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Tryptophan Mutants in *Arabidopsis*: The Consequences of Duplicated Tryptophan Synthase β Genes

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The cruciferous plant *Arabidopsis thaliana* has two closely related, nonallelic tryptophan synthase β genes (*TSB1* and *TSB2*), each containing four introns and a chloroplast leader sequence. Both genes are transcribed, although *TSB1* produces >90% of tryptophan synthase β mRNA in leaf tissue. A tryptophan-requiring mutant, *trp2-1*, has been identified that has about 10% of the wild-type tryptophan synthase β activity. The *trp2-1* mutation is complemented by the *TSB1* transgene and is linked genetically to a polymorphism in the *TSB1* gene, strongly suggesting that *trp2-1* is a mutation in *TSB1*. The *trp2-1* mutants are conditional: they require tryptophan for growth under standard illumination but not under very low light conditions. Presumably, under low light the poorly expressed gene, *TSB2*, is capable of supporting growth. Genetic redundancy may be common to many aromatic amino acid biosynthetic enzymes in plants because mutants defective in two other genes (*TRP1* and *TRP3*) also exhibit a conditional tryptophan auxotrophy. The existence of two tryptophan pathways has important consequences for tissue-specific regulation of amino acid and secondary metabolite biosynthesis.

INTRODUCTION

Plants synthesize amino acids, purines, and pyrimidines, yet plant auxotrophs are extremely rare. Recently, several tryptophan auxotrophs of *Arabidopsis thaliana* were uncovered (Last and Fink, 1988). One of the auxotrophic mutants (*trp1-1*) appeared to lack the enzymatic activity corresponding to the second step in the tryptophan pathway, the anthranilate phosphoribosyl (PR)-transferase. The *trp1-1* mutant was characterized extensively because it had an easily scorable fluorescence phenotype due to the accumulation of anthranilic acid derivatives.

Why was it possible to obtain tryptophan auxotrophs in *A. thaliana* and not in other plants? The use of a positive selection facilitated the identification of rare Trp^- plants. However, other explanations for the absence of amino acid auxotrophs must be considered, including inviability of absolutely auxotrophic plants and genetic redundancy for amino acid biosynthetic enzyme genes. Genetic redundancy, a common feature of eukaryotic genomes, can confound the search for recessive auxotrophic mutations because the presence of multiple isozymes for the same biosynthetic reaction would require multiple mutations to produce the auxotrophic phenotype. Although the existence of auxotrophic mutations in *A. thaliana* suggested that the biosynthetic genes in this species were present in single copy, we needed to test this suggestion by direct hybridization studies. The availability of a clone for the *A. thaliana* tryptophan synthase β subunit, which catalyzes the final step in tryptophan biosynthesis (*TSB1*, see Berlyn et al., 1989), encouraged us to characterize additional tryptophan auxotrophs with the hope of identifying a tryptophan synthase β mutant and estimating gene copy number.

In this report we show that *trp2-1* is a tryptophan synthase β-deficient tryptophan auxotroph of *A. thaliana*. That *trp2-1* is a mutation in *TSB1*, the previously identified tryptophan synthase β structural gene, is shown by genetic complementation in transgenic plants and restriction fragment length polymorphism (RFLP) mapping. Surprisingly, there is a second tryptophan synthase gene, *TSB2*, unlinked to *TSB1*. The duplication of biosynthetic genes in *A. thaliana* has important evolutionary, functional, and practical implications for plant molecular genetics.

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RESULTS

There Are Two Tryptophan Synthase β Genes in *A. thaliana*

Previous DNA gel blot analysis showed that *TSB1* hybridized to two genomic fragments (Berlyn et al., 1989), one representing *TSB1* and a second unidentified segment. [The original and newly isolated genes will be referred to as *TSB1* and *TSB2* (tryptophan synthase β subunit), respectively, to conform with standard *A. thaliana* genetic nomenclature.] The second tryptophan synthase β gene was isolated with the *TSB1* gene as a probe, and, as shown in Figure 1, hybridized to both *TSB1* and *TSB2*. The 3′ half of the second gene was isolated by screening a size-fractionated genomic sublibrary of EcoRI-digested *A. thaliana* DNA with the original tryptophan synthase β gene as hybridization probe. The rest of the second gene was isolated by screening a λEMBL3 genomic library with the 2.5-kb EcoRI fragment as probe.

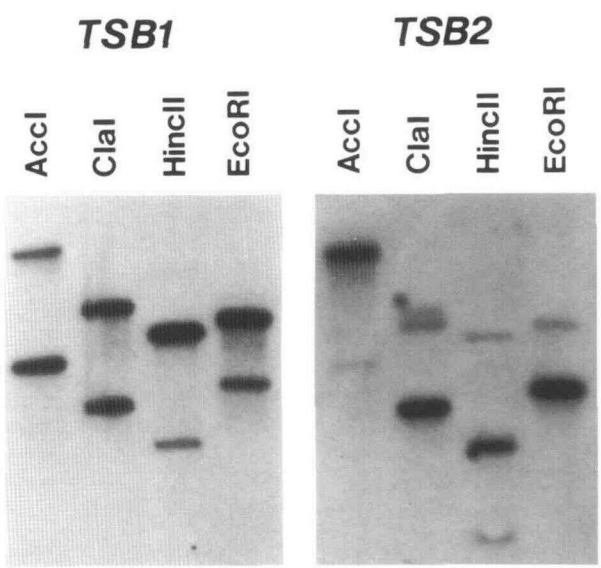


Figure 1. Genomic DNA Gel Blot Hybridization with the Two Tryptophan Synthase β Genes.

Genomic DNA of *A. thaliana* was digested with the restriction endonuclease indicated above each lane, and duplicate gels were blotted to Zeta Probe (Bio-Rad). Hybridization was done overnight at 65°C in buffer containing 225 mM NaCl, 1% SDS, 10% dextran sulfate, and 0.5 mg/mL herring sperm DNA, and the most stringent wash was performed at 50°C in 15 mM NaCl, 1.5 mM sodium citrate, and 1% SDS buffer. Left, hybridization was done with a *TSB1* probe [the 0.8-kb SacI-XhoI fragment from the cDNA clone pMBT3 (Berlyn et al., 1989)]. Right, the *TSB2* genomic DNA included in clone pRLT1 (contained on a 2.5-kb EcoRI fragment that includes exons three through five) was used as a hybridization probe.

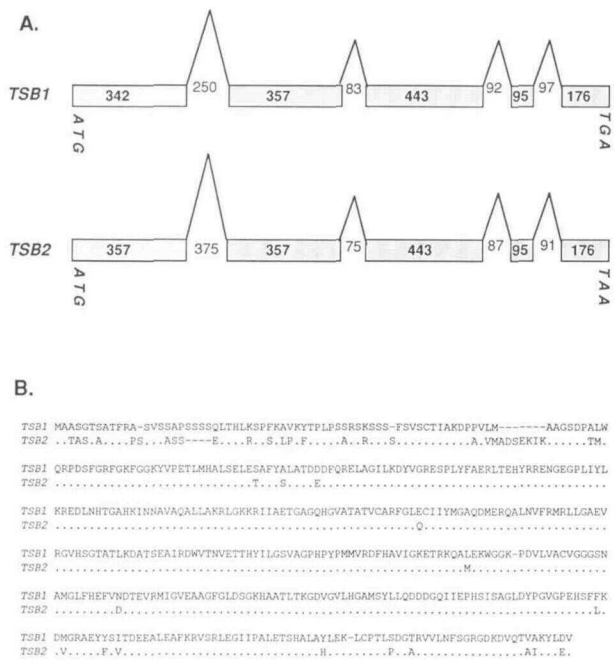


Figure 2. Structures of the Two Tryptophan Synthase β Protein Coding Regions.

(A) Positions and sizes of exons and introns. Exons are shown as boxes and introns as thin lines with their relative positions and sizes (in nucleotides) indicated.

(B) Comparison of amino acid sequences of *TSB1* (GenBank accession no. M23872) and *TSB2* (GenBank accession no. M62824). The entire *TSB1* coding sequence is shown, in one-letter code. Dots in the *TSB2* sequence indicate identity with the *TSB1* sequence, and dashes indicate where gaps were used to maximize sequence similarity. The first two lines represent the relatively divergent amino-terminal extension absent in microbial tryptophan synthase β genes.

DNA sequence analysis of the coding region of the genomic clone indicated that it contains a second tryptophan synthase β gene (*TSB2*, GenBank accession no. M62824). Figure 2A shows that the exon and intron structures of these loci are virtually identical. Comparison of *TSB1* and *TSB2* DNA sequences reveals >95% amino acid (Figure 2B) and 85% nucleotide identity in the region homologous to microbial tryptophan synthase β subunit genes. The presumed chloroplast target sequences at the amino termini show reduced, but significant, amino acid sequence similarity. There is no DNA sequence similarity among the four introns seen beyond the 5′ splice site conserved elements, canonical AG dinucleotide at the 3′ splice site, and an overall high A+T composition. The sequences of the transcribed, untranslated regions are highly divergent.

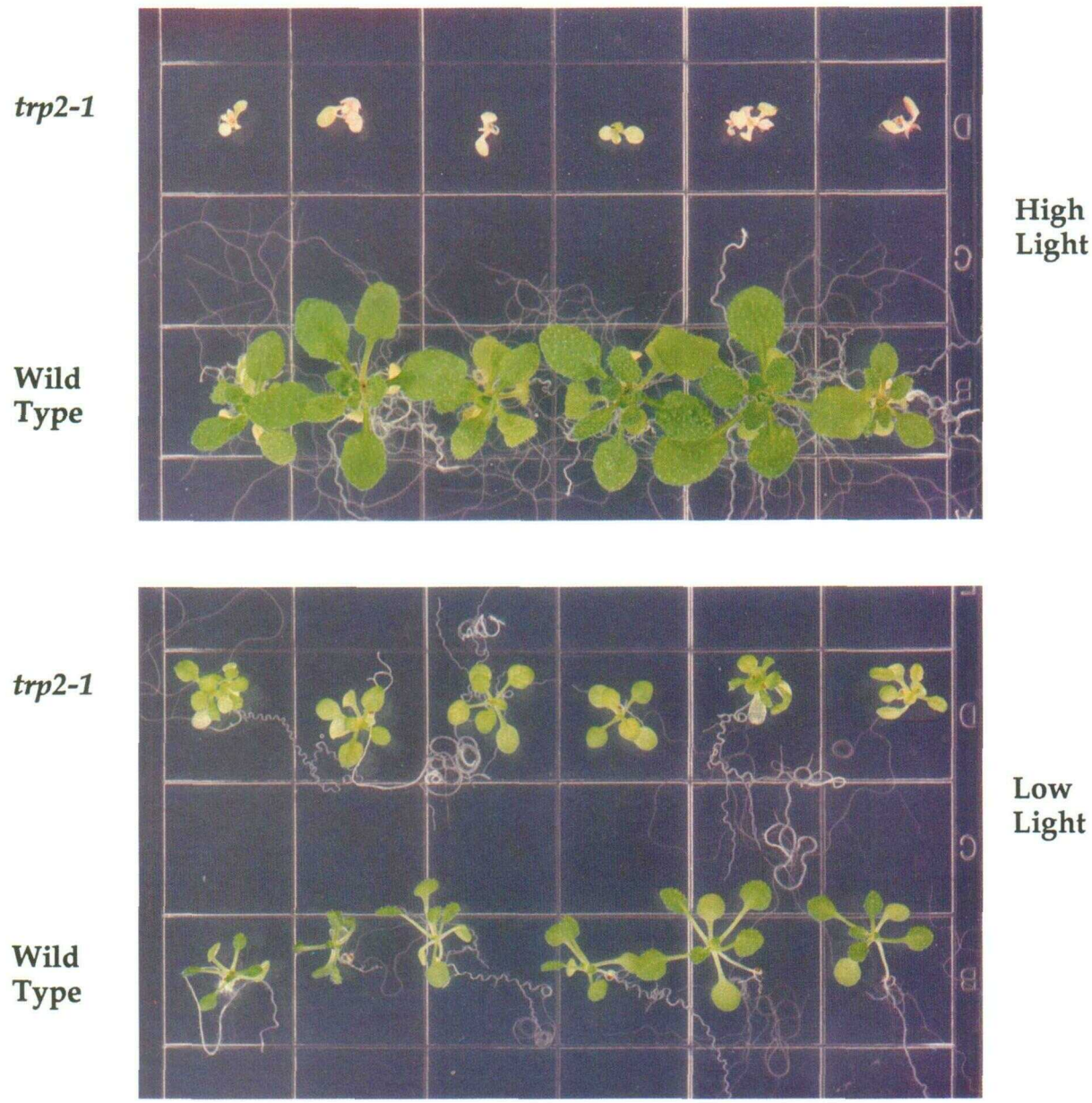


Figure 3. The *trp2-1* Mutant Has a Light-Level Conditional Phenotype.

The two panels represent the phenotypes under high light (top) or very low light (bottom). Both panels show plants grown under sterile conditions in the absence of tryptophan. When grown under normal culture conditions (High Light), the mutant seeds germinate but do not form green true leaves or roots. In contrast, sibling plants can grow without tryptophan under very low photosynthetically active light levels. This residual tryptophan biosynthesis detected in the slowly growing plants under low light is believed to result from the low-level expression of the *TSB2* gene.

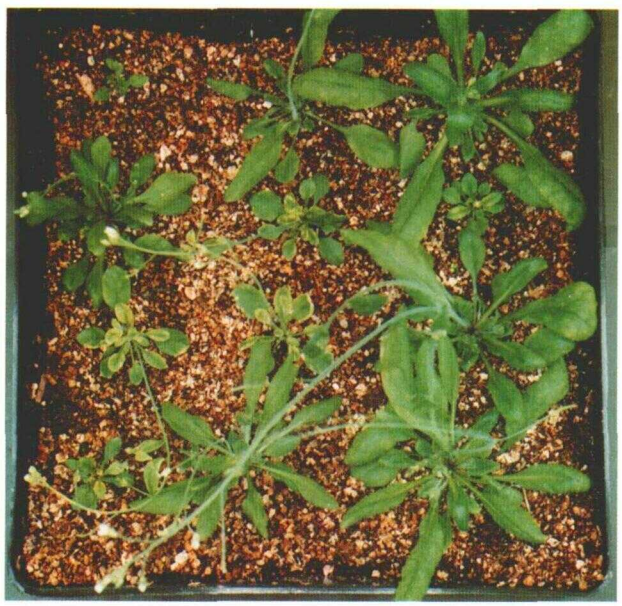


Figure 4. Morphological Characteristics of the *trp2-1* Mutant.

Seeds segregating for the Trp$^-$ phenotype (derived by self-fertilization of a *TRP2/trp2-1* heterozygote) were grown under sterile conditions on medium containing 50 μM tryptophan for 10 days and transferred to a nonsterile soil mixture hydrated with 50 μM tryptophan and grown for 4 more weeks. Note the small size, rounded leaf morphology, and light-green color of the six Trp$^-$ plants.

trp2-1 Is a Recessive Mutation Affecting Tryptophan Synthase Function

The *trp2-1* tryptophan auxotrophic mutant was identified as resistant to 5-methylanthranilic acid + tryptophan (Last and Fink, 1988). Some aspects of the *trp2-1* phenotype are similar to those of the previously described *trp1-1* mutant (Last and Fink, 1988). Figure 3 (top) shows that seeds homozygous for *trp2-1* fail to develop true leaves and roots when germinated on agar medium without tryptophan. As compared with wild-type plants, mature *trp2-1* plants grow much more slowly in soil mix containing 50 μM to 200 μM tryptophan and have altered leaf morphology and coloration, as shown in Figure 4. Although mature *trp2-1* plants are more bushy than the wild type, they are larger and less bushy than *trp1-1* plants and have normal fertility. Whereas isogenic *trp1-1* and Columbia wild-type plants produce dark-brown seeds, a *trp2-1/trp2-1* plant produces yellow-green mature seeds early in its development and light-brown seeds in older plants.

Growth experiments suggested that *trp2-1* might be a mutation in a gene encoding the tryptophan synthase β subunit. As shown in Figure 5, tryptophan synthase β is required to convert indole to tryptophan. Therefore, tryptophan synthase β mutants should fail to grow on indole as a precursor of tryptophan, whereas mutants defective in earlier steps should grow on indole in place of tryptophan (Last and Fink, 1988; R. Last, unpublished results). Tryptophan synthase β mutants should also be resistant to 5-fluoroindole because they are unable to convert it into the toxic fluorotryptophan analog. Consistent with a tryptophan synthase β defect, *trp2-1/trp2-1* plants failed to grow on minimal nutrient medium supplemented with 50 μM indole and are resistant to 5-fluoroindole in the presence of tryptophan. Unlike *trp1-1* plants, *trp2-1* does not accumulate fluorescent anthranilate compounds. Extracts prepared from *trp2-1* produce a pink color in response to $FeCl_3$-sulfuric acid reagent, suggesting the accumulation of one or more indolic compounds (Yanofsky, 1956). These results are consistent with a defect in tryptophan synthase β subunit activity in the *trp2-1* mutant.

Dominance was tested by scoring the phenotype of the F_1 progeny from crosses in which pollen from the original M_3 Trp$^-$ line (*trp2-1/trp2-1*) was used to fertilize a Trp$^+$ line of the *A. thaliana* Columbia ecotype (Col-0). Table 1 shows that these F_1 progeny had a Trp$^+$ phenotype on sterile minimal medium, suggesting that the *trp2-1* mutation is recessive to *TRP2*$^+$. Approximately one-quarter of the F_2 progeny derived by self-pollination of heterozygous (*TRP2/trp2-1*) plants appeared to be homozygous for *trp2-1* (failed to grow vigorously after germination on sterile minimal medium). This result is consistent with a monogenic, recessive trait conferring tryptophan auxotrophy. Segregation of the Trp$^-$ phenotype persisted through six consecutive outcrosses to Trp$^+$ Columbia ecotype plants.

A complementation test showed that the *trp2-1* mutation is functionally distinct from *trp1-1*. As shown in Table 2, pollen from a *trp1-1/trp1-1* plant crossed with a *trp2-1/trp2-1* individual yielded F_1 progeny that grew normally on sterile medium lacking tryptophan. The F_1 plants (*TRP1/trp1-1*; *TRP2/trp2-1*) were self-pollinated, and the resultant F_2 seeds were germinated on sterile nutrient medium lacking tryptophan to determine whether the two muta-

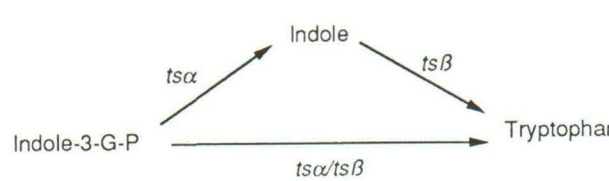


Figure 5. The Reactions That Convert Indole Glycerol-Phosphate to Tryptophan in Plants.

Tryptophan synthase [L-serine hydrolyase (adding indoleglycerol phosphate); EC 4.2.1.20] catalyzes conversion of indoleglycerol phosphate to tryptophan, the final step in tryptophan biosynthesis. The wild-type enzyme consists of two functional domains: the α subunit (TSα) catalyzes the conversion of indoleglycerol phosphate to indole, whereas the β subunit (TSβ) catalyzes the conversion of indole plus serine to tryptophan.

Table 1. Results of Crosses with the *trp2-1* Mutant

Cross[a]	Type	Total	Trp$^+$	Trp$^-$	Trp$^-$ (%)	χ^{2b}
trp2-1/trp2-1 × *TRP2/TRP2*	F_1	25	25	0	0	
TRP2/trp2-1 × *TRP2/trp2-1*	F_2	864	662	202[c]	23.4	1.2[d]

[a] Pollen from the M_3 *trp2-1* mutant was crossed with a Trp$^+$ strain homozygous for the recessive tricome-deficient *gl-1* trait to yield a pubescent F_1 strain, indicating that the cross was successful. Several F_1 plants were allowed to self-pollinate to yield F_2 progeny.
[b] The χ^2 value was calculated on the basis of the expectation of 25% Trp$^-$ progeny.
[c] The F_3 progeny from the putative Trp$^-$ F_2 plants required tryptophan to grow, verifying that the F_2 plants were tryptophan auxotrophs.
[d] $P > 0.05$.

tions were linked. Three phenotypic classes were observed among the F_2 progeny (Table 2): vigorous growth (Trp$^+$), poor growth without blue fluorescence (*trp2-1/trp2-1* single homozygous mutants), and blue fluorescence with concomitant poor growth (*trp1-1/trp1-1* single homozygous mutants and the *trp1-1/trp1-1;trp2-1/trp2-1* double mutant). The 9:3:4 phenotypic ratio of these classes indicates that the *trp1-1* and *trp2-1* mutations are unlinked.

trp2-1 Is Deficient in Tryptophan Synthase β Activity

Direct assays of tryptophan biosynthetic enzyme activity in leaf extracts confirmed that *trp2-1* homozygotes contained reduced levels of tryptophan synthase β activity. Table 3 presents the results of assays for four enzymes in the pathway. Tryptophan synthase β activity, assayed as the pyridoxal phosphate-dependent disappearance of indole, from *trp2-1* homozygous plants is ~15% of that of wild type. Similar results, shown in Figure 6A, were obtained by monitoring the production of radioactive tryptophan from indole by incorporation of radioisotopically labeled serine. The defect in activity was not caused by the presence of an inhibitor in the mutant extracts because wild-type synthase activity was not inhibited by addition of mutant extract (data not shown). The reduction of tryptophan synthase β activity was not due to a general regulatory defect because the *trp2-1* mutant had activity equal to or greater than that of wild type for the first three enzymes in the pathway (Table 3). Figure 6B shows that more than 98% of the detectable tryptophan synthase β activity in Columbia wild-type plants was in the chloroplast fraction, consistent with the presence of a presumed chloroplast target sequence at the amino terminus of each protein coding region. Although this procedure enriches for chloroplasts, the presence of other organelles in the chloroplast fraction cannot be excluded.

In Vivo Evidence for *trp2-1* Residual Tryptophan Synthase β Activity

A conditional tryptophan requirement was observed for *trp2-1* plants, establishing an in vivo significance for the residual enzyme activity observed for *trp2-1*. Under light conditions that promote rapid growth of wild-type plants [photosynthetically active radiation (PAR) fluences of 75 to 225 μmol photons$\cdot m^{-2}\cdot sec^{-1}$], *trp2-1* mutants had an absolute tryptophan requirement (Figure 3, top). However, when propagated under conditions of reduced light levels that dramatically reduced the growth rate of wild type (15 to 25 μmol PAR$\cdot m^{-2}\cdot sec^{-1}$), *trp2-1* lost its tryptophan dependence (Figure 3, bottom). The reduced tryptophan biosynthetic activity in *trp2-1* plants was adequate to sustain the mutant under very low light growth conditions but not under high light. The *trp1* and *trp3* auxotrophic mutants described earlier (Last and Fink, 1988) also exhibited a light-conditional tryptophan requirement (R. Last, unpublished observations).

The Two Tryptophan Synthase β Structural Genes Are Expressed at Different Levels

The simplest explanation for the properties of *trp2-1* is that it has a mutation in one of the two tryptophan synthase genes, either *TSB1* or *TSB2*, and the residual activity of the mutant is due to continued expression of the other, unmutated gene. This explanation requires that both genes be expressed but that one be expressed at a much higher level than the other. To test this hypothesis, the mRNA level for each *TSB* gene was measured in a quantitative S1 nuclease protection assay. The probes employed discriminate between mRNAs from the two genes because they include upstream nontranslated regions that have highly divergent nucleotide sequences. These probes also allow mapping of the *TSB* mRNA 5′ ends. In leaf tissue from plants grown at 100 μmol PAR$\cdot m^{-2}\cdot sec^{-1}$, the steady-state level of *TSB1* mRNA was at least 10-fold higher than that of *TSB2* mRNA, as shown in Figure 7A. These quantitative results are supported by S1 nuclease protection experiments with gene-specific oligonucleotide probes and from primer extension experiments employing *TSB* gene-specific primers (R. Last, data not shown). The great difference in levels of expression of the two genes (about 10-fold) correlates well with the reduction in tryp-

Table 2. Results of Crosses between *trp1-1* and *trp2-1*

Cross[a]	Type	Total	Trp$^+$	Trp$^-$[b]		χ^{2c}
				Blue Fluo	Nonfluo	
trp1-1/trp1-1 × *trp2-1/trp2-1*	F_1	142	142	0	0	
trp1-1/TRP1;trp2-1/TRP2 × *trp1-1/TRP1;trp2-1/TRP2*	F_2	233	137	45	51	4.3[d]

[a] Pollen from a *trp1-1* mutant was crossed with a *trp2-1* strain to yield the F_1 seed. Several F_1 plants were allowed to self-pollinate to yield F_2 progeny.
[b] "Blue fluo" indicates blue-fluorescent, Trp$^-$ phenotypic progeny (homozygous for *trp1-1* and unknown *TRP2* genotype); "nonfluo" describes nonfluorescent Trp$^-$ plants (homozygous for *trp2-1* and either heterozygous or homozygous wild-type for *TRP1*).
[c] The χ^2 value was calculated on the basis of the expectation of 9/16 Trp$^+$, 3/16 nonblue-fluorescent, Trp$^-$ and 4/16 blue fluorescent, Trp$^-$ progeny (9:3:3:1).
[d] $P > 0.05$.

tophan synthase activity in *trp2-1*, suggesting that *trp2-1* might be a *TSB1* structural gene mutation.

The level of neither *TSB* mRNA is altered by growth under very low light levels (15 μmol PAR $\cdot$ m^{-2} $\cdot$ sec^{-1}, Figure 7B). The absence of an effect of light suggests that the loss of the tryptophan requirement of *trp2-1* plants in low light is not due to differences in mRNA accumulation of *TSB* genes in response to light intensity. We have not determined whether there are developmental or tissue-specific differences in *TSB* gene expression.

trp2-1 Is a Mutation in the More Highly Expressed Tryptophan Synthase β Gene (*TSB1*)

Two lines of evidence demonstrate that *trp2-1* is a *TSB1* structural gene mutation: the wild-type *TSB1* gene complements the *trp2-1* mutation, and the cloned *TSB1* gene maps genetically to the site of the *trp2-1* mutation. Complementation of *trp2-1* was observed when a *trp2-1* mutant strain was transformed with pBINTSB1, a binary *Agrobacterium* plasmid that contains the *TSB1* gene and flanking DNA on a 4.2-kb EcoRI fragment (Berlyn et al., 1989) in plasmid pBin19 (Bevan, 1984). Several transformants (R_1, or first regenerated generation) were obtained by selecting for shoot regeneration on kanamycin medium. Figure 8 shows the result of a genomic DNA gel blot hybridization experiment demonstrating that *TSB1* DNA has integrated at a single ectopic location in the *A. thaliana* genome in this transgenic line. Consistent with the Trp$^+$ phenotype, assay of tryptophan synthase β activity in extracts from this Trp$^+$ regenerant indicated that the transformant had increased levels of tryptophan synthase β activity, as shown in Figure 6C.

An RFLP linked to the cloned *TSB1* gene was used to test for linkage of *TSB1* to *trp2-1*. A *TSB1* locus RFLP was revealed by DNA gel blot hybridization of BglII-digested genomic DNA from Columbia (the genetic background of the Trp$^-$ mutation, designated *trp2-1*$^{COL-0}$) and a Trp$^+$ line of different genetic background, the AA-0 ecotype (*TRP2*$^{AA-0}$). The segregation of this polymorphism was followed by crossing pollen from the *TRP2*$^{AA-0}$/*TRP2*$^{AA-0}$ ecotype to *trp2-1*$^{COL-0}$/*trp2-1*$^{COL-0}$ plants, and pollen from the Trp$^+$ F_1 progeny (*TRP2*$^{AA-0}$/*trp2-1*$^{COL-0}$) was testcrossed to the original *trp2-1*$^{COL-0}$/*trp2-1*$^{COL-0}$ mutant. Many of the T_1 testcross plants were sickly and could not be tested for their phenotype (see subsequent section); only healthy plants that were clearly Trp$^+$ were analyzed for linkage.

Eighteen of the healthy Trp$^+$ testcross progeny plants that were self-pollinated showed Trp$^-$ segregating among their T_2 progeny. These T_1 Trp$^+$ testcross progeny plants were, therefore, *TRP2/trp2-1*. Among the progeny of these 18 heterozygotes, true breeding Trp$^+$ and Trp$^-$ T_2 plants were identified by self-crossing and examining the Trp phenotype of T_3 progeny. T_3 plants were then analyzed for the RFLP segregation. If *trp2-1* were a mutation in the *TSB1* gene, the expectation would be that Trp$^-$ T_3 progeny (*trp2-1*$^{COL-0}$/*trp2-1*$^{COL-0}$) should have only the Columbia polymorphism, whereas Trp$^+$ progeny (*TRP2*$^{AA-0}$/*trp2-1*$^{COL-0}$ or *TRP2*$^{AA-0}$/*TRP2*$^{AA-0}$) should have at least one chromosome bearing the AA-0 polymorphism at *TSB1*. All 18 Trp$^-$ lines showed only the Columbia polymorphism, as is shown for a representative sample of segregants in Figure 9. In contrast, all 18 Trp$^+$ lines have at least one AA-0 polymorphic fragment. There is no evidence for recombination between the *trp2-1* mutation and the *TSB1* polymorphism, a result consistent with the hypothesis that *trp2-1* is a mutation in this tryptophan synthase β subunit gene.

Mapping studies, summarized in Figure 10, indicate that *TSB1* (*TRP2*) and *TSB2* are unlinked because the two genes are on different chromosomes. *TSB1* is located at 90 map units (mu) on the Goodman Laboratory RFLP map (S. Hanley and H. Goodman, personal communication) and at 147 mu on chromosome 5 on the Meyerowitz laboratory RFLP map (S. Kempin and E. Meyerowitz, personal communication). *TSB2* maps at 65 mu on chromosome 4 (S.

Table 3. Specific Activities of Tryptophan Biosynthetic Enzymes

Strain	Anthranilate synthase	Anthranilate transferase	Anthranilate isomerase	Tryptophan synthase-β
Wild type	2.3 ± 0.5	153 ± 33	179 ± 21	715 ± 92
trp2-1/trp2-1	3.6 ± 0.5	187 ± 49	191 ± 30	127 ± 78

Enzymes were assayed from crude plant extracts (see Methods). Specific activities (mean ± SD) are reported in picomoles of anthranilate converted per minute per microgram of chlorophyll *a* + *b* or as picomoles of indole converted per minute per microgram of protein for tryptophan synthase β.

Kempin and E. Meyerowitz, personal communication). Yeast artificial chromosomes (YACs) containing these genes were isolated by hybridization to an *A. thaliana* YAC library (provided by E. Grill and C. Somerville, Michigan State University). A single, previously unmapped YAC clone containing the *TSB1* gene (YAC13-G2) was identified by cross-hybridization to a fragment of the 5′ nontranslated region of the *TSB1* clone. Four YAC clones (YACs 4-C1, 21-3H, 21-31, and 21-41) cross-hybridized with the 3′ half of the *TSB2* gene. YAC4-C1 had previously been placed on chromosome 4 at 65 mu (E. Grill, personal communication).

The AA-0 Ecotype Contains Modifiers of the *trp2-1* Mutation

Several anomalies arose in crosses between the Columbia and AA-0 ecotypes. In the testcross where the Trp^+ F_1 progeny ($TRP2^{AA\text{-}0}/trp2\text{-}1^{COL\text{-}0}$) were crossed to the original $trp2\text{-}1^{COL\text{-}0}/trp2\text{-}1^{COL\text{-}0}$ mutant, one expects equal numbers of Trp^+ and Trp^- testcross (T_1) progeny. We were unable to verify this prediction because many of the testcross progeny grew poorly on medium with or without tryptophan, making it impossible to score the Trp^- phenotype. The poor growth was clearly a result of outcrosses to the AA-0 ecotype because control crosses in which all strains had the Columbia background ($TRP2^{COL\text{-}0}/trp2\text{-}1^{COL\text{-}0}$ × $trp2\text{-}1^{COL\text{-}0}/trp2\text{-}1^{COL\text{-}0}$) yielded robust Trp^+ and Trp^- progeny in a 1:1 ratio. The absence of clear Trp^- progeny from the outcross could result from modifiers of the Trp^- phenotype inherited from the AA-0 parent.

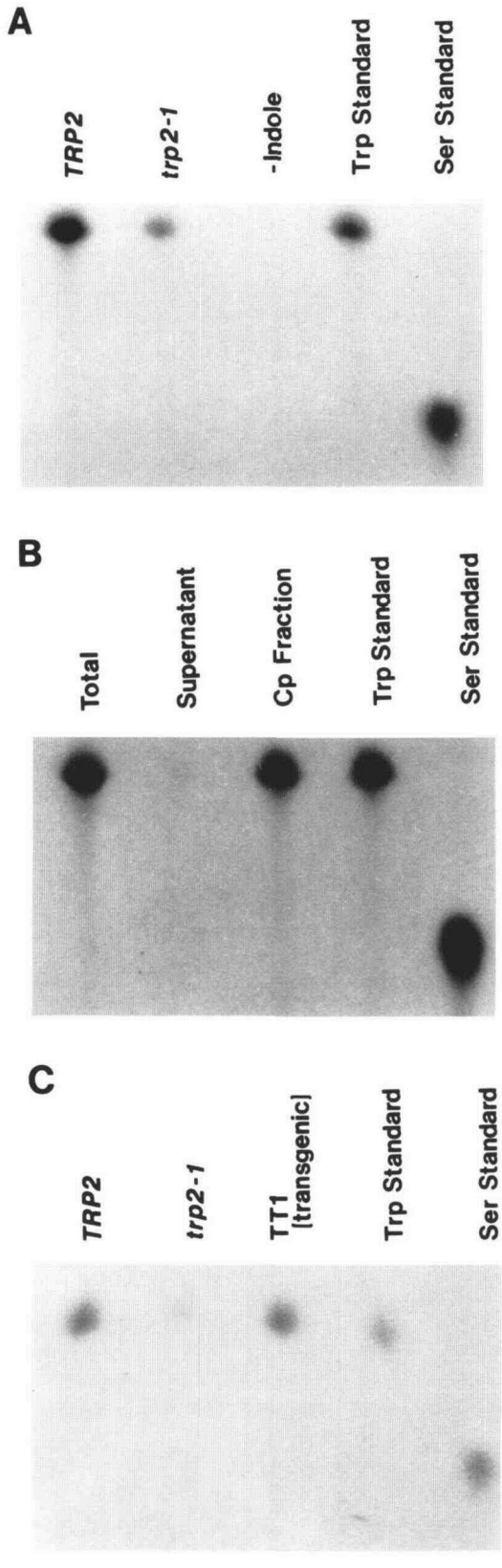
Some of the Trp^+ progeny resulting from the healthy testcross progeny also showed anomalies indicative of modifiers. As indicated in the previous section, a number of the T_2 Trp^+ plants were judged to be homozygous because upon selfing they gave only Trp^+ T_3 progeny; these T_3 plants all should have been $TRP2^{AA\text{-}0}/TRP2^{AA\text{-}0}$. Unexpectedly, the T_3 progeny of half of the putative homozygote Trp^+ T_2 lines, predicted to be homozygous for the AA-0 polymorphism, showed segregation for the polymorphism ($TRP2^{AA\text{-}0}$ and $trp2\text{-}1^{COL\text{-}0}$). Despite the fact that the T_2 plants must have been genotypically heterozygous for the *trp2-1* mutation ($TRP2^{AA\text{-}0}/trp2\text{-}1^{COL\text{-}0}$), none of their T_3 progeny manifested the tryptophan auxotrophy of the *trp2-1* mutation. This result is consistent with the hypothesis that modifiers from the AA-0 genetic background are still segregating in the cross. These modifiers might suppress the Trp^- phenotype or render *trp2-1/trp2-1* T_3 seeds inviable. Further experiments are necessary to understand the basis for the failure of Trp^- to segregate normally in these outcrosses.

DISCUSSION

Two Highly Conserved *TSB* Genes in *A. thaliana*

A. thaliana has two genes encoding the tryptophan synthase β subunit. The two genes were probably derived one from the other by duplication because they share >85% nucleotide and 95% amino acid identity in the catalytically active region of the protein. Both *TSB* genes contain chloroplast transit peptide-like sequences at their amino termini, consistent with the association of the majority of *A. thaliana* tryptophan synthase β activity with the chloroplast fraction (Figure 6B). The lower level of amino acid sequence conservation seen in the presumptive chloroplast transit region is characteristic of other plant aromatic amino acid biosynthetic enzymes [compare the chloroplast transit sequences of 3-*enol*-pyruvoylshikimate-5-phosphate (EPSP) synthase genes from the solanaceous plants petunia and tomato (Gasser et al., 1988)].

Neither the *TSB1* nor *TSB2* gene is adjacent or fused to the α subunit gene, suggesting that the β subunit is synthesized independently of the α subunit (Berlyn et al., 1989). The arrangement in *A. thaliana* is unlike that in the eukaryotes yeast and *Neurospora*, where the two activities reside on a single polypeptide (Zalkin and Yanofsky, 1982; Burns and Yanofsky, 1989). The *GCN4* binding site core 5′-TGACTC-3′ is present upstream from the transcription initiation site of both *TSB* genes, and AP1 and SP1 transcription factor core binding sites are present upstream of the *TSB2* transcribed region. Determining whether these



sequences play any role in the expression of the genes will require further analysis.

trp2-1, a Tryptophan Synthase β-Deficient Mutant of *A. thaliana*

Precursor and inhibitor feeding studies and enzyme assays of leaf extracts (Table 3 and Figure 6A) indicate that this mutant is specifically deficient in tryptophan synthase β subunit activity. Complementation of *trp2-1* to Trp^+ by introduction of a *TSB1* transgene and demonstration that *TSB1* and *trp2-1* are tightly linked provide strong evidence that the *TRP2* locus encodes *TSB1*. The ability to complement *trp2-1* with *TSB1* DNA should be very useful as a system to select for transgenic *A. thaliana* plants and for the analysis of *cis*-acting elements involved in regulation of amino acid biosynthesis in plants.

The analysis of our mutants indicates that the *A. thaliana* tryptophan pathway is probably identical to the well-defined pathway found in microorganisms. Analysis of the *trp2-1* mutant provides direct in vivo evidence in plants that tryptophan synthase β activity is required for tryptophan biosynthesis. This extends the list of enzymes shown to be required for in vivo tryptophan production, including anthranilate phosphoribosyltransferase (EC 2.4.2.18), defective in the *A. thaliana trp1-1* mutant (Last and Fink, 1988), and tryptophan synthase α subunit activity, absent from VIIIB9, a tryptophan requiring cell culture variant of *Hyoscyamus muticus* (Fankhauser et al., 1990).

Influence of Genetic Background on Gene Expression

The difficulty in recovering Trp^- segregants upon crossing the *trp2-1* mutant of Columbia ecotype with $TRP2^+$ of

Figure 6. Autoradiograms Showing Results of Tryptophan Synthase Enzyme Assays.

This assay monitors the production of L-[^{14}C]tryptophan products from L-[3-^{14}C]serine in a crude extract. Lanes containing ^{14}C-tryptophan product (Trp Standard) and serine substrate (Ser Standard) markers are included for comparison in all cases. Thin-layer chromatography migration proceeded from bottom to top.

(A) Comparison of activity in whole-leaf extracts from *trp2-1* mutant and $TRP2^+$ plants. The lanes from left to right are wild-type extract (*TRP2*), mutant extract (*trp2-1*), and wild-type extract without indole substrate (−Indole).

(B) Tryptophan synthase assays from subcellular fractions. The lanes from left to right indicate the extract produced from comparable samples of whole-leaf protoplasts (Total), post-chloroplast supernatant (Supernatant), and chloroplast-enriched fraction (Cp Fraction).

(C) The *TSB1* transgene restores normal activity to *trp2-1*. Whole-leaf extracts were assayed from plants of the following genotypes (from left to right): control (*TRP2*), untransformed (*trp2-1*), and the *trp2-1* mutant containing the *TSB1* transgene (TT1 [transgenic]).

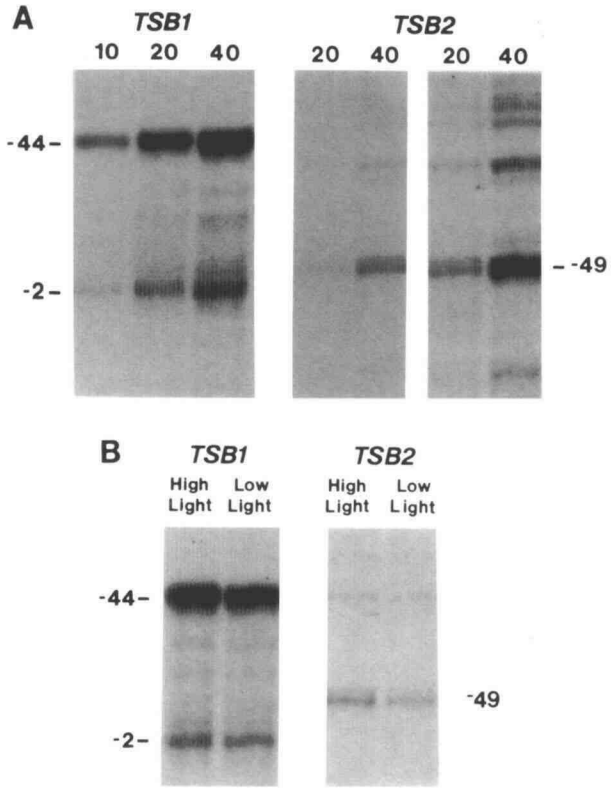


Figure 7. Messenger RNA Levels from the *TSB1* and *TSB2* Genes.

A quantitative S1 nuclease protection assay was employed to distinguish between the highly conserved mRNAs. Uniformly labeled, single-stranded probes extend upstream of the divergent transcribed, nontranslated regions, allowing the analysis of mRNA 5′ ends. The numbers on the sides indicate the positions of the RNA 5′ ends with respect to the first inframe methionine codon.
(A) Comparison of abundance of *TSB1* and *TSB2* transcripts under standard growth conditions. The figure shows an RNA titration experiment using leaf RNA isolated from 3-week-old plants grown under standard light levels (100 μmol of PAR·m^{-2}·sec^{-1}). The right *TSB2* panel is from a fourfold longer exposure than the *TSB1* experiment or the *TSB2* left panel. Quantities of total leaf RNA in micrograms are indicated above the lanes.
(B) Levels of *TSB* transcripts under varying light conditions. RNA samples from plants grown under standard light levels (100 μmol PAR·m^{-2}·sec^{-1}) (High Light, left lanes) or very low light levels (15 μmol PAR·m^{-2} sec^{-1}) (Low Light, right lanes) were assayed from *TSB1* (left panel) or *TSB2* (right panel) transcript levels.

AA-0 ecotype indicates that these geographical races of *A. thaliana* have genetic incompatibilities that influence genetic segregation or gene expression. The observation that plants heterozygous for RFLP markers at *TSB1* behaved as true-breeding Trp^+ plants is consistent with the hypothesis that the AA-0 genetic background either distorts the segregation of *trp2-1* or modifies the penetrance of the Trp^- phenotype. The influence of genetic background upon the expression of auxotrophic mutant phenotypes is a well-documented phenomenon in many organisms. For example, even closely related strains of yeast may differ in a nonMendelian element called ψ; some strains are ψ^+ and some are ψ^-. In the ψ^+ background, some nonsense mutations such as *trp5-48* are suppressed and no longer lead to an auxotrophic requirement (Sherman, 1982). For example, a cross of *trp5-48*ψ^- by *TRP5*ψ^+ yields all Trp^+ progeny even though half of the haploid meiotic products contain the *trp5-48* mutation. Whether the factors responsible for the failure of segregation of *trp2-1* in our *A. thaliana* outcrosses are Mendelian or nonMendelian has not been determined.

The use of potentially highly divergent varieties for RFLP mapping in *A. thaliana* and other plants makes it important to have information about the genetic backgrounds of

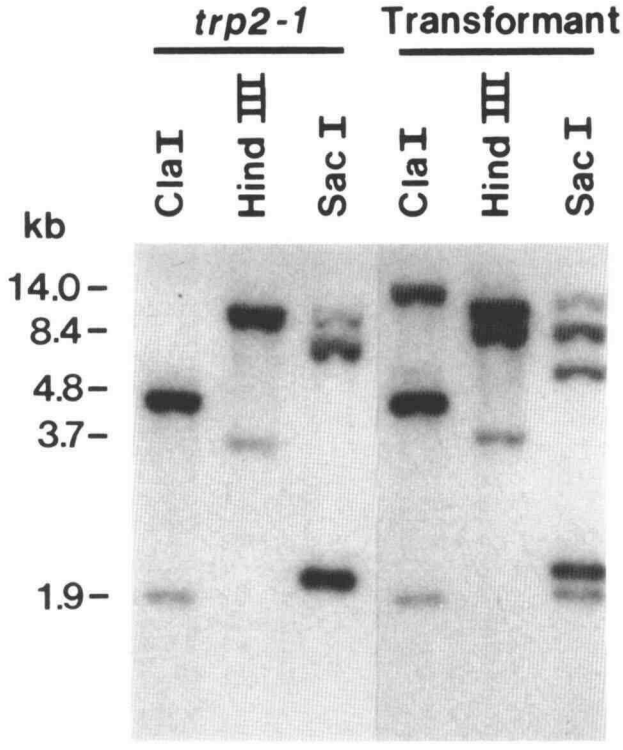


Figure 8. Genomic DNA Gel Blot Analysis of Transgenic Line TT1.

The *TSB1* 4.2-kb EcoRI fragment from pMBT2 (Berlyn et al., 1989) was the hybridization probe. The restriction endonuclease used to digest the DNA is shown above each lane, and the position of DNA size markers is indicated on the left in kilobases. The lanes marked Transformant correspond to TT1, a Trp^+ line derived by introducing the *TSB1* gene into the *trp2-1* genetic background. The signals unique to digests of transformant DNA represent the transgene and the weaker signals common to both genotypes correspond to the *TSB2* locus.

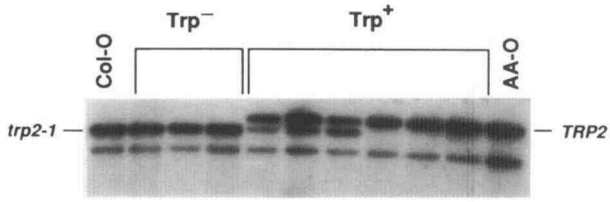


Figure 9. RFLP Analysis Showing Cosegregation of *TSB1* and *trp2-1*.

A BglII genomic DNA gel blot hybridized with a *TSB1* cDNA probe allows detection of the *TSB1* polymorphism. Parental Col-0 (*trp2-1*) and AA-0 (*TRP2*) polymorphic fragments are shown in the far left and right lanes, respectively. DNA of representative Trp⁻ plants (*trp2-1*/*trp2-1*; lanes 2 to 4) and Trp⁺ plants (lanes 5 to 10) are shown. Differences in the relative intensities of the polymorphic bands in lanes 5 to 7 are not significant.

plants used in crossing experiments. Not all polymorphic strains show unexpected genetic segregation patterns. For example, the *trp2-1* mutation is fully penetrant and segregates normally in crosses between Trp⁻ Columbia strains and the Trp⁺ Landsberg ecotype (R. Last, unpublished observations), even though these strains have extensive RFLPs (Chang et al., 1988; Nam et al., 1989).

Influence of Genetic Redundancy on Mutant Phenotype

The tryptophan requirement of *trp2-1* in high light intensity and the absence of the requirement in low light intensity might be related to the presence of two copies of the gene encoding tryptophan synthase and the differential expression of the two genes. We presume that in high light when the plants are growing rapidly, they require a functional *TSB1* gene. Under these growth conditions, the minor *TSB2* isoenzyme is simply unable to supply sufficient tryptophan to permit the growth of the *trp2-1* mutant. Under low light, where the plant grows slowly, the low amount of *TSB2* mRNA might be sufficient to sustain growth in the absence of tryptophan supplementation. Light-level conditional phenotypes are also seen for *trp1* auxotrophs, deficient in anthranilate PR-transferase (Last and Fink, 1988), and *trp3* mutants altered in an as-yet-unidentified step between anthranilate and tryptophan. Our working hypothesis is that, by analogy to *trp2-1*, the residual tryptophan biosynthetic activity in the other *A. thaliana* tryptophan auxotrophs is due to the presence of a second gene.

An alternative explanation is that *trp2-1* is leaky or alters the developmental regulation of *TSB1*. In this scenario, residual expression of *TSB1* would contribute to the low but detectable leaf tryptophan synthase β enzyme activity and low light level prototrophy of the *trp2-1* mutant. The observation that tryptophan auxotrophs are uncovered at very low frequency compared with other recessive traits in *A. thaliana* (Last and Fink, 1988; R. Last, unpublished observations) is consistent with the hypothesis that only specific types of missense mutations cause viable Trp⁻ mutants.

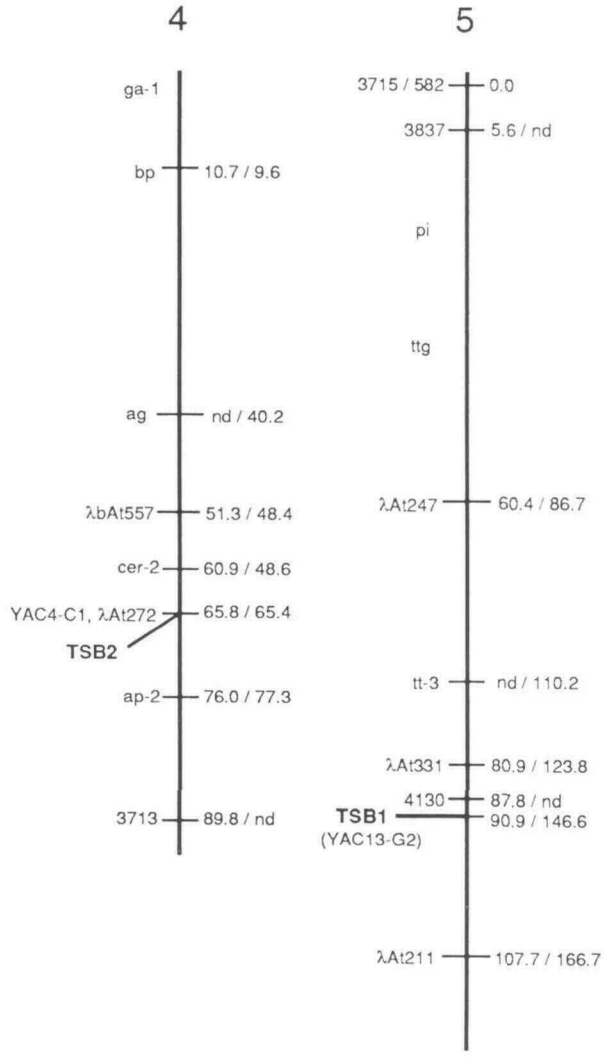


Figure 10. Schematic Representation of the Locations of the Tryptophan Synthase Genes on Chromosomes 4 and 5 of *A. thaliana*.

Symbols to the left of the vertical lines indicate the positions of mapped visible and RFLP genetic markers. Map distances in centimorgans are indicated to the right of the markers in pairs of numbers; the numbers on the left are taken from the RFLP map compiled by Nam et al. (1989); the numbers on the right are from the map compiled by Chang et al. (1988). An effort was made to include all markers that have been mapped by both groups.

Redundancy May Imply Divergent Function or Expression

Although all the evidence is not in, there are indications that every step in plant aromatic amino acid biosynthesis may be duplicated. There appear to be two copies of genes for EPSP synthase (Klee et al., 1987), deoxy-D-arabino-D-heptulosonatephosphate synthase (B. Keith and G. Fink, unpublished results), and anthranilate synthase (K. Niyogi and G. Fink, unpublished results). Genetic redundancy for aromatic amino acid biosynthetic enzymes is not limited to *A. thaliana*. The EPSP synthase gene also appears to be duplicated in petunia (Gasser et al., 1988). Recent data indicate that there are two tryptophan synthase β subunit genes in maize (A. Wright and K. Cone, University of Missouri, personal communication). Unlike the situation in *A. thaliana*, in maize only the double mutant (*orp1;orp2*), defective for both copies of the tryptophan synthase β subunit genes, shows a Trp^- phenotype.

Although the phenotypes of the *A. thaliana* and maize tryptophan mutants suggest functional redundancy of the duplicated tryptophan genes, it is possible that the duplicated genes do not have completely overlapping functions. The aromatic amino acid biosynthetic pathway produces a number of important "secondary" products, including the hormone indole-3-acetic acid, or auxin (Bandurski and Nonhebel, 1987), and indoleglucoscinolates (Hogge et al., 1988) from the tryptophan branch, and a diverse group of compounds from phenylalanine including phytoalexins, and flavonoids (Hahlbrock and Scheel, 1989). The duplication of genes for the aromatic pathway in plants could be a reflection of the dual fates of aromatic amino acids: protein synthesis and secondary metabolism (Bryan, 1990). One might imagine that the existence of two pathways would permit independent regulatory control. Precedence for gene duplication to meet the exigencies of dual function exists even in microorganisms. For example, *Pseudomonas aeroginosa* has duplicated genes for anthranilate synthase. One of these enzymes functions in the biosynthesis of tryptophan and the other synthesizes secondary compounds called pycocyanins (Essar et al., 1990).

The two postulated tryptophan pathways may be segregated either at the cellular level or at the tissue level. For example, one tryptophan pathway might be present in the cytosol and one in the chloroplast. However, the fact that both *TSB1* and *TSB2* genes are predicted to have chloroplast leader sequences suggests that both tryptophan synthases may be in the chloroplast. Furthermore, preliminary cell fractionation studies indicate that the majority of the tryptophan synthase β activity is present in the chloroplast (Figure 6B). Of course, it is possible that the two pathways are in the chloroplast, but each is located in a different compartment within the organelle. Alternatively, the two pathways might be expressed in different tissues and organs during plant development. Transgenic plants containing easily scored indicator genes fused to the *TSB1* and *TSB2* promoters should help to resolve some of these questions.

Isolation of Plant Amino Acid Auxotrophs Despite Redundant Genes

The characteristics of *A. thaliana* tryptophan mutants suggest several approaches to circumvent the problem of gene redundancy. The light-conditional Trp^- phenotype associated with these mutants suggests looking for plant amino acid auxotrophs under growth conditions where very active protein synthesis is required. This phenotypic dependence on environmental conditions reinforces the need to perform mutant isolations using highly reproducible culture conditions. If information on the expression of biosynthetic genes is available, mutant isolation protocols can be biased to favor expression of one of the redundant genes. A second consideration is that success in isolating auxotrophs may depend on genetic background. Although the low level of *TSB2* in the Columbia background is insufficient to support the growth of a *tsb1* mutant (the *trp2-1* mutant) in high light, the low-level expression of this gene might be sufficient in the AA-0 background. Alternatively, the level of expression of the *TSB2* gene may be higher in the AA-0 background. One might predict that it would be extremely difficult to obtain tryptophan mutants in the AA-0 background.

METHODS

Plant Lines and Culture Conditions

Arabidopsis thaliana lines homozygous for *pgm* (Caspar et al., 1985), *gl1-1*, and *trp2-1* were derived from the Columbia ecotype. The AA-0 ecotype was obtained from Dan Voytas and Fred Ausubel, Harvard University. Plant culture conditions were identical to those described (Last and Fink, 1988), unless indicated in the text. Leaf tissue from plants that had not yet started to produce elongated flower stalks was used for biochemistry and molecular biology.

DNA and RNA Manipulations

Total DNA was isolated from leaves by the method of Cone (1989). DNA manipulations and analysis were performed using standard protocols (Ausubel et al., 1989). Blot hybridization probes were labeled by the random primer method (Feinberg and Vogelstein, 1983). RNA was isolated from leaf tissue using the aurintricarboxylic acid procedure described by Nagy et al. (1989).

The complete *A. thaliana TSB2* gene was isolated in two steps. To isolate the 3′ half of the *TSB2* gene, the 0.8-kb SacI-XhoI fragment from *TSB1* cDNA clone pMBT3 (Berlyn et al., 1989) was used to screen a library of 2.4-kb to 3.5-kb EcoRI genomic fragments from the Columbia ecotype cloned into λGT10 (Promega Biotec). The insert from one positive phage clone (λRLT1) was cloned into the EcoRI site of pUC118 (Vieira and Messing, 1987) in both orientations to generate pRLT1 and pRLT2. This

genomic clone was used to screen a Columbia ecotype λEMBL3 library by hybridization. A 5.0-kb BamHI fragment that overlaps with pRLT1 and contains the 5′ half of the *TSB2* gene from the positive phage clone λRLT2 was cloned in both orientations into BamHI-cleaved pUC118 to create pRLT3 and pRLT4.

Dideoxy chain-termination sequencing reactions were run with modified T7 DNA polymerase (Tabor and Richardson, 1987), following the Sequenase protocol (United States Biochemical). Single-stranded plasmid DNAs from pUC118 recombinants were used as sequencing templates (Vieira and Messing, 1987). The DNA sequence of the *TSB2* gene 3′ half was obtained from a series of nested deletions (Henikoff, 1984) from the 2.5-kb EcoRI fragment in pRLT1 and pRLT2. The DNA sequence of the 5′ half of the gene was determined using a series of oligonucleotides with single-stranded plasmid DNA from pRLT3 and pRLT4 and by subcloning specific fragments into appropriate pBluescript plasmids (Stratagene). The oligonucleotide sequencing primers were designed based on the known *TSB1* or *TSB2* DNA sequences. The *TSB2* nucleotide sequence has been submitted to GenBank.

Yeast colony blot hybridizations were performed as described by Rose et al. (1990). The YAC library provided by Erwin Grill and Chris Somerville (Michigan State University) was plated on 24 individual plates, and colony lifts from each of these plates were made on 137-mm nylon membranes (Biotrans, ICN) and probed with fragments of the two genes encoding tryptophan synthase β. The *TSB1* probe consisted of the 5′ untranscribed 1.7-kb EcoRI-NdeI fragment from pMBT3 (Berlyn et al., 1989), and the *TSB2* probe consisted of the 3′ 2.4-kb EcoRI fragment from pRLT1.

S1 Nuclease Protection Assays

S1 assays were performed with uniformly labeled single-stranded probes from the divergent 5′ *TSB* gene ends by modification of the procedure of Ausubel et al. (1989). Single-stranded probes were generated by oligonucleotide-primed, Klenow fragment-mediated DNA synthesis in the presence of a single ^{32}P-labeled nucleoside triphosphate and excess cold nucleoside triphosphates. Synthesis of the antisense *TSB1*-specific probe employed oligonucleotide TSB1PE (5′-GTCAGATCCGGCGGCCATGAGAACAGGCGG-3′) and single-stranded sense DNA template produced from phagemid pMBT2 (Berlyn et al., 1989). *TSB2*-specific antisense probe was synthesized using oligonucleotide TSB2PE (5′-CCAGCGGCCTTAATCTTCTCCGAGTCCGCC-3′) and single-stranded template from pRLT3. After the synthesis reaction, the *TSB1* and *TSB2* reaction products were cleaved with restriction endonucleases SspI and BspHI, respectively. The digestion products were purified on a denaturing agarose gel to yield 330-nucleotide (*TSB1*) and 560-nucleotide (*TSB2*) antisense strand DNA probes.

Variable quantities of total *A. thaliana* RNA were hybridized with 5×10^4 cpm (Cherenkov) probe in 80% formamide S1 hybridization buffer at 49°C for 11 hr. After the hybridization, 0.3 mL of room temperature S1 digestion buffer containing 300 units S1 (Bethesda Research Laboratories) was added and the tubes were immediately placed in a 37°C water bath for a 45-min incubation. The products were visualized on 6% acrylamide/urea sequencing gels with a DNA sequence ladder and end-labeled DNA size standards.

Tryptophan Biosynthetic Enzyme Assays

Anthranilate synthase (EC 4.1.3.27), anthranilate PR-transferase (EC 2.4.2.18), and PR-anthranilate isomerase activities were assayed as described in Last and Fink (1988). Concentrations of chlorophyll *a* plus *b* were determined by measurement of the A_{654} of extracts in 90% ethanol (Winterman and Demots, 1965). Protein concentration was measured by the method of Bradford (1976).

Tryptophan Synthase β Assays

Two different methods were employed for measuring tryptophan synthase β activity. A nonradioactive colorimetric assay measured indole substrate disappearance (Yanofsky, 1955; Greenberg and Galston, 1959), whereas a radioisotopic assay monitored L-tryptophan production from reaction of the substrates L-[3-^{14}C]serine and unlabeled indole (a modification of the procedure of Chen and Boll, 1968). All steps were performed at 4°C, unless indicated otherwise.

Plant extracts were prepared by grinding 8 g of leaf tissue to a paste with a prechilled mortar and pestle containing 2 mL of 0.1 M potassium phosphate buffer (pH 8.2), 600 mg of 100-μm glass beads, and 600 mg of polyvinylpolypyrrolidone (Sigma). Homogenates were sonicated for 6 × 10 sec and cleared by centrifugation at 12,000*g* for 15 min. The resultant supernatant fraction was used as the enzyme extract.

The first assay was a colorimetric assay in which 60 μmol of L-serine, 0.2 μmol of indole, 80 μmol of potassium phosphate (pH 8.2), and 10 μg of pyridoxal phosphate in a total of 0.6 mL were incubated with 0.4 mL of plant extract with gentle agitation at 30°C. The reaction was stopped by the addition of 0.1 mL of 0.2 M sodium hydroxide after 90 min. The residual indole was extracted into 4 mL of toluene by gently vortexing (vigorous agitation created a permanent emulsion). After centrifugation for 15 min at 1500*g*, 0.5 mL of the toluene layer was added to 2 mL of ethanol and 1 mL of Ehrlich's Reagent (Sigma). The color was allowed to develop for 30 min at room temperature and the product was measured spectrophotometrically at 540 nm.

Assay of the synthesis of radiolabeled tryptophan from L-[3-^{14}C]serine is a more sensitive and specific tryptophan synthase β assay. The colorimetric assay described above was increased in volume twofold and a mixture of 0.014 μmol of L-[3-^{14}C]serine (58 mCi/mmol; ICN) and 72 μmol of unlabeled serine was used. The reaction was stopped with 0.1 mL of 0.2 M NaOH and the reaction products were further purified. The mixture was first concentrated by ultrafiltration with a Centricon-3 centrifugal microconcentrator (Amicon) and then applied to a Dowex 50-H^+ column (0.6 cm × 4 cm). The column was washed with 5 mL of H_2O and the compounds binding to the matrix were eluted with 8 mL of a 2 M ammonium hydroxide solution. This eluate was neutralized with hydrochloric acid and loaded onto a C_{18} cartridge (Waters Associates). The cartridge was washed with 3 mL of H_2O and the amino acids were eluted with 1 mL of acetonitrile. After drying, the purified reaction products were taken up in water and separated by cellulose thin-layer chromatography (Analtech, MN300) using ammonia:isopropyl alcohol:water (2:7:1) as solvent. The radioactive tryptophan band was visualized by autoradiography and identified by an adjoining L-[3-^{14}C]tryptophan standard (Du Pont-New England Nuclear). The tryptophan spots were then cut out, eluted into H_2O, quantified by liquid scintillation, and rerun

under the same chromatographic conditions to separate them from an abundant radioactive indole derivative that was also produced in boiling-inactivated extracts.

Under the conditions of these assays, the rate of disappearance of indole (assay one) or tryptophan accumulation (assay two) was linear with respect to time and amount of plant extract. Mixing equal amounts of *trp2-1* mutant and wild-type extracts produced tryptophan synthase activity comparable with wild-type alone.

Chloroplast Isolation

Intact chloroplasts were isolated by a modification of the method of Somerville et al. (1981), with all steps performed at 4°C unless otherwise indicated. Three grams of starchless leaves from the *pgm* mutant in buffer containing macerase and cellulysin were subjected to three rounds of vacuum infiltration. Digestion was allowed to proceed at room temperature for 3 hr to 4 hr, at which time most of the protoplasts had fallen to the bottom of the Erlenmeyer flask leaving only the epidermis and vascular elements floating in the medium. The washed chloroplasts were resuspended in the recommended buffer lacking sodium bicarbonate. The chloroplasts were ruptured by resuspension in ice-cold 0.1 M potassium phosphate buffer (pH 8.2) and sonication (six times for 10 sec each). The post-chloroplast supernatant was desalted by passage through a NAP-25 Sephadex G-25 column (Pharmacia) before use in the enzyme assay.

Plant Transformation

The 4.2-kb EcoRI fragment containing the *TSB1* gene and flanking sequences (Berlyn et al., 1989) was cloned into the binary vector pBIN19 (Bevan, 1984) to create pBINTSB1. The *TSB1* gene and NPTII marker gene are transcribed divergently in this plasmid. *Agrobacterium tumefaciens* strain AGL1, provided by Dr. Robert Ludwig (University of California, Santa Cruz), was transformed with pBinTRPB1 DNA (An et al., 1988).

A. thaliana root explants were transformed and regenerated by modification of the method of Valvekens et al. (1988). For both transformation and regeneration, the explants, calli, and plantlets were cultivated at low light intensities (25 to 50 μmol PAR·m^{-2}·sec^{-1}) in media solidified with 0.75% Phytagar (Gibco). Plant containers from Flow Laboratories (9.5 × 9.5 × 70 cm) were used for growth of larger plants. All plant culture media included 50 μM L-tryptophan to enable the *trp2-1* mutants to grow, whereas 10 μM acetosyringone (Aldrich) was added to the 0.5/0.05 callus-inducing medium (Valvekens et al., 1988) during *A. tumefaciens* cocultivation. To induce root formation, the regenerated shoots were cut from the callus tissue, dipped in a rooting hormone mixture (0.2% 1-naphtaleneacetamide, 0.1% indole-3-butyric acid; Rockland Chemical Co.), grown on GM plates (Valvekens et al., 1988) until roots were observed, and then transferred to soil. Kanamycin resistance of progeny seedlings was assayed by germination on Murashige and Skoog (1962) plates containing 25 μg/mL kanamycin.

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