Tryptophan-Requiring Mutants of the Plant *Arabidopsis thaliana*

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Although amino acid auxotrophs are among the most frequently isolated mutations in microorganisms, no mutants that require amino acids have been isolated at the whole plant level. Tryptophan-requiring mutants of the cruciferous plant *Arabidopsis thaliana* have now been isolated by selecting for resistance to 5-methylanthranilic acid. The tryptophan requirement of one mutant, *trp1-1*, results from a defect in the second step of the tryptophan pathway catalyzed by anthranilate phosphoribosyl transferase. Mutant *trp1-1* plants are highly fluorescent and aromatic because they accumulate anthranilic acid and anthranilate β-glucoside. Plants homozygous for the *trp1-1* mutation exhibit a syndrome of morphological defects suggestive of a defect in the biosynthesis, metabolism, or localization of a tryptophan derivative such as auxin. All of these morphological phenotypes cosegregate with the tryptophan requirement as a simple Mendelian recessive trait.

THE ABSENCE OF PLANT AMINO ACID AUXOTROPHS IS SURprising because plants synthesize the 20 essential amino acids and can be cultured on a minimal medium that contains calcium, magnesium, potassium, nitrate, phosphate, sulfate, and trace minerals. Although amino acid requiring tissue culture lines have been isolated from a number of species, most variants were not regenerated into plants. Those variants that were successfully regenerated yielded infertile plants, with the exception of one fertile methionine-requiring *Nicotiana plumbaginifolia* mutant (*1–5*). However, this regenerant failed to transmit the requirement to its progeny as a simple Mendelian trait (*5*).

Two formal explanations can account for the absence of plant amino acid auxotrophs. One model posits that mutations leading to a requirement for an amino acid are lethal. A plant auxotroph would be an embryo lethal if the embryo could not obtain sufficient amounts of the amino acid from the surrounding maternal tissue. Similarly, auxotrophy would be lethal to the germinating seedling if the roots were unable to import the required amino acid from the surrounding medium. A second explanation is that plants have multiple structural genes for each step in amino acid biosynthesis. In such a genetically redundant system, induction of rare multiple mutations or alterations in regulatory genes would be required for amino acid auxotrophy.

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These considerations have led us to attempt the isolation of amino acid auxotrophs in the small cruciferous plant *Arabidopsis thaliana*. Experiments on the *A. thaliana* isoleucine-valine pathway suggest that these amino acids are permeable and that at least one gene in the pathway is present in a single copy per haploid genome (*6*). This finding coupled with the fact that *A. thaliana* has the smallest genome of characterized dicotyledonous plants (*7*) led us to believe that neither amino acid permeability nor genetic redundancy would be absolute barriers to the isolation of auxotrophs in this organism. Furthermore, *A. thaliana* has several features that should facilitate the identification of auxotrophic mutants. Mutagenized seed can be assayed for auxotrophy by plating on a simple, defined minimal medium. The genetic basis for the auxotrophic phenotype of any putative mutation can be determined rapidly because of the facile genetic system and short life cycle (*7*). Despite the positive attributes of *A. thaliana* for mutational studies no amino acid auxotrophs have been uncovered in previous experiments (*1*).

The isolation of tryptophan auxotrophs in *A. thaliana* was attempted because the biochemistry and physiology of both the bacterial and fungal pathways have been worked out in enormous detail. Although the biochemical pathway from shikimate to tryptophan is identical in *Escherichia coli* and yeast, the arrangement of genes and their regulation are dramatically different in the two organisms (*8*).

Little is known about the sequence of reactions, the arrangement of genes, or the regulation of expression of the tryptophan pathway in plants. This pathway is of additional interest in plants because tryptophan is thought to be the precursor of the plant hormone indole-3-acetic acid (the auxin, IAA). Although the pathway for the biosynthesis of IAA from tryptophan has been determined by mutant analysis in the bacterium *Agrobacterium tumefaciens* (*9*), the exact sequence of reactions has not been determined in plants (*10*). The availability of tryptophan auxotrophs in *A. thaliana* would permit the elucidation of the tryptophan and IAA biosynthetic pathways and provide a genetic basis for the study of hormone action.

A strategy for the isolation of tryptophan auxotrophs in plants. Amino acid auxotrophs might have been overlooked either because they were very rare or because they grew so slowly that they were overgrown by the surrounding plants. The scarcity of a mutation creates a technical barrier to its isolation because of the large number of manipulations required to test a mutagenized population for the presence of auxotrophs. Unlike microorganisms, mutagenized population of *A. thaliana* cannot be replica-plated to diagnostic media to test directly for auxotrophy. Rather, seed arising from individually propagated M_2 (*11*) plants must be harvested and tested on medium with and without amino acid supplementation. Under this scheme, if a mutation occurs at 10^{-4} per M_2 seed, one would have to propagate 10^4 M_3 families for differential screening.

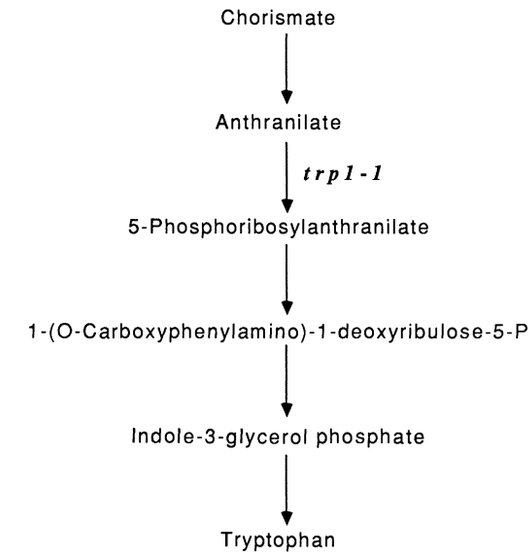


Fig. 1. The tryptophan biosynthetic pathway in bacteria and fungi. The intermediates and final product of the tryptophan biosynthetic pathway are diagrammed (*8*). The location of the defective enzyme activity in the *trp1-1* mutant is indicated.

The problems caused by slow growth and infrequency of mutants could be averted by a positive selection that permits the growth of auxotrophs but not wild type.

We therefore adapted a scheme previously shown to give *E. coli* tryptophan auxotrophs a growth advantage over wild-type. *E. coli* tryptophan-requiring (Trp^-) mutants can be selected from a population of Trp^+ cells on a medium containing a mixture of 5-methylanthranilic acid (5MA) and tryptophan (*12*). The growth of Trp^+ cells is inhibited because they convert the 5MA to the toxic compound 5-methyltryptophan, whereas Trp^- auxotrophs grow because they are blocked in the pathway from anthranilate to tryptophan and fail to metabolize the 5MA properly (see Fig. 1). The levels of both tryptophan and 5MA must be adjusted so that there is sufficient tryptophan to support the growth of the Trp^- cells, but not so much that the inhibition by 5MA is overcome. A similar strategy has been used in *Saccharomyces* to enrich for uracil auxotrophs with a mixture of 5-fluoroorotate and uracil (*13*) and for lysine auxotrophs with a mixture of alpha-aminoadipate and lysine (*14*).

The growth of *A. thaliana* is inhibited by 5MA, and this inhibition is overcome by the addition of exogenous anthranilate or tryptophan (*15*). This result suggests that the plant takes up 5MA and converts it to a toxic tryptophan derivative. The relative concentrations of tryptophan and 5MA that would allow auxotrophs to grow while wild type remained inhibited were determined empirically. Populations of 500 to 1000 M_2 *A. thaliana* seed mutagenized with ethylmethanesulfonate (*16*) were germinated on nutrient agar plates containing 500 to 600 μM 5MA and 40 to 50 μM tryptophan. These concentrations do not completely inhibit wild type, but slow its growth so that faster growing variants can be detected. Variants with better than average leaf and root growth after 2 to 3 weeks on selective medium were then grown for 2 weeks on sterile nutrient agar plates containing 50 μM tryptophan. These plants were allowed to complete the life cycle on nonsterile soil mix that contained 50 μM tryptophan (*17*). Of 180,000 M_2 seeds tested, 55 putative mutants were identified that appeared to grow better than wild type on the 5MA-tryptophan mixture. Of these, 43 survived to maturity and yielded seed within 4 months of growth (*18*).

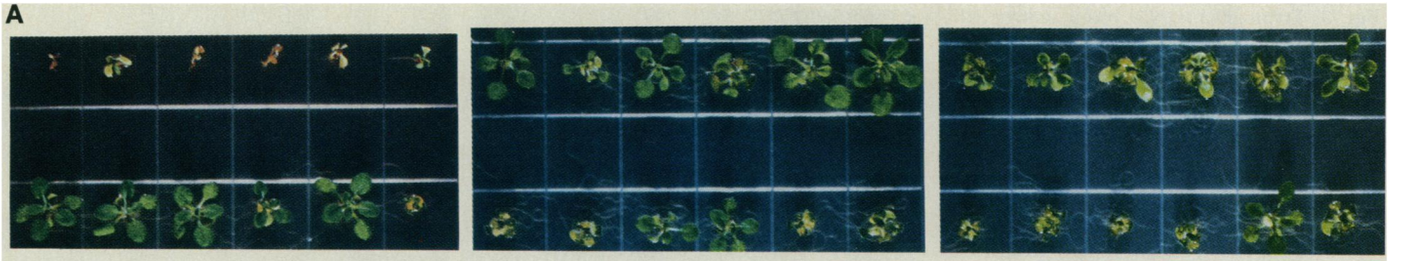
Growth tests on M_3 seed (derived from self-pollination of the M_2 plants) allowed preliminary classification of four of the resistant lines as tryptophan auxotrophs, one of which is described below. The M_3 seed from this 5MA-resistant line formed abnormally light-colored leaves and short roots on sterile agar minimal nutrient medium. The small seedlings showed little or no post-germination growth, presumably because seed tryptophan reserves were rapidly exhausted (Fig. 2A, left, and B). Most of these tryptophan-starved plants recovered when transferred to tryptophan-containing agar medium. The M_3 seed germinated on sterile tryptophan medium grew as well as did wild type during early stages of development (Fig. 2A, right, and B). The nutritional requirement for tryptophan persists after germination as shown by transfer of seedlings germinated on tryptophan to minimal medium (*19*). The mutant was not rescued by addition of any other amino acid, vitamin, or hormone (*20*).

Tryptophan auxotrophy is caused by a mutation at a single locus. The genetic basis for the tryptophan requirement was determined by crossing a wild-type Trp^+ strain of *A. thaliana* with pollen from the tryptophan-requiring strain (Table 1). The F_1 seeds resulting from this cross grew like wild type on sterile minimal medium, indicating that the Trp^+ phenotype is dominant to Trp^-. Of the F_2 seeds plated on sterile minimal medium, 25 percent germinated but failed to grow vigorously. The Trp^+ phenotype of the F_1 and the 3:1 (Trp^+:Trp^-) segregation in the F_2 are consistent with a monogenic recessive trait conferring tryptophan auxotrophy. The conclusion that *trp1-1* is a single Mendelian trait was supported by a test cross in which pollen from a Trp^- M_3 plant (*trp1-1/trp1-1*) was crossed to a F_1 heterozygote (*TRP1*$^+$*/trp1-1*) (Table 1). We call the mutation that causes the tryptophan requirement in these *A. thaliana* lines *trp1-1* to indicate that it is a recessive allele of the *TRP1* gene.

To confirm that the failure of progeny to show substantial growth on minimal medium resulted from a tryptophan requirement, we transplanted the putative Trp^- F_2 progeny to tryptophan-containing medium. The majority of the putative Trp^- plants recovered and grew into maturity when supplemented with tryptophan (in Fig. 3, compare the small plant in the left panel to the plant in the right panel). The F_3 progeny from these putative Trp^- F_2 plants required tryptophan for growth, verifying that the F_2 plants were tryptophan auxotrophs.

Strains carrying the *trp1-1* mutation lack phosphoribosyl transferase activity. The 5MA selection procedure can yield mutants affected in any of the enzymatic steps leading from anthranilate to tryptophan. If we assume that the pathway of tryptophan biosynthesis in plants is identical to that found in bacteria and fungi (Fig. 1), certain predictions can be made about the ability of intermediates in the pathway to supplement the classes of auxotrophs expected. Microorganisms are permeable to two of the intermediates, anthranilate and indole. These compounds can satisfy the nutritional requirement of some mutants. Anthranilic acid should not supplement any 5MA-resistant mutants because resistance requires that the auxotrophic defect be in the conversion of anthranilic acid to tryptophan. Indole should supplement all mutants except those blocked in the tryptophan synthase B activity. The *trp1-1* mutant is supplemented by indole but not anthranilate, consistent with a defect between anthranilate and indole-3-glycerol phosphate or in the tryptophan synthase A activity (Fig. 2).

Direct enzyme assays on crude extracts of wild-type and mutant plants indicate that the *trp1-1* mutation causes a defect in the anthranilate phosphoribosyl (PR)-transferase activity (E.C. 2.4.2.18), which catalyzes the formation of *N*-5′-phosphoribosylanthranilate from PRPP and anthranilate (Table 2). The defect in activity is not caused by the presence of an inhibitor in the mutant extracts because mixing mutant and wild-type extracts does not cause inhibition of wild-type transferase activity. The absence of



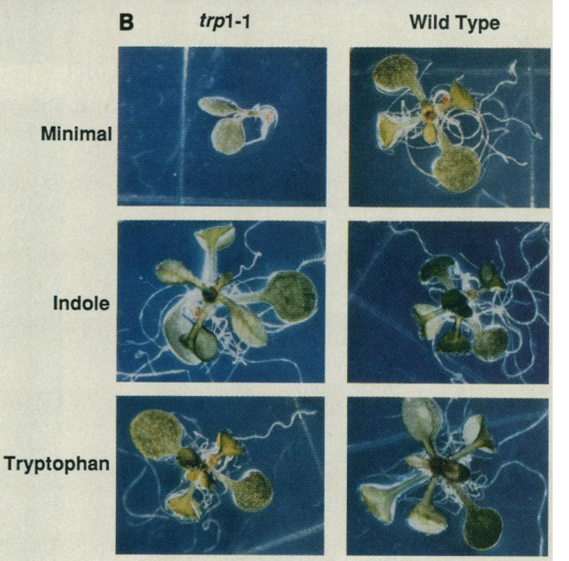


Fig. 2. The *trp1-1* growth defect is supplemented by indole and tryptophan. Surface-sterilized *trp1-1* mutant seeds (top rows) and wild-type seeds (bottom rows) were grown for 2 weeks on sterile plant nutrient agar. (**A**) Plates contained (left) 50 μ*M* anthranilate, (center) 50 μ*M* indole, or (right) 50 μ*M* tryptophan. Mutant plants grown on minimal medium were indistinguishable from those grown in the presence of anthranilate. (**B**) Photomicroscopy of *trp1-1* mutant and wild-type plants grown on sterile minimal nutrient medium or medium supplemented with 50 μ*M* indole or tryptophan.

anthranilate PR-transferase activity is not the result of a general regulatory defect in tryptophan biosynthetic enzyme activities since the *trp1-1* mutant strains have anthranilate synthase and PR-anthranilate isomerase activities equal to that of wild type (Table 2).

Plants carrying the *trp1-1* mutation are fluorescent. Plants carrying the *trp1-1* mutation exhibit a striking amethyst fluorescence under either longwave or shortwave ultraviolet light (see cover photo). The fluorescence is observed soon after germination and persists until after the plants reach maturity. In addition, the adult mutant plants have a sweet aroma that is absent from wild-type plants. The fluorescence phenotype cosegregates with the tryptophan requirement in F_2 populations, showing that both phenotypes are caused by the same or very tightly linked mutations.

The fluorescence of the Trp^- plants results from the accumulation of a covalently modified tryptophan biosynthetic intermediate. Paper chromatography of ethyl acetate extracts of mutant plants revealed a major fluorescent compound with an R_F of 0.6 in both acidic and basic butanol solvent systems; these chromatographic properties are characteristic of anthranilate β-glucoside (*21*). Brief β-glucosidase treatment of the ethyl acetate extracts from *trp1-1* plants caused conversion to a form that comigrates with authentic anthranilate in both chromatographic solvent systems (*22*). The plants also accumulate small amounts of a second fluorescent compound that has chromatographic properties identical to anthranilate. The accumulation of anthranilate β-glucoside and anthranilate supports the assigned position of the *trp1-1* mutant block in the tryptophan biosynthetic pathway (Fig. 1). The PR-transferase mutants in bacteria and fungi accumulate anthranilate (*23*). Apparently the *A. thaliana trp1-1* mutant converts most of the excess anthranilic acid to the β-glucoside (*24*).

The fluorescence phenotype cosegregates with the Trp^- phenotype, suggesting that the anthranilate β-glucoside accumulation is caused by the anthranilate PR-transferase defect (*25*). The *trp1-1* M_2 plant was derived from heavily mutagenized M_1 seed and was likely to have harbored mutations that were not associated with the tryptophan auxotrophy. Both the test cross and F_2 analysis were performed to assay the segregation of all the phenotypes associated with the M_2, M_3, and M_4 lines. The purpose of the crosses was to segregate away any traits that were not a consequence of the *trp1-1* mutation. The segregation analysis is more meaningful when performed on F_2 progeny, where both parents are heterozygous, than on test cross progeny because in the self-pollination of the F_1 there is the opportunity for segregation of incidental mutations away from *trp1-1* in both parental meioses; whereas, in the test cross segregation can only occur in the meiosis of one of the parents (since the other parent is homozygous).

Table 1. Results of crosses with the *trp1-1* mutant. Pollen from the M_3 *trp1-1* mutant was crossed with a Trp^+ strain homozygous for a recessive tricome-deficient *gl1-1* homozygous strain to yield a pubescent F_1 strain, indicating that the cross was successful. This F_1 was allowed to self-pollinate to yield F_2 progeny, or crossed with M_3 *trp1-1/trp1-1* pollen to yield the test cross.

Cross	Type	Total	Trp^+	Trp^-	Trp^- (%)	χ^2*
trp1-1/trp1-1 × *TRP1/TRP1*	F_1	52	52	0	0	
TRP1/trp1-1 × *TRP1/trp1-1*	F_2	768	573	195†	25	0.06‡
trp1-1/trp1-1 × *TRP1/trp1-1*	Test cross	53	28	25	53	0.17‡

*The chi-square values were calculated on the basis of the expectation of 25 and 50 percent Trp^- progeny for the F_2 and test cross, respectively. †A single putative Trp^- plant that was not fluorescent and had wild-type morphology was shown to be Trp^+ by progeny testing. ‡$P > 0.05$.

Morphological consequences of *trp1-1* mutation. Tryptophan does not restore the wild-type phenotype to adult plants that are homozygous for the *trp1-1* mutation. During early stages of growth on tryptophan nutrient agar medium, the plants grow at a normal rate and look similar to wild type; by 3 to 4 weeks after germination the mutant is dramatically smaller than wild-type siblings. At this time the mutant also begins to display a characteristic array of morphological differences from wild type. The rosette leaves of the *trp1-1* mutant are small and crinkled and have a smaller petiole than wild-type plants of comparable age. The mutant also shows an unusual bushiness: uncrowded wild-type plants grow three to five flowering stalks, whereas the mutant has many more (Fig. 4A). During the first 2 months of growth, each plant makes more than 50 small, thin flower bolts. These bolts contain many small flower buds that shed only small amounts of pollen (Fig. 4B), and are infertile as a female parent even when crossed with wild-type pollen. After 2 to 3 months most plants make larger, thicker bolts that produce a small number of fertile flowers of normal appearance (Fig. 4C). These

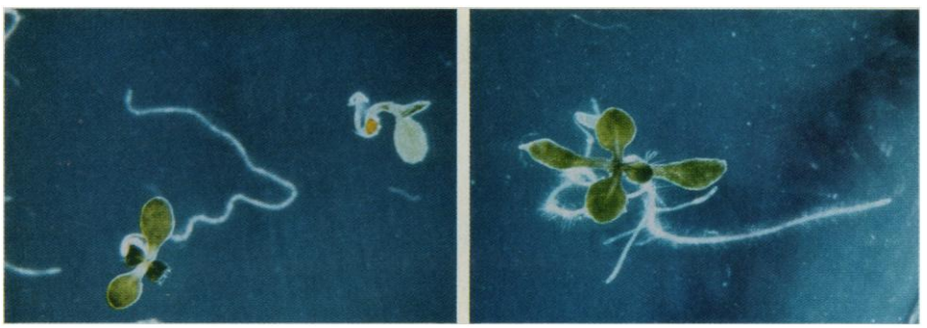


Fig. 3. Tryptophan-starved *trp1-1* mutant seedlings can be rescued by transfer to tryptophan. (Left) Surface-sterilized F_2 seeds were grown on minimal nutrient agar for 6 days. At this time the Trp^+ plant (left plant) is distinguished from its Trp^- sibling (right plant) by its long root, dark green cotyledon leaves, and emerging true leaves. (Right) The Trp^- F_2 plant was moved from minimal to 50 μM tryptophan medium and grown for 7 days. The plant grew an extensive root network, and developed healthy green leaves.

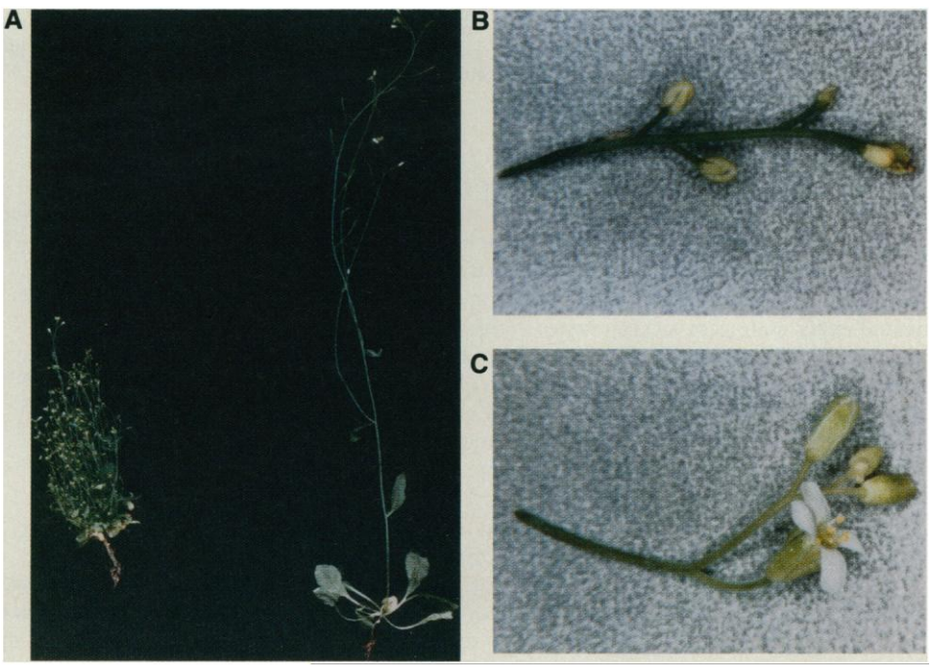


Fig. 4. Morphological characteristics of the *trp1-1* mutant. (**A**) A Trp^- M_4 plant was germinated and grown for 2 weeks on sterile medium containing 50 μM tryptophan. It was then transferred to nonsterile soil mixture containing 50 μM tryptophan and grown for an additional 8 weeks. This *trp1-1* plant is shown on the left, next to a 4-week-old wild-type plant grown without tryptophan. Note the small size, bushy morphology, and many small infertile flowers characteristic of this mutant. The mutant plant has its first set of flowers with normal morphology located at the top of the plant. (**B**) A higher magnification photograph of the small infertile flowers. (**C**) The same magnification of the mutant flowers that appear later in development. These are indistinguishable from wild type.

morphological features are not a consequence of the tryptophan in the medium because $TRP1^+$ plants fail to show these alterations when grown on the same medium. All of these unusual phenotypic traits cosegregate with the tryptophan requirement in F_2 progeny, showing that they are a consequence of the *trp1-1* mutation.

Useful properties of the *trp1-1* mutant. We have devised a selection that makes it possible to isolate tryptophan auxotrophs in *A. thaliana*. The utility of such mutants in unraveling basic problems in the genetics, biochemistry, and physiology of plants was apparent even in the process of characterizing the mutant strains. For example, *trp1-1* has proved to be a useful genetic marker because it can be used both for screens and selections. On medium containing tryptophan, Trp^- plants can be easily distinguished from Trp^+ plants by their fluorescence and unusual morphology. The fluorescence of the mutant provides an easily scored visual marker throughout the life cycle of the plant, permitting the isolation of tryptophan auxotrophs without 5MA selection. Rare Trp^+ plants can be selected out of a large population of Trp^- plants on minimal medium, providing a powerful tool for reversion and recombination analyses.

The *trp1-1* mutant also illustrates the value of auxotrophs in identifying the sequence of reactions in tryptophan biosynthesis. Analysis of this mutant provides the first direct in vivo evidence in plants that conversion of anthranilate to *N*-5′-phosphoribosylanthranilate is an obligate step in tryptophan biosynthesis. The mutant also provides some insight into the regulation of tryptophan biosynthetic enzymes in *A. thaliana*. The high levels of anthranilate synthase and anthranilate PR-isomerase activities and the accumulation of fluorescent compounds when the mutant is grown on tryptophan show that tryptophan does not completely repress the expression of *A. thaliana* anthranilate synthase, as it does in *E. coli*. The growth of the mutant on indole suggests that the latter part of the *A. thaliana* tryptophan pathway could be similar to that found in bacteria and fungi. Furthermore, the viability of the homozygous *trp1-1/trp1-1* mutant suggests that the surrounding maternal tissue might be capable of supplying the nascent embryo with enough tryptophan to sustain its growth and development. Alternatively, the mutation might be sufficiently leaky to permit production of a viable embryo.

Even in the presence of tryptophan the *trp1-1* mutant has a number of unusual phenotypes: slow growth, small crinkled leaves, bushy morphology, and production of large numbers of small, infertile flowers during the first 2 to 3 months of growth. Three other tryptophan auxotrophs that we have isolated also show increased bushiness and slow growth (*26*). Although these abnormalities may result indirectly from the slow growth of the plants, we favor the hypothesis that the altered morphology of the *trp1-1* mutant results from a perturbation of tryptophan biosynthesis. For example, tryptophan auxotrophy may lead to a defect in the biosynthesis of a plant hormone such as auxin, thought to be derived from tryptophan. Auxin has been implicated in the control of many plant developmental processes including apical dominance, cell size,

Table 2. Specific activities of tryptophan biosynthetic enzymes. Enzymes were assayed from crude plant extracts (*30*). Specific activities ($x \pm$ S.D.) are reported in picomoles of anthranilate per minute per microgram of chlorophyll A plus B. Concentrations of chlorophyll A plus B were determined by measurement of the A_{654} of extracts in 90 percent ethanol (*31*). All numbers are the average of values from two independent experiments.

Strain	Anthranilate synthase	Anthranilate transferase	Anthranilate isomerase
Wild type	3.0 ± 0.4	160 ± 130	220 ± 100
trp1-1	3.7 ± 0.1	ND*	220 ± 60

*There was no detectable loss of anthranilate from reactions containing *trp1-1* extracts.

cell division rate, and flower development. Auxin-resistant *A. thaliana* mutants altered in the *axr1* locus have morphological defects similar to those we have described for *trp1-1* (*16*). A defect in auxin-mediated growth phenomena could result from an insufficient supply of tryptophan or from inhibition of auxin synthesis or utilization by the intermediates accumulated by the *trp1-1* mutant.

The failure of exogenous tryptophan to restore normal growth and morphology to the *trp1-1* strains is puzzling. The simplest explanation is that the plants are still slightly starved for tryptophan even when the amino acid is present in the medium. Growth of *trp1-1* plants to maturity on medium containing higher tryptophan concentrations (50, 100, or 200 μ*M*) with or without foliar feeding with tryptophan did not eliminate the observed growth defects (*27*). One possible explanation is that tryptophan uptake is inefficient and therefore unable to provide sufficient amino acid for normal rates of protein synthesis or hormone production. Alternatively, exogenous and endogenous tryptophan may not have equal access to intracellular compartments or to specific tissues, so that once the exogenous tryptophan is internalized it cannot be utilized efficiently.

The morphological defects, slow growth, or infrequency of the *trp1-1* mutant could explain why auxotrophs have been so difficult to isolate in plants. This mutant grows so slowly, takes so long to set seed, and has such an unusual morphology that it might easily be overlooked in a mutant hunt that did not include the 5MA selection. Screens that included complex mixtures of amino acids could have precluded the recovery of tryptophan-requiring mutants because *trp1-1* plants grow even more slowly on medium containing equimolar quantities of all three aromatic amino acids than they do on tryptophan alone (*28*). A significant number of putative M_2 5MA-resistant mutants did not survive transplantation to tryptophan-supplemented soil mix, suggesting that these plants might be Trp^- auxotrophs that require different culture conditions for growth. Even with the selection for 5MA-resistant plants tryptophan auxotrophs were rare, occurring at a frequency of 10^{-4} to 10^{-5} per M_2 seed. Other recessive mutations obtained from similar mutagenesis procedures (for example, *A. thaliana* lipid and starch biosynthetic mutants) occur at frequencies of 2×10^{-3} per M_2 seed (*29*).

In addition to their value as genetic markers in crosses and transformation experiments, the tryptophan auxotrophs provide a new system for the study of plant molecular biology and may provide new information about the biochemistry of hormone action. Analysis of the growth and development of tryptophan auxotrophs should provide insights that will permit the isolation of auxotrophs in other biosynthetic pathways.

REFERENCES AND NOTES

1. For a recent comprehensive review of plant amino acid requiring mutants see P. McCourt and C. R. Somerville, in *The Biochemistry of Plants*, D. Davies, Ed. (Academic Press, New York, 1987), vol. 15, pp. 32–64. Although mutants that respond favorably when supplemented with a specific amino acid have been isolated in whole plants, these mutants are able to synthesize the amino acid and, thus, are not amino acid auxotrophs. These include the chlorophyll-deficient *xan-b*[61] mutant of barley [J. B. Land and G. Norton, *Genet. Res.* **15**, 135–137 (1970)] and the collapsed endosperm *pro-1* mutants of maize [M. L. Racchi, G. Gauazzi, C. Dierks-Ventling, P. J. King, *Z. Pflanzenphysiol.* **101**, 303 (1981)].
2. V. A. Sidorov and P. Maliga, *Mol. Gen. Genet.* **186**, 328 (1982).
3. C. Gebhardt *et al.*, *Planta* **159**, 18 (1983).
4. R. B. Horsch and J. King, *ibid.*, p. 12.
5. I. Negrutiu, D. DeBrouwer, R. Dirks, M. Jacobs, *Mol. Gen. Genet.* **199**, 430 (1985).
6. G. W. Haughn and C. Somerville, *ibid.* **204**, 430 (1986).
7. G. P. Redei, *Annu. Rev. Genet.* **9**, 111 (1975); E. M. Meyerowitz and R. E. Pruitt, *Science* **229**, 1214 (1985); R. E. Pruitt and E. M. Meyerowitz, *J. Mol. Biol.* **187**, 169 (1986); E. M. Meyerowitz, *Annu. Rev. Genet.* **21**, 93 (1987).
8. R. Landick and C. Yanofsky, in *Escherichia coli and Salmonella typhimurium*, F. C. Niedhardt *et al.*, Eds. (American Society for Microbiology, Washington, DC, 1987), vol. 2, pp. 1276–1301; E. W. Jones and G. R. Fink, in *The Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression*, J. N. Strathern, E. W. Jones, J. R. Broach, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982), pp. 181–299.
9. G. Schroder, S. Waffenschmidt, E. Weiler, J. Schroder, *Eur. J. Biochem.* **138**, 387 (1984); L. Thomashow, S. Reeves, M. Thomashow, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 5071 (1984); M. F. Thomashow, S. Hugly, W. G. Buchholz, L. S. Thomashow, *Science* **231**, 616 (1986).
10. R. S. Bandurski and H. M. Nonhebel, in *Advanced Plant Physiology*, W. B. Wilkins, Ed. (Pitman, Marshfield, MA, 1987), pp. 1–20
11. The designation M_2 refers to the progeny that result from self-pollination of the plants derived from ethylmethanesulfonate-mutagenized seeds. Mutations that were induced in M_1 seeds will be homozygous in M_2 plants. Thus, recessive mutations can be identified in the M_2 plants. The M_3 plants are derived from self-pollination of M_2 plants, and M_4 plants from M_3 individuals.
12. G. Zurawski, D. Elseviers, G. V. Stauffer, C. Yanofsky, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5988 (1978).
13. J. D. Boeke, F. LaCroute, G. R. Fink, *Mol. Gen. Genet.* **197**, 345 (1984).
14. B. B. Chattoo *et al.*, *Genetics* **93**, 51 (1979).
15. Anthranilate or L-tryptophan (at 50 μ*M*) reversed the growth inhibition by 100 μ*M* 5MA on wild-type *A. thaliana*.
16. M. A. Estelle and C. Somerville, *Mol. Gen. Genet.* **206**, 200 (1987).
17. Seedlings of *Arabidopsis thaliana* Columbia wild type and its derivatives were grown aseptically in Parafilm-sealed petri plates (100 mm in diameter, 25 mm high) with 25 ml of plant nutrient medium containing 0.5 percent sucrose-solidified with 0.75 percent Difco agar (*6*). Seeds were surface-sterilized in 30 percent Clorox and 0.02 percent Triton X-100 for 6 minutes and washed three times with sterile H_2O. In 5MA selections, sterile seeds were sown randomly in 2.5 ml of 50°C sterile 0.75 percent agar in water. The growth of individual seeds was tested either by germination on one sterile medium and then transferring the seedling to the test medium, or by sowing the seeds individually in an ordered array on the test medium. Plants on petri dishes were grown at 23° to 25°C at 80- to 120-μE illumination. Plants were grown to maturity on nonsterile Peat-Lite soil mixture (W. R. Grace, Inc.). The soil mix was hydrated with reverse-osmosis purified water or tryptophan in water, and plants were watered by subirrigation with water twice a week. Tryptophan (50 μ*M*) was included in the water once every 2 weeks when appropriate. Plants in soil mix were grown at 23° to 25°C in 80 percent relative humidity or at ambient room humidity and temperature with 80- to 100-μE illumination. All plants were grown under constant illumination [5MA, Pfaltz and Bauer, Inc., Waterbury, CT; indole and L-tryptophan, Sigma Chemical].
18. The selection for 5MA-resistant mutants is not absolute and requires some explanation. The growth of wild type is only partially inhibited so that the 5MA-resistant mutants must be identified as plants that grow above the canopy of wild type. The wild-type background growth varied from plate to plate. One of the most important variables in the amount of background growth of plants was the density of seeds plated. In regions of high local seed density, a high percentage of wild-type plants escaped the selection (escapers). Plates with low seed density also contained escaper plants after 3 weeks or more. M_2 plants were chosen for propagation and progeny testing only if they were significantly more healthy than neighboring plants. The *trp1-1* M_2 plant had large roots and showed good true leaf growth at 18 days after plating the M_2 seeds.
19. Surface-sterilized wild-type or *trp1-1* M_4 seeds were grown at 24°C for 3 days on nutrient medium containing 50 μ*M* tryptophan. Twenty-five of each type of seedling were moved to minimal or tryptophan medium and assayed for root growth by the method of Estelle and Somerville (*16*). Four days after transfer, *trp1-1* seedlings grown on minimal medium had roots of 7.2 ±1.6 mm (SD), whereas wild-type roots were 22 ± 4.6 mm long. Wild-type and mutant seedlings grown with tryptophan both had roots of 12 ± 2.6 mm. The *trp1-1* plants growing without tryptophan failed to make true leaves and showed little further root growth under these conditions.
20. The nutritional requirement of M_4 seeds of the tryptophan requiring line was not satisfied when the following nutrients were added to sterile nutrient agar: 50 μ*M* phenylalanine, 50 μ*M* tyrosine, or a mixture of the two; a mixture of 250 μ*M* isoleucine and valine plus 62 μ*M* leucine, and histidine; a mixture of 500 μ*M* lysine and 250 μ*M* threonine and arginine plus 125 μ*M* methionine; a vitamin mixture containing 10 μ*M* biotin, 280 μ*M* *myo*-inositol, 20 μ*M* nicotinic acid, 20 μ*M* *p*-aminobenzoic acid, 12 μ*M* pyridoxine-HCl, and 1.5 μ*M* thiamine; 10 μ*M* gibberellic acid (GA_3); 0.25 μ*M* naphthaleneacetic acid (NAA); 1 or 20 n*M* 2,4-dichlorophenoxyacetic acid (2,4-D); 1, 10, 100, or 500 n*M* IAA.
21. M. Singh and J. M. Widholm, *Biochem. Genet.* **13**, 357 (1975).
22. A 200-mg sample of 2-month-old *trp1-1* mutant was homogenized in 500 μl of 10 m*M* tris-HCl, *p*H 7.5, in a small, ground-glass tissue homogenizer. The solution was clarified by centrifugation and the supernatant was extracted into an equal

volume of ethyl acetate. The ethyl acetate was removed at reduced pressure and resuspended in 22 μl of H_2O. A 5-μl sample was mixed with an equal volume of 1 mg of β-glucosidase (Sigma, almond type I) per milliliter of distilled H_2O, and the mixture was incubated for 5 minutes at 37°C. Paper chromatography developed in a mixture of water-saturated 1-butanol and glacial acetic acid (95:5), or 1-butanol saturated with 3 percent NH_4OH in water (*21*), showed that, after 5 minutes of β-glucosidase treatment, most or all of the anthranilate β-glucoside fluorescence was converted to a species that comigrated with anthranilate. When it was desirable to assay anthranilate as well as the β-glucoside the ethyl acetate extractions were done in 50 m*M* sodium citrate, *p*H 4.5.

23. O. H. Smith and C. Yanofsky, *Methods Enzymol.* **5**, 794 (1962).
24. There is a maize mutant that is fluorescent blue under ultraviolet light and accumulates large quantities of anthranilate β-glucoside as well as smaller amounts of anthranilate. This mutant does not require tryptophan (*21*).
25. All six adult Trp^- F_2 progeny tested contained a blue fluorescent compound (R_F = 0.6) that was converted by β-glucosidase treatment to a compound that was chromatographically identical to anthranilate.
26. All four of the *A. thaliana* tryptophan auxotrophs isolated by 5MA selection show increased bushiness and smaller size compared to wild-type plants, but the morphological changes are less pronounced in the other three mutants (R. Last and G. Fink, in preparation). The common phenotypes of all Trp^- strains might result from the inability of these mutants to synthesize tryptophan. Only the *trp1-1* mutant accumulates high levels of blue fluorescent compounds, and has abnormally small, sterile flowers. It is not known whether the differences in specific properties or variations in the degree of phenotypic severity in the mutants are due to differences in leakiness of the alleles or are specific to the affected steps.
27. Wild-type and *trp1-1* mutant plants were grown in nonsterile Peat Lite soil mixture supplemented with 50, 100, or 200 μ*M* tryptophan. The plants were watered twice a week, and tryptophan was added to the water every 2 weeks. Every 2 days half of the individuals were sprayed with water and the other half were sprayed with a 300 μ*M* tryptophan solution. Under these conditions the tryptophan spray had no effect on the growth rate or morphology of the mutant plants, while the tryptophan spray of the wild-type plants grown on 200 μ*M* tryptophan caused marked growth inhibition.
28. Growth of *trp1-1* seedlings was inhibited when germinated on sterile agar medium containing 25 μ*M* phenylalanine, tyrosine, and tryptophan as compared with the growth of seeds on tryptophan alone. True leaf development was severely inhibited by this mixture, perhaps due to competition for a common uptake system. A similar, but less pronounced effect, was observed when 50 μ*M* amino acids were used.
29. J. Browse, P. McCourt, C. R. Somerville, *Science* **227**, 763 (1985); T. Caspar, S. C. Huber, C. Somerville, *Plant Physiol.* **79**, 11 (1985).
30. Tryptophan biosynthetic enzymes were assayed by modification of the method of Widholm [*Biochim. Biophys. Acta* **320**, 217 (1973)]. All steps in the preparation of plant and bacterial extracts were done at 4°C. Plant extracts were prepared by grinding 2 g of plant material to a paste in a mortar and pestle containing 2 ml of grinding buffer [200 m*M* tris-HCl, *p*H 7.5, 0.2 m*M* EDTA, 8 m*M* $MgCl_2$, 0.2 m*M* dithiothreitol (DTT), and 60 percent glycerol], 200 mg of 0.1-μm glass beads, and 200 mg of polyvinylpolypyrrolidone. The suspension was cleared by centrifugation for 10 minutes at 10,000 rpm (SS-34 rotor). A 2.5-ml portion of the supernatant was loaded on a NAP-25 column (Sephadex G-25; Pharmacia) that had been equilibrated in column buffer (50 m*M* tris-HCl, *p*H 7.5, 0.05 m*M* EDTA, 2 m*M* $MgCl_2$, 0.05 m*M* DTT, and 5 percent glycerol). Bacterial extracts were prepared by a modification of the method of Smith and Yanofsky (*23*). Fresh *E. coli* cells (2 g) from an overnight culture grown under tryptophan limitation were washed once in 0.9 percent saline and treated for 30 minutes with 3.0 mg of lysozyme in 5 ml of 100 m*M* sodium phosphate buffer, *p*H 7.8, containing 5 m*M* EDTA. The bacterial suspension was then sonicated with six pulses of 10 seconds each, and the extract was cleared by centrifugation for 30 minutes at 40,000 rpm (70 Ti rotor). A 2.5-ml sample of the supernatant was subjected to chromatography on a NAP-25 column that had been equilibrated in 100 m*M* phosphate buffer, *p*H 7.8. Anthranilate synthase activity (E.C. 4.1.3.27) was measured as the chorismate-dependent production of anthranilate in a 2.0-ml reaction containing 1.0 ml of column buffer, 0.1 μmol chorismic acid, 20 μmol glutamine, 2.0 μmol $MgCl_2$, and 25 μmol tris-HCl, *p*H 8.0. The reaction was started by addition of plant extract to the reaction mixture and terminated by addition of 0.2 ml of 1.0*M* HCl. The anthranilate produced was then extracted into 2.0 ml of ethyl acetate, and the extraction mixture was clarified by low-speed centrifugation at room temperature. The anthranilate produced was quantified with a Perkin-Elmer 650-10S fluorescence spectrophotometer (excitation 340 nm, emission 400 nm) with anthranilate solutions as standards. These conditions yielded linear production of anthranilate when 25 to 50 μl of plant extract was used in 15- to 30-minute reactions. Anthranilate PR-transferase (E.C. 2.4.2.18) and PR-anthranilate isomerase were assayed by monitoring the 5-phosphorylribose 1-pyrophosphate (PRPP)–dependent disappearance of anthranilate. The assays were performed in the presence of *E. coli* mutant extracts lacking the activity to be measured, but otherwise able to convert anthranilate to the nonfluorescent compound indole-3-glycerol phosphate (made from *E. coli* strains containing *trpD9923* and *trpC9830* for transferase and isomerase assays, respectively). The reaction contained 8 nmol of anthranilate, 100 nmol of PRPP, 2 μmol of $MgCl_2$, 25 μmol of tris-HCl, *p*H 8.0, 25 or 50 μl of plant extract, and 50 μl of bacterial extract in a final volume of 1.1 ml. The reactions were run at 30°C for 15 or 30 minutes. Under these conditions the rate of disappearance of anthranilate was linear with respect to time and amount of plant extract, and the plant extract appeared to be limiting for the enzyme activity being measured. Mixing equal volumes of wild-type and mutant extracts produced wild-type transferase activity [anthranilate, chorismate (barium salt), PRPP (sodium salt), and polyvinylpolypyrrolidone were from Sigma].
31. J. F. G. M. Winterman and A. Demots, *Biochim. Biophys. Acta* **109**, 448 (1965).
32. We are grateful to Charles Yanofsky for *E. coli* strains, for suggesting the use of 5MA for selection of tryptophan auxotrophs and for providing advice and information on the lore of the tryptophan pathway; Mike Christman, Marc Learned, Peter McCourt, and Barbara Ruskin for critical reading of the manuscript. Supported by NSF grant DCB-8416894 (G.R.F.) and NSF postdoctoral research fellowship DCB-8508804 (R.L.L.).

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