA1* from *amt-1*. Sequence analysis revealed a single base-pair change in the mutant relative to the wild-type sequence of *ASA1* (GenBank accession No. M92354). This mutation results in the change of G to A at position 4430 of *ASA1* and would result in the substitution of an Asn residue in place of Asp at position 341 of the predicted amino acid sequence of the ASA1 protein (Fig. 3).

Alignment of the predicted ASA1 amino acid sequence with the related sequences of plant, yeast, and bacteria indicates that the Asp at position 341 of the *ASA1* protein is conserved in Arabidopsis ASA2, *R. graveolens* AS α1 and AS α2, *Bacillus subtilis* TrpE, and *E. coli* PabB, although not in *Salmonella typhimurium* (Band et al., 1984; Goncharoff and Nichols, 1984; Caligiuri and Bauerle, 1991; Niyogi and Fink, 1992; Bohlmann et al., 1995).

**Table III.** *Comparison of AS enzyme activity, and levels of ASA1 mRNA and protein in wild-type and mutant extracts*

Values are presented in relation to the value for wild-type (Columbia) leaf, which was assigned a value of 1.00 in each of the assays. AS activity was expressed on a nmol mg$^{-1}$ protein h$^{-1}$ basis (Kreps and Town, 1992), and mRNA and protein levels were determined by scanning both RNA blots and immunoblots with a PhosphorImager.

| Tissue | AS Enzyme Activity | ASA1 mRNA | ASA1 Protein |
|---|---|---|---|
| Wild-type leaves | 1.00 | 1.00 | 1.00 |
| *amt-1* leaves | 2.39 | 0.86 | 0.89 |
| Wild-type callus | 5.92 | 2.52 | 2.13 |
| *amt-1* callus | 8.85 | 2.77 | 1.96 |

## Effect of Trp Analogs on Root Growth

While our sequencing work was in progress, we examined the effects of the structurally related inhibitors αMT, 5MA, and 6MA on root growth on both our isolate, *amt-1*, and on LIA2, one of the 6MA-resistant mutants isolated by the Last group that had a phenotype similar to *amt-1*. Our results indicated that *amt-1* and LIA2 showed a similar degree of resistance to αMT compared to sensitive wild-type (Columbia) seedlings (Table IV). We found that 6MA and 5MA were considerably more toxic to all genotypes tested than αMT. However, both of the mutants tested were more resistant to 6MA and 5MA than the wild type. Our previous report that *amt-1* was as sensitive as wild type to 5MA, with 50% inhibitory concentrations of 7 and 8 μM, respectively (Kreps and Town, 1992), was misleading because we did not use a range of concentrations low enough to distinguish between the responses of wild type and mutant. The use of yellow Plexiglas light screens in the present study to prevent possible photodegradation of media components may also have contributed to the observed differences in analog sensitivity in the two studies. Li and Last (1996) reported that their 6MA-resistant mutants LIA1–3 were resistant to 300 μM 6MA. In our hands, both our mutant (*amt-1*) and theirs (LIA2) showed 97 to 98% inhibition of root growth after 10 d on 100 μM 6MA but had

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1 TrpE  (S.t)   --------------- --------------- --------------- --------------- --------------- ---------------     0
2 ASA2  (A.t)   MSAVSISAVKSDFFT VEAIAVTHHRTPHPP HF-----PSLRFPLS L----KSPP--ATSL NLVA-GSKLLHFSRR LPSIKCSYTPS----    74
3 ASα2  (R.g)   ------------MIT LNVETPPLTRSQLPS TFRVSSAASVNFNDR V----ATSRWRPNSL SLTT-SSY------R LRTLKCAASAS----    63
4 ASA1  (A.t)   --------------- ------------MSS SMNVATMQALTFSRR LLPSVASRYLSSSSV TVTGYSGRSSAYAPS FRSIKCVSVSPEASI    63
5 Asα1  (R.g)   --------------- --------------M SAAATSMQSLKFSNR LVP--PSRRLSPVPN NVT--CNNLPKSAAP VRTVKCCASSWNSTI    57

                                                                                                 ●●●
1 TrpE  (S.t)   --------------- --------------- ---MQTPKPTLELLT CDAAYRENPTALFHQ VC-----GDRPATLL LESADIDSKDDLKSL    52
2 ASA2  (A.t)   ---------LDLSE- -----------EQFTK FKKASEKGNLVPLFR C--VFSDHLTPILAY RCLVKEDDRDAPSFL FESVEPGSQ------   136
3 ASα2  (R.g)   ---------TSASTS ASPSPSPSLVDQSAN FHEASKKGNLIPLYR C--IFSDHLTPVLAY RCLVKEDDRDAPSFL FESVEPGSQ------   136
4 ASA1  (A.t)   --------------- ---------VSDTKK LADASKSTNLIPIYR C--IFSDQLTPVLAY RCLVKEDDREAPSFL FESVEPGSQ------   121
5 Asα1  (R.g)   NGAAATTNGASAASN GASTTTTTYVSDATR FIDSSKRANLVPLYR C--IFADHLTPVLAY RCLVQEDDKETPSFL FESVEPG-R------   138

                                                                                                ○
1 TrpE  (S.t)   LLVDSALRITALGDT VTIQALSDNGASLLP LLDTALPAGVENDVL PAGRVLRFPPVSPLL DEDARLCSLSVFDAF RLLQGVVNIPTQERE   142
2 ASA2  (A.t)   --SSNIGRYSVVGAQ PTIEIVAKG--NVVT VMDHGASLRTEEEV- --------------- -DDPMMVPQKIMEEW N--PQGI----DELP   199
3 ASα2  (R.g)   --ASSIGRYSVVGAQ PAIEIVAKE--NMVT ILDHEGGQRTEQFV- --------------- -EDPMDVPRRIMEGW K--PQLI----DELP   199
4 ASA1  (A.t)   --MSSVGRYSVVGAQ PAMEIVAKE--NKVI VMDHNNETMTEEFV- --------------- -EDPMEIPRKISEKW NPDPQLV----QDLP   186
5 Asα1  (R.g)   --ISTVGRYSVVGAH PVMEVIAKD--NMVT VMDHEKGSLVEEVV- --------------- -DDPMEIPRRISEDW K--PQII----DDLP   201

                                                           ○
1 TrpE  (S.t)   AMFFGG---LFAYDL VA-------GFEALP HLEAGNNCPDYCFYL AGTLMVIDHQKKST- ---RIQASLFTASDR EKQRLNARLAYLSQQ   218
2 ASA2  (A.t)   EAFCGGWVGYFSYDT VRYVEKKKLPFSNAP --EDDRSLPDVNLGL YDDVIVFDHVEKKAY VIHWVRIDKDRSVEE NFREGMNRLESLTSR   287
3 ASα2  (R.g)   EAFCGGWVGYFSYDT VRYVEKKKLPFFSAP --TDDRNLPDVHLGL YDDVIVFDHVEKKAF VIHWVRLDQYSSVAE AYNDGMNRLENLVSR   287
4 ASA1  (A.t)   DAFCGGWVGFFSYDT VRYVEKRKLPFSKAP --EDDRNLPDMHLGL YDDVVVFDHVEKKAY VIHWIRLDGSLPYEK AYSNGMQHLENLVAK   274
5 Asα1  (R.g)   EAFCGGWVGFFSYDT VRYVEKKKLPFSKAP --QDDRNLADMHLGL YNDVIVFDHVEKKVY VIHWVRLNQQSSEEK AYAEGLEHLERLVSR   289

                                                                                      ↓            ●●   ●●
1 TrpE  (S.t)   LTQPAPPLPVTPVPD MRCE--------CNQ SDDAFGAVVRQLQKA IRAGEIFQVVPSRRF SLPC-PSPLAAYYVL KKSNPSPYMFFMQDN   299
2 ASA2  (A.t)   IQDQKPPKMPTGFIK LRTQLFGPKLEKSTM TSEAYKEAVVEAKEH ILAGDIFQIVLSQRF ERRTFADPFEIYRAL RIVNPSPYMAYLQVR   377
3 ASα2  (R.g)   VHDIVPPKLRSGSIK LHTRHFGPKLERSSM TSEAYKEAVLEAKEH ILAGDIFQIVLSQRF ERRTFADPFEIYRSL RIVNPSPYMTYLQAR   377
4 ASA1  (A.t)   LHDIEPPKLAAGNVN LQTRQFGPSLDNSNV TCEEYKEAVVKAKEH ILAGDIFQIVLSQRF ERRTFADPFEVYRAL RVVNPSPYMGYLQAR   364
5 Asα1  (R.g)   VQDENTPRLAPGSID LHTGHFGPPLKKSNM TCEEYKMAVLAAKEH IQAGDIFQIVLSQRF ERRTFADPFEVYRAL RVVNPSPYMTYMQAR   379

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1 TrpE  (S.t)   DFTLFGASPESSLKY DAASRQIEIYPIAGT RPRGRRADGTLDRDL DSRIELDMRTDHKEL SEHLMLVDLARNDLA RICTPGSRYVADLTK   389
2 ASA2  (A.t)   GCILVASSPEILLR- -SKNRKITNRPLAGT VRRGKTPKE------ DLMLEKELLSDEKQC AEHIMLVDLGRNDVG KVSKPGSVEVKKLKD   459
3 ASα2  (R.g)   GCILVASSPEILTR- -VKKRKITNRPLAGT IRRGKTRKE------ DLVFEKELLNDEKQC AEHIMLVDLGRNDVG KVSEPGSVKVEKLMN   459
4 ASA1  (A.t)   GCILVASSPEILTK- -VKQNKIVNRPLAGT SKRGKNEVE------ DKRLEKELLENEKQC AEHIMLVDLGRNDVG KVTKYGSVKVEKLMN   446
5 Asα1  (R.g)   GCVLVASSPEILTR- -VKKNKIVNRPLAGT ARRGRTTEE------ DEMLETQLLKDAKQC AEHVMLVDLGRNDVG KVSKSGSVKVEKLMN   461

                            ○                                                             ○     ●
1 TrpE  (S.t)   VDRYSYVMHLVSRVV GELRHDLDALHAYRA CMNMGTLSGAPKVRA MQLIADAEGQRRGSY GGAVGYFTAHGDLDT CIVIRSALVENG---   476
2 ASA2  (A.t)   IEWFSHVMHISSTVV GELLDHLTSWDALRA VLPVGTVSGAPKVKA MELIDELEVTRRGPY SGGFGGISFNGDMDI ALALRTMVFPTNTRY   549
3 ASα2  (R.g)   IEHYSHVMHISSTVT GELLDHLTSWDALRA ALPVGTVSGAPKVKA MEIIDKLEVTRRGPY GGGFGGISFTGDLDI ALALRTMVFQTATRY   549
4 ASA1  (A.t)   IERYSHVMHISSTVT GELQDGLTCWDVLRA ALPVGTVSGAPKVKA MELIDELEPTRRGPY SGGFGGVSFTGDMDI ALSLRTIVFPTACQY   536
5 Asα1  (R.g)   VERYSHVMHISSTVT GELQDNLSCWDALRA ALPVGTVSGAPKVKA MELIDELEVNRRGPY SGGFGGISFTGDMDI ALALRTIVFQTGTRY   551

                                                                        ○
1 TrpE  (S.t)   --------------- IATVQAGAGIVLDSV PQSEADETRNKARAV LRAIATAHHAQETF- --------------- ---------------   520
2 ASA2  (A.t)   DTLYSYKHPQRRREW IAHIQAGAGIVADSN PDDEHRECENKAAAL ARAI---DLAESSFL EAPEFTTITPHINNI ---------------   621
3 ASα2  (R.g)   DTMYSYKDVDKRREW IAHLQAGAGIVADSD PADEQRECENKAAAL ARAI---DLAESSFI EK------------- ---------------   608
4 ASA1  (A.t)   NTMYSYKDANKRREW VAYLQAGAGVVADSD PQDEHCECQNKAAGL ARAI---DLAESAFV KK------------- ---------------   595
5 Asα1  (R.g)   DTMYSYKNATKRRQW VAYLQAGAGIVADSD PDDEHRECQNKAAGL ARAI---DLAESAFV NKSSS---------- ---------------   613
```

**Figure 3.** Alignment of predicted amino acid sequences of *ASA1* and *ASA2* proteins from Arabidopsis (A.t.) (Niyogi and Fink, 1992) with AS $\alpha$ subunit sequences of *R. graveolens* (R.g.) (Bohlmann et al., 1995) and *S. typhimurium* (S.t.) TrpE. The G to A mutation in the *ASA1* gene of *amt-1* would cause a change of Asp at position 341(↓) in the wild-type protein to Asn. The amino acid residues affected in mutants of the *Salmonella* enzyme (Caligiuri and Bauerle, 1991) are indicated by filled circles (●) for strongly feedback-resistant mutants and by open circles (○) for feedback-resistant mutations having more subtle effects.

much greener and healthier cotyledons than wild-type seedlings in which root growth was totally inhibited. The apparent discrepancy between our characterization of 6MA resistance and that of Li and Last is thus due partly to the end points used to evaluate growth (measured root length versus "growth"). Differences in the growth conditions used in the two studies (light intensity and quality, culture medium, etc.), which could affect both plant physiology and inhibitor stability, may also be involved.

## DISCUSSION

In a previous report, *amt-1*, a mutant of *A. thaliana* that is resistant to the Trp analog $\alpha$MT was isolated and partially characterized (Kreps and Town, 1992). This mutant is similar to some Trp-analog-resistant variants in other species (Scott et al., 1979; Ranch et al., 1983) because it contains high levels of Trp and exhibits a higher specific activity of AS that is more resistant to feedback inhibition by Trp. Cell cultures of the mutant also showed higher AS activity and Trp levels than callus initiated from wild-type seed. It is interesting that the respective callus cultures of mutant and wild type had higher AS activity and Trp levels than whole plants (Kreps and Town, 1992).

In this study, the mutation in *amt-1* was mapped to a position on chromosome V at or close to *ASA1*, a gene encoding the $\alpha$ subunit of AS. Since mutant extracts exhibited higher AS (holoenzyme) activity than wild-type ex-

**Table IV.** *Effect of Trp analogs on root growth of various Arabidopsis genotypes*

The values presented indicate concentrations of compound required to inhibit growth of the respective genotypes by 50% ($IC_{50}$) and are the means ± SE for two or more experiments.

| Strain | $IC_{50}$ | | |
|---|---|---|---|
| | αMT | 6MA | 5MA |
| | | $IC_{50}$ μM | |
| Wild type | 15 ± 1 | 0.5 ± 0.1 | 1.2 ± 0.1 |
| *amt-1* | 96 ± 16 | 2.8 ± 0.1 | 2.9 ± 0.4 |
| LIA2 | 97 ± 30 | 2.3 ± 0.7 | 2.3 ± 0.5 |

tracts and showed increased resistance to feedback inhibition and the mutation mapped to *ASA1*, enzyme activity of the α subunit was measured in an assay in which ammonia was used as the amino donor for the AS enzyme reaction. Mutant extracts exhibited higher ammonia-dependent AS activity compared to the corresponding wild-type extracts, again suggesting that there may be an alteration in the α subunit protein of the mutant. An alternative explanation for elevated AS activity in the mutant could involve greater amounts of *ASA1* transcript and/or ASA1 protein in the mutant. RNA gel blot analysis showed that mutant and wild-type leaf tissue accumulated similar amounts of ASA1 transcript. Similarly, mutant and wild-type callus cultures exhibited no difference in the amount of *ASA1* transcript, although both mutant and wild-type callus had approximately 2.5 times more *ASA1* transcript than did leaf tissue. Immunoblot analysis revealed that the respective tissues (callus or leaf) of mutant and wild type had similar levels of ASA1 protein. However, consistent with the increased RNA, callus extracts contained higher amounts of ASA1 protein than leaf extracts. Increased amounts of *ASA1* mRNA and protein may at least partially account for increased AS activity (both Gln and ammonia dependent) in callus compared with plants. This increase in *ASA1* expression in callus may be analogous to wound or pathogen response in Arabidopsis, which is known to cause an increase in *ASA1* mRNA levels (Niyogi and Fink, 1992).

Since our data from enzyme analysis, genetic mapping, and gene expression studies indicated that the basis for the mutant phenotype was likely caused by a change in the *ASA1* structural gene, sequencing of *ASA1* from *amt-1* was undertaken. Consistent with this prediction, a single base-pair change was detected that would be expected to cause a single amino acid substitution at position 341 (Asp to Asn) in the mutant protein. Separately and independently, Li and Last (1996) identified the identical substitution in three independently isolated 6MA-resistant mutants. This mutation in the *ASA1* gene is now designated *trp5–1* in all four mutant isolates to indicate the identity of the base change and to conform to Arabidopsis nomenclature. Our observation that *amt-1* and LIA2 both possess elevated levels of free Trp, have AS activity with increased resistance to feedback inhibition, and show a similar spectrum of Trp analog resistance is not surprising in view of the identity of the mutant alleles in these two strains. The differences between levels of free Trp and the degree of Trp analog resistance reported between *amt-1* and the LIA mutants is most likely due to differences in plant physiology (e.g. light conditions) as well as to the way in which "resistance" was evaluated in the two studies.

The Asp residue at position 341 of the wild-type *ASA1* protein sequence is conserved in the only other plant enzymes sequenced to date, Arabidopsis *ASA2* and *R. graveolens* AS α1 and AS α2, as well as in some bacteria (Band et al., 1984; Goncharoff and Nichols, 1984; Niyogi and Fink, 1992; Bohlmann et al., 1995). However, this alteration does not correspond to any of the amino acids identified by Caligiuri and Bauerle (1991) as affecting feedback sensitivity in the *Salmonella* enzyme, and in fact the aspartate residue is not conserved in this enzyme.

In a recent report, αMT-resistant lines of *Lemna gibba* were isolated that appear to be similar to this group of Arabidopsis *trp5–1* mutants in terms of the elevated levels of Trp and AS activity and the resistance of mutant AS to feedback inhibition by Trp (Tam et al., 1995). Like the *trp5–1* mutants (Kreps and Town, 1992; Li and Last, 1996), these *Lemna* lines, MTR-1 and MTR-2, exhibit cross-resistance to the Trp analog 5-methyltryptophan. However, the molecular basis for the mutations in these lines has not been reported. It is interesting that these *Lemna* lines also show elevated IAA turnover.

In summary, we have characterized a Trp-analog-resistant mutant of Arabidopsis at the physiological, biochemical, and molecular levels. This mutant has altered Trp metabolism that appears to be due to a single base-pair change in a gene encoding the α subunit of the predominant form of AS. These studies of AS along with investigations of other mutants in the Trp pathway could further our understanding of the regulation of the metabolism of Trp, IAA, and secondary products in plants.

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## LITERATURE CITED

**Band L, Shimotsu H, Henner DJ** (1984) Nucleotide sequence of the *Bacillus subtilis trpE* and *trpD* genes. Gene **27:** 55–65

**Bohlmann J, DeLuca V, Eilert U, Martin W** (1995) Purification and cDNA cloning of anthranilate synthase from *Ruta graveolens*: modes of expression and properties of native and recombinant enzymes. Plant J **7:** 491–501

**Bradford MM** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem **72:** 248–254

**Caligiuri MG, Bauerle R** (1991) Identification of amino acid residues involved in feedback regulation of the anthranilate synthase complex of *Salmonella typhimurium*. Evidence for an amino terminal regulatory site. J Biol Chem **266:** 8328–8335

**Carlson JE, Widholm JM** (1978) Separation of two forms of anthranilate synthetase from 5-methyltryptophan-susceptible and

-resistant cultured *Solanum tuberosum* cells. Physiol Plant **44:** 251–255

**Church GM, Gilbert W** (1984) Genomic sequencing. Proc Natl Acad Sci USA **81:** 1191–1195

**Goncharoff P, Nichols BP** (1984) Nucleotide sequence of *Escherichia coli pabB* indicates a common evolutionary origin of *p*-aminobenzoate synthetase and anthranilate synthetase. J Bacteriol **159:** 57–62

**Gudelsky JT, Kufel A, Town CD** (1993) Isolation and characterization of Arabidopsis TrpG cDNAs by complementation of a mutant of *S. cerevisiae* (abstract No. 57). Program and abstracts of lectures and posters for the fifth international conference on *Arabidopsis* research. The Ohio State University, Columbus, p 69

**Hütter R, Niederberger P, DeMoss JA** (1986) Tryptophan biosynthetic genes in eukaryotic microorganisms. Annu Rev Microbiol **40:** 55–77

**Keller J, Bancroft I** (1991) CTAB (cetyltrimethylammonium bromide) plant DNA extraction (medium to large scale). *In* D Flanders, C Dean, eds, *Arabidopsis*: The Compleat Guide. Agricultural and Food Research Council Plant Molecular Biology *Arabidopsis* Programme, Norwich, UK

**Kosambi DD** (1944) The estimation of map distances from recombination values. Ann Eugen **12:** 172–175

**Kreps JA, Town CD** (1992) Isolation and characterization of a mutant of *Arabidopsis thaliana* resistant to $\alpha$-methyltryptophan. Plant Physiol **99:** 269–275

**Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

**Last RL, Fink GR** (1988) Tryptophan requiring mutants of the plant *Arabidopsis thaliana*. Science **240:** 305–310

**Li J, Last RL** (1996) The *Arabidopsis thaliana trp5* mutant has a feedback-resistant anthranilate synthase and elevated soluble tryptophan. Plant Physiol **110:** 51–59

**Maniatis T, Fritsch EF, Sambrook J** (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

**Murashige T, Skoog F** (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 473–497

**Niyogi KK, Fink GR** (1992) Two anthranilate synthase genes in *Arabidopsis*: defense-related regulation of the tryptophan pathway. Plant Cell **4:** 721–733

**Niyogi KK, Last RL, Fink GR, Keith B** (1993) Suppressors of *trp1* fluorescence identify a new *Arabidopsis* gene, *TRP4*, encoding the anthranilate synthase $\beta$ subunit. Plant Cell **5:** 1011–1027

**Poulsen C, Bongaerts RJM, Verpoorte R** (1993) Purification and characterization of anthranilate synthase from *Catharanthus roseus*. Eur J Biochem **212:** 431–440

**Poulsen C, Verpoorte R** (1991) Roles of chorismate mutase, isochorismate synthase and anthranilate synthase in plants. Phytochemistry **30:** 377–386

**Ranch JP, Rick S, Brotherton JE, Widholm JM** (1983) Expression of 5-methyltryptophan resistance in plants regenerated from resistant cell lines of *Datura inoxia*. Plant Physiol **71:** 136–140

**Rochester DE, Winer JA, Shah DM** (1986) The structure and expression of maize genes encoding the major heat-shock protein, hsp 70. EMBO J **5:** 451–458

**Rogers SO, Bendich AJ** (1988) Extraction of DNA from plant tissues. *In* SB Gelvin, RA Schilperoort, eds, Plant Molecular Biology Manual. Kluwer Academic, Dordrecht, The Netherlands, pp A6/1–A6/11

**Scott AI, Mizukami H, Lee S-L** (1979) Characterization of a 5-methyltryptophan resistant strain of *Catharanthus roseus* cultured cells. Phytochemistry **18:** 795–798

**Somerville CR, Ogren W** (1982) Isolation of photorespiration mutants of *Arabidopsis thaliana*. *In* M Edelman, RB Hallick, NH Chua, eds, Methods in Chloroplast Molecular Biology. Elsevier Biomedical Press, New York, pp 129–139

**Stasinopoulos TC, Hangarter RP** (1990) Preventing photochemistry in culture media by long-pass light filters alters growth of cultured tissues. Plant Physiol **93:** 1365–1369

**Suiter KA, Wendel JF, Case JS** (1983) Linkage-1: a PASCAL computer program for the detection and analysis of genetic linkage. J Hered **74:** 203–204

**Tam YY, Slovin JP, Cohen JD** (1995) Selection and characterization of $\alpha$-methyltryptophan-resistant lines of *Lemna gibba* showing a rapid rate of indole-3-acetic acid turnover. Plant Physiol **107:** 77–85

**Widholm JM** (1977) Relation between auxin autotrophy and tryptophan accumulation in cultured plant cells. Planta **134:** 103–108

**Zhao J, Last RL** (1995) Immunological characterization and chloroplast localization of the tryptophan biosynthetic enzymes of the flowering plant *Arabidopsis thaliana*. J Biol Chem **270:** 6081–6087