5-Fluoroindole Resistance Identifies Tryptophan Synthase Beta Subunit Mutants in *Arabidopsis thaliana*

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ABSTRACT

A study of the biochemical genetics of the *Arabidopsis thaliana* tryptophan synthase beta subunit was initiated by characterization of mutants resistant to the inhibitor 5-fluoroindole. Thirteen recessive mutations were recovered that are allelic to *trp2-1*, a mutation in the more highly expressed of duplicate tryptophan synthase beta subunit genes (*TSB1*). Ten of these mutations (*trp2-2* through *trp2-11*) cause a tryptophan requirement (auxotrophs), whereas three (*trp2-100* through *trp2-102*) remain tryptophan prototrophs. The mutations cause a variety of changes in tryptophan synthase beta expression. For example, two mutations (*trp2-5* and *trp2-8*) cause dramatically reduced accumulation of TSB mRNA and immunologically detectable protein, whereas *trp2-10* is associated with increased mRNA and protein. A correlation exists between the quantity of mutant beta and wild-type alpha subunit levels in the *trp2* mutant plants, suggesting that the synthesis of these proteins is coordinated or that the quantity or structure of the beta subunit influences the stability of the alpha protein. The level of immunologically detectable anthranilate synthase alpha subunit protein is increased in the *trp2* mutants, suggesting the possibility of regulation of anthranilate synthase levels in response to tryptophan limitation.

PAST studies of the biochemistry and genetic regulation of amino acid biosynthesis in plants were hampered by the paucity of useful mutants (LAST 1993). This is in contrast to microorganisms, where amino acid auxotrophs are among the most frequently isolated general class of mutants. The phenylalanine, tyrosine and tryptophan biosynthetic pathways of plants are particularly interesting because they produce precursors to a variety of secondary metabolites, in addition to amino acids for protein synthesis. For example, the tryptophan branch provides precursors to indolic secondary metabolites, including the hormone indole-3-acetic acid (WRIGHT *et al.* 1991; NORMANLY *et al.* 1993), antimicrobial phytoalexins (TSUJI *et al.* 1993), glucosinolates (HAUGHN *et al.* 1991) and alkaloids (CORDELL 1974).

In contrast to the pathways leading to other amino acids, tryptophan biosynthesis is proving to be especially well suited to genetic analysis in *Arabidopsis thaliana* (LAST and FINK 1988; LAST *et al.* 1991; ROSE *et al.* 1992; NIYOGI *et al.* 1993; reviewed in ROSE and LAST 1994) and other plants (FANKHAUSER *et al.* 1990; WRIGHT *et al.* 1992). As shown in Figure 1, structural gene mutations in Arabidopsis are available that cause defects in four of the seven proteins that convert chorismate into tryptophan, and the structural genes affected in these mutants are characterized. These are the anthranilate synthase beta subunit (step 1 in Figure 1) *trp4* mutants (NIYOGI *et al.* 1993), phosphoribosylanthranilate transferase (step 2 in Figure 1) *trp1* mutants (LAST and FINK 1988; ROSE *et al.* 1992), and the tryptophan synthase alpha subunit (TSA; step 5 in Figure 1) *trp3* mutants (unpublished) and the beta subunit (TSB; step 6 in Figure 1) *trp2-1* mutant (LAST *et al.* 1991). Although mutations are not described for the remaining three proteins, Arabidopsis genes were characterized for anthranilate synthase alpha subunit (ASA; step 1 in Figure 1) (NIYOGI and FINK 1992), PR-anthranilate isomerase (step 3 in Figure 1) (LI *et al.* 1995b) and indole-3-glycerol phosphate synthase (step 4 in Figure 1) (LI *et al.* 1995a).

The enzyme tryptophan synthase is an especially attractive target for detailed biochemical genetic analysis in plants because the structure and function of bacterial tryptophan synthase are very well understood (MILES 1991). In bacteria, the enzyme exists as an $\alpha_2\beta_2$ heterotetramer, which catalyzes the conversion of indole-3-glycerol phosphate and serine to tryptophan and glyceraldehyde-3-phosphate. However, each subunit can less efficiently catalyze a half-reaction:

α-subunit activity:

Indole-3-glycerol phosphate $\leftrightarrow$ **Indole**

+ D-glyceraldehyde-3-phosphate

β_2-subunit activity:

Indole + L-Serine $\rightarrow$ L-tryptophan + H_2O

Although the reactions carried out by fungal TS are

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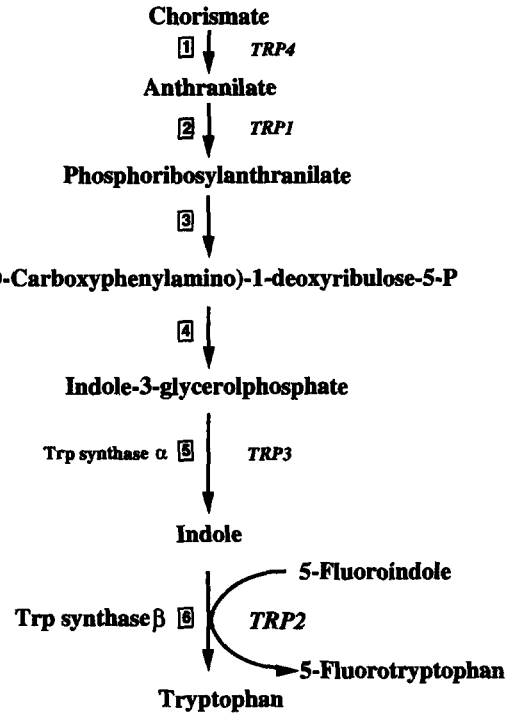

FIGURE 1.—The tryptophan biosynthetic pathway in Arabidopsis. The enzymatic steps are in boxed numbers, as discussed in the text, and the four genes for which mutations are available are indicated. The presumed TSB-mediated conversion of 5-fluoroindole to 5-fluorotryptophan is also indicated.

identical, the two subunits are fused into a single polypeptide: in *Neurospora crassa* and *Saccharomyces cerevisiae* the amino-terminal domain is homologous to the alpha subunit and the carboxyl-terminus is homologous to the beta subunit (BURNS and YANOFSKY 1989; ZALKIN and YANOFSKY 1982).

Two lines of evidence indicate that the plant enzyme is composed of separate subunits, reminiscent of prokaryotes. The Arabidopsis (BERLYN *et al.* 1989; LAST *et al.* 1991) and *Zea mays* (maize) (WRIGHT *et al.* 1992) beta subunit genes do not contain alpha subunit coding information. Similarly, the Arabidopsis alpha subunit gene is devoid of sequences homologous to the beta subunit (E. R. RADWANSKI, J. ZHAO and R. L. LAST, unpublished data). Biochemical studies also suggest that the active enzyme from *Nicotiana tabacum* (tobacco) and *Pisum sativum* (pea) is composed of alpha and beta subunit activities (DELMER and MILLS 1968; CHEN and BOLL 1971; NAGAO and MOORE 1972).

Analysis of the Arabidopsis *trp2-1* mutant, which is deficient in TSB subunit activity, revealed it to be relatively insensitive to the toxic effects of 5-fluoroindole (LAST *et al.* 1991). This observation suggests that 5-fluoroindole toxicity results from conversion to 5-fluorotryptophan by wild-type TSB protein. In this respect, Arabidopsis responds to 5-fluoroindole in the same way as do the fungi *S. cerevisiae* (MIOZZARI *et al.* 1977) and *Coprinus cinereus* (TILBY 1978).

We identified Arabidopsis mutants by selection for growth on 5-fluoroindole. Genetic, molecular and immunological analyses are presented for the 13 mutants that contain recessive *trp2* alleles. These lines are shown to range from severely affected tryptophan-requiring auxotrophs to leaky mutants that grow in the absence of exogenous amino acid. The TSB subunit mRNA and protein accumulate to varying extents, ranging from approximately 5% to 150% of the wild type. Thus, the selection for 5-fluoroindole resistance yielded an allelic series of Arabidopsis *trp2* mutations.

MATERIALS AND METHODS

Plant strains and growth conditions: The Columbia ecotype of Arabidopsis was used as the wild-type line throughout this work. For all biochemical assays, rosette leaves of 3-week-old plants grown in soilless mix (Peat-lite Redi Earth from W. R. Grace, or Cornell Mix) were harvested into liquid nitrogen and stored at −80° until extraction. Unless otherwise specified, plant growth was at 22° under continuous illumination. For sterile culture, plants were grown in 100-mm-diameter Petri plates sealed with parafilm (American National Can, Greenwich, CT) on sterile plant nutrient medium with 0.5% sucrose (PNS) (HAUGHN and SOMERVILLE 1986) solidified with 0.75% Bacto agar. Plants were grown in soilless mix under 80–100 μmol/m^2/sec photosynthetically active radiation (PAR) from mixed cool-white and incandescent lights or from MVR-400/U multivapor metal halide bulbs (General Electric).

Mutagenesis: Mutagenesis was performed on batches of 50,000 seeds, and the self-fertilized progeny M_2 seeds were harvested from separate pools of 5000 M_1 plants. EMS mutagenesis was performed as described, using 0.25–0.30% (v/v) EMS (Sigma) (ESTELLE and SOMERVILLE 1987). Gamma irradiation (30 krad) was performed in a ^{135}Cs source (Gammacell 1000; AECL Industrial, Ontario, Canada) on seeds that had imbibed in water for 4 hr at room temperature. Nitrosomethylurea (NMU, Sigma) mutagenesis was done by a modification of HAGEMANN (1982). Seeds were imbibed overnight at room temperature in water and then treated at room temperature for 4 hr with 150–200 μM NMU in a pH 5.0 buffer (0.0485 M citric acid and 0.103 M sodium phosphate dibasic), with gentle shaking. The seeds were washed with 500 ml water 10 times, and the NMU contaminated solutions and solids were detoxified overnight with 5 M NaOH. Seeds that were mutagenized with diepoxybutane (DEB; Sigma) were imbibed overnight in water at room temperature and then treated with 22 mM DEB in water for 5 hr at room temperature, with gentle shaking. The seeds were washed five times with 1 liter of water each time, before planting. The DEB solution was detoxified overnight with 2% sulfuric acid, and the other contaminated materials were treated with 1% H_2SO_4 for 24–48 hr.

Mutant selections and genetic manipulations: In selection experiments, M_2 seeds were surface sterilized (LAST and FINK 1988) and suspended at 500 seeds/ml in 0.1% agar. Aliquots of 500 seeds were mixed with 4.0 ml of molten 50° agar and poured onto the surface of 100-mm-diameter Petri plates containing 25 ml PNS supplemented with 200 μM 5-fluoroindole plus 40 μM tryptophan (to permit growth of tryptophan auxotrophic plants), and the plates were incubated at 100 μmol/m^2/sec PAR. Putative resistant plants were identified after 14–21 days, transferred to PNS containing 50 μM tryptophan for 5–7 days to recover, and then transplanted to soilless mix to self-pollinate. The 5-fluoroindole resistance phenotype of M_3, F_1 or F_2 seeds was rescreened by planting 100 seeds per plate

containing 100 μM 5-fluoroindole plus 40 μM tryptophan, and the phenotype was scored after 14–21 days growth. Tryptophan auxotrophy was assayed by comparing growth at 180 μmol/m²/sec PAR of seeds on minimal PNS medium containing or lacking tryptophan (LAST and FINK 1988; LAST *et al.* 1991; ROSE *et al.* 1992).

Nucleic acid and protein isolation and detection: Plant genomic DNA (CONE 1989) and total RNA (DEVRIES *et al.* 1988) were isolated by published methods. Genomic DNA (AUSUBEL *et al.* 1994) and RNA gel electrophoresis and blot transfers (PRUITT and HANSON 1991), and blot hybridizations (CHURCH and GILBERT 1984) were performed as described. The gel-purified *TSB1* cDNA and genomic probes were a 0.8-kb *Sad-Xho*I and a 4.2-kb *Eco*RI fragment, respectively (BERLYN *et al.* 1989). The *TSA1* cDNA probe, which encodes the Arabidopsis TSA subunit, was a 1.2-kb *Eco*RI fragment from pERR65 (E. R. RADWANSKI, J. ZHAO and R. L. LAST, unpublished data). DNA probes were labeled with ^{32}P using the Megaprime DNA Labeling Kit (Amersham, Arlington Heights, IL), and unincorporated nucleotides were removed with Nuctrap Push Columns (Stratagene, La Jolla, CA). The abundance of TSA and TSB mRNA was normalized to 26S rRNA as previously described (PRUITT and LAST 1993). Production of rabbit antisera, protein sample preparation, protein gel electrophoresis, immunoblotting and Phosphoimager radioactivity quantification were performed as described by ZHAO and LAST (1995).

DNA sequence analysis of *trp2* mutant alleles: The *TSB1* genes were cloned from *Eco*RI-digested *trp2-5* and *trp2-8* mutant total DNA, which was separated by preparative agarose gel electrophoresis. DNA was isolated from the region spanning an estimated size range of 3–6 kb and purified with Glassmilk silica beads (Bio 101 Inc., La Jolla, CA). We confirmed that the gel slice was not contaminated with *TSB2* DNA by testing a sample using Southern blot hybridization with the *TSB1* genomic probe, which hybridizes efficiently with the *TSB2* locus as demonstrated in LAST *et al.* (1991). The size-selected DNA was then ligated into *Eco*RI-digested and alkaline phosphatase-treated λZAPII, packaged with Gigapack Gold, and infected into *Escherichia coli* XL1-Blue MRF', using the recommendations of the manufacturer (Strategene). Nitrocellulose plaque hybridizations were performed with digoxigenin-labeled *TSB1* genomic probe and detected with alkaline phosphatase conjugated anti-digoxigenin FAB (Genius System, Boehringer Mannheim Corp., Indianapolis, IN). The recombinant pZAPII plasmid was excised using M13 helper phage ExAssist (Stratagene) and purified with a Qiagen-tip 20 column (Qiagen Incorporated, Chatsworth, CA). Double-stranded plasmid DNA was sequenced with *TSB1* oligonucleotides using the DyeDeoxy Terminator Sequencing Kit (Applied Biosystems Incorporated, Foster City, CA) and the Thermolyne TEMP-TRONIC DB66925 thermocycler. The DNA fragments were purified with CentriSep spin columns (Princeton Separations, Adelphia, PA) and then loaded onto an Applied Biosystems 373A Automated DNA Sequencer.

DNA sequence of *trp2-10* was obtained by PCR amplification of total DNA. The 5′ half of the *TSB1* gene was amplified with primers AB3 and AB4 and the 3′ half of the gene was amplified with primers AB5 and AB6. Single stranded template was obtained by the method of HIGUCHI and OCHMAN (1989), and DNA sequence was obtained by manual sequencing methods using Sequenase as specified by the manufacturer (U.S. Biochemical). The MacVector software (IBI, New Haven, CT) was used for sequence analysis. The accuracy of the mutant DNA sequence was verified by sequencing the altered region on both strands. The oligonucleotides used are listed in Table 1.

TABLE 1

Sequencing primers

Primer	Sequence
KP 30	5′GGAAGAAGAGGAAGGAGC3′
KP 31	5′GAAAGGATATTTCAAGGAGAAGAATA3′
KP 32	5′AACGACCCACAACGCAAAATGTCTCA3′
KP 33	5′GGTTCAATGTCTCATTCATGTCCTTTG3′
KP 34	5′GTTCAGTAACAAAGTTTATCACATTTA3′
KP 35	5′CTTTTAAATGTGATAAACTTTGTTACT3′
KP 36	5′CATTCACAAATTCATGGAAGAGTCCC3′
KP 37	5′GGTAAAGAAACAAGGAAACAAGCTTG3′
KP 38	5′TGGCTAACTGAGTAGACTTATTATGG3′
KP 39	5′AGTCTACTCAGTTAGCCACGGCTGC3′
KP 40	5′GTTATGAAACAAAGAACAGATTTTCAA3′
KP 41	5′TCTGTTCTTTGTTTCATAACTTACCT3′
KP 42	5′CAAAAAGGATTTCATTCTGTCTGAATAACT3′
AB 1	5′CCTGTAATGCTCCGTAAG3′
AB 2	5′TCGGTGTGGAAGCAGCGG3′
AB 3	5′GAGAGCTTTCTAGGCAATCCCGAG3′
AB 4	5′CTTCTTCCCCAACCGCTTAGCAAG3′
AB 5	5′GAGGCTTACGGAGCATTACAGG3′
AB 6	5′TGGGATTTCAGAAGAGGCAGAG3′

RESULTS

Isolation of 5-fluoroindole resistant mutants: Previous 5-methylanthranilate resistance selections yielded *trp2-1*, a recessive Arabidopsis mutation shown to cause reduced TSB subunit activity due to a mutation in *TSB1*, the more highly expressed of duplicate TSB genes (LAST *et al.* 1991). This mutant was found to be resistant to 5-fluoroindole, indicating that direct selection for 5-fluoroindole resistance would yield mutants altered in TSB activity. We reasoned that these mutants might include a wide variety of *trp2* structural gene alterations, as well as mutations in other genes that less directly influence TSB activity. This hypothesis was tested by subjecting a total of 390,000 M_2 seeds (obtained by self-pollination of plants derived from mutagenesis of seeds with EMS, NMU, DEB or gamma irradiation) to selection on medium supplemented with both 5-fluoroindole and tryptophan. A total of 132 putative resistant mutants that exhibited good root growth and true leaf development were transferred to soil. These plants were allowed to self-pollinate, and the M_3 progeny were retested for inhibitor resistance and tryptophan requirement.

Nineteen independently derived 5-fluoroindole-resistant mutants were chosen for further analysis because they exhibited strong inhibitor resistance in the M_3 (see Table 2). Two additional 5-fluoroindole-resistant lines, which were originally identified as resistant to 6-methylanthranilate (*trp2-11* and FIR 17H2) (J. LI and R. LAST, unpublished results), were also included in this collection. Of these 21 mutants, 10 are tryptophan-requiring auxotrophs under our standard high-light growth conditions, whereas 11 lines are capable of growth on sterile minimal medium lacking tryptophan under all conditions tested. As described below, 13 of the 5-fluoroindole-resistant lines (including all of the auxotrophs and three prototrophs) are recessive

TABLE 2

Mutant selection

New allele designation	$5FI^R$ isolate designation	Mutagen	Tryptophan phenotype
trp2-2	11A1	EMS	–
trp2-3	13H2	EMS	–
trp2-4	11D2	EMS	–
trp2-5	11E1	EMS	–
trp2-6	11F1	EMS	–
trp2-7	13A9	EMS	–
trp2-8	17H8	Gamma	–
trp2-9	1F10*a*	NMU	–
trp2-10	10C4	EMS	–
trp2-11	150102	NMU	–
trp2-100	11B4	EMS	+
trp2-101	12D1	EMS	+
trp2-102	14B3	EMS	+
	13B1[a]	EMS	+
	13H4[a]	EMS	+
	17G3[a]	Gamma	+
	17H2[a]	Gamma	+
	1A5	NMU	+
	1A5*a*	DEB	+
	1D8	NMU	+
	14E9	EMS	+

[a] The indicated isolates were shown to contain recessive Mendelian traits that complement *trp2*.

trp2 alleles. Phenotypic characterization of the remaining eight prototrophic mutations indicated that four are recessive Mendelian mutations that complement the *trp2* tester alleles (A. J. BARCZAK, unpublished results). Preliminary data from complementation crosses among these four recessive mutants revealed a minimum of two complementation groups. One of these alleles (isolate 13H4) failed to complement the TSA-deficient *trp3-1* mutation and is likely to be defective in the alpha subunit. Crosses of the remaining four mutants with wild type yielded F_2 progeny that could not be reliably scored for 5-fluoroindole resistance, hampering their further characterization.

As was previously observed with the *trp2-1* mutation, the auxotrophic plants are smaller than the wild type when grown on nonsterile soilless potting mix with or without tryptophan supplementation. However, growth of these new mutants is generally more robust than that of the original *trp2-1* mutant (A. J. BARCZAK, unpublished results). Growth of the three prototrophic mutants in soil is less severely affected than that of the auxotrophs, consistent with the hypothesis that these are leakier *trp2* alleles.

Genetic characterization of the 5-fluoroindole-resistant mutants: The results of crosses to wild-type Arabidopsis indicate that 5-fluoroindole resistance and tryptophan auxotrophy are conferred by recessive monogenic traits in the mutants tested. As shown in Tables 3 and 4, the F_1 progeny derived from crosses with nine of the auxotrophic mutants displayed the wild-type phenotype (tryptophan prototrophy and 5-fluoroindole sensitivity). One-quarter of the self-cross F_2 progeny were of mutant phenotype (tryptophan requiring or 5-fluoroindole resistant). F_2 progeny were not obtained for the *trp2-9* cross to wild type because the F_1 plants died before setting seed, apparently due to a problem with the batch of soil used to grow these individuals. One of the auxotrophic mutants (*trp2-7*) was very difficult to cross and was not outcrossed to the wild type. Although the F_2 obtained from the crosses of *trp2-8* and *trp2-11* were not tested for tryptophan auxotrophy, they were scored for 5-fluoroindole resistance, as shown in Table 4.

Results obtained with the prototrophic mutants indicate that these mutations are also recessive, as the F_1 progeny were uniformly 5-fluoroindole sensitive (Table 5). Surprisingly, analysis of the *trp2-100* F_2 cross yielded an unexpectedly high number of 5-fluoroindole-resistant progeny (33%). When calculated for an expected 3:1 segregation ratio, these data result in a chi-square value of 32.1, which does not fit the expectation at $P = 0.05$. F_2 analysis of the other two prototrophic alleles (*trp2-101* and *trp2-102*) yielded the expected 3:1 ratio. As this cross was only performed once, the significance of this skewed segregation pattern is unclear.

Allelism tests were performed to ask whether the analogue resistance and tryptophan requirement were caused by auxotrophic *trp2* mutations, with the results shown in Table 6. In each case it was demonstrated that the heteroallelic F_1 plants were resistant to 5-fluoroindole (and unable to grow in the absence of tryptophan if the new allele was auxotrophic), demonstrating allelism with the tester mutation. Consistent with the hypothesis that the new mutations are in *trp2*, tight linkage between each pair of mutations was demonstrated by the absence of recombinant wild-type F_2 progeny. This analysis was initially complicated by the observation that it was difficult to obtain successful crosses using the severely affected *trp2-1* auxotrophic mutant as the tester line. To facilitate complementation crosses, two of the new auxotrophic 5-fluoroindole-resistant mutants were used in this analysis. First, the *trp2-2* and *trp2-3* mutations were shown to be allelic to *trp2-1*, and then the *trp2-6* mutant was crossed to *trp2-2*, demonstrating allelism (Table 6). The remaining mutants were then crossed with *trp2-1*, *trp2-3* or *trp2-6* to complete the complementation and linkage analyses.

Tryptophan synthase gene product accumulation in *trp2* mutants: It was previously shown that *trp2-1* is an allele of *TSB1*, the more highly expressed of duplicate TSB subunit structural genes in Arabidopsis (LAST *et al.* 1991). RNA blot hybridization and immunoblot analysis were used to ask whether any of the new *trp2* alleles were altered in accumulation of TSA or TSB gene products. Analysis of the levels of these mRNAs and proteins in the 5-fluoroindole-resistant mutants revealed a number of interesting differences. Figure 2 shows that the *trp2-8* and *trp2-5* mutants had the most dramatically reduced

TABLE 3

Results from crosses with wild type: tryptophan auxotrophy

Cross[a]	Type	Total	Trp^+	Trp^-	% Trp^-
TRP2/TRP2 × *trp2-2/trp2-2*	F_1	6	6	0	0
	F_2	421	337	84	20
TRP2/TRP2 × *trp2-3/trp2-3*	F_1	13	13	0	0
	F_2	426	319	107	25
TRP2/TRP2 × *trp2-4/trp2-4*	F_1	6	6	0	0
	F_2	429	320	109	25.4
TRP2/TRP2 × *trp2-5/trp2-5*	F_1	12	12	0	0
	F_2	382	271	111	29.1
TRP2/TRP2 × *trp2-6/trp2-6*	F_1	6	6	0	0
	F_2	423	323	100	23.6
TRP2/TRP2 × *trp2-8/trp2-8*	F_1	10	10	0	0
TRP2/TRP2 × *trp2-9/trp2-9*	F_1	9	9	0	0
TRP2/TRP2 × *trp2-10/trp2-10*	F_1	8	8	0	0
	F_2	400	296	104	26
TRP2/TRP2 × *trp2-11/trp2-11*	F_1	7	7	0	0

[a] Pollen from the M_3 5-fluoroindole resistant mutant was crossed with wild type to generate F_1 seeds. The heterozygous F_1 plants were allowed to self-pollinate to yield F_2 progeny.

accumulation of TSB mRNA (7% and 34% of wild type, respectively). In contrast, *trp2-9* and *trp2-10* plants had modest, but reproducible, enhancement of TSB RNA (28% and 40% higher than the wild type, respectively). In all cases, TSA mRNA abundance is the same as, or slightly higher than, that of the wild type (data not shown). Although the results obtained for TSA mRNA quantitation were consistent throughout three independent experiments, we are cautious about drawing strong conclusions about the significance of the enhanced mRNA levels because the TSA mRNA hybridization signal is very weak in total RNA.

As shown in Figures 3, bottom, and 4B, quantitative immunoblot analysis with polyclonal antibodies revealed that TSB protein levels in the *trp2* mutants vary from very low (13% of wild type for *trp2-5* and 15% for *trp2-8*) to significantly greater than that of wild type (50–60% higher for *trp2-10*, *trp2-100* and *trp2-102*). The observation that the *trp2-5* and *trp2-8* mutants produce small amounts of TSB protein is consistent with the dramatic reductions of mRNA (Figure 2). None of the mutants accumulate stable nonsense peptides of lower molecular weight or proteolytic fragments that are detectable with the polyclonal antisera.

TABLE 4

Results of crosses with wild type: 5-fluoroindole resistance

Cross[a]	Type	Total	FIS[b]	FIR[c]	%FIR
TRP2/TRP2 × *trp2-2/trp2-2*	F_1	14	14	0	0
	F_2	263	201	62	23.6
TRP2/TRP2 × *trp2-3/trp2-3*	F_1	18	18	0	0
	F_2	283	218	65	23
TRP2/TRP2 × *trp2-4/trp2-4*	F_1	12	12	0	0
	F_2	276	211	65	23.6
TRP2/TRP2 × *trp2-5/trp2-5*	F_1	19	19	0	0
	F_2	263	208	55	23.6
TRP2/*TRP2* × *trp2-6/trp2-6*	F_1	13	13	0	0
	F_2	441	321	120	27.2
TRP2/TRP2 × *trp2-8/trp2-8*	F_1	8	8	0	0
	F_2	896	684	212	23.7
TRP2/TRP2 × *trp2-9/trp2-9*	F_1	6	6	0	0
TRP2/TRP2 × *trp2-10/trp2-10*	F_1	16	16	0	0
	F_2	625	460	165	26.4
TRP2/TRP2 × *trp2-11/trp2-11*	F_1	14	14	0	0
	F_2	594	434	160	26.9

[a] Pollen from the M_3 5-fluoroindole resistant mutant was crossed with wild type to generate F_1 seeds. The heterozygous F_1 plants were allowed to self-pollinate to yield F_2 progeny.

[b] 5-Fluoroindole sensitive.

[c] 5-Fluoroindole resistant.

TABLE 5

Results of crosses with wild type: prototrophic mutants

Cross[a]	Type	Total	FIS[b]	FIR[c]	%FIR
TRP2/TRP2 × *trp2-100/trp2-100*	F_1	18	18	0	0
	F_2	1088	728	360	33.1
TRP2/TRP2 × *trp2-101/trp2-101*	F_1	10	10	0	0
	F_2	1252	923	329	26.2
TRP2/TRP2 × *trp2-102/trp2-102*	F_1	12	12	0	0
	F_2	1223	898	325	26.6

[a] Pollen from the M_3 5-fluoroindole resistant mutant was crossed with wild type to generate F_1 seeds. The heterozygous F_1 plants were allowed to self-pollinate to yield F_2 progeny.
[b] 5-Fluoroindole sensitive.
[c] 5-Fluoroindole resistant.

Despite the fact that these mutants have lesions in a TSB gene and are presumably wild type for TSA, the steady-state TSA protein concentrations are altered in the majority of the *trp2* mutants (Figures 3, top, and 4A). Figure 5 illustrates a trend between the level of accumulation of mutant TSB subunits and steady-state concentration of the wild-type TSA protein. At one extreme, *trp2-5* and *trp2-7* accumulate ~50% of the wild-type TSA subunit, whereas *trp2-10* and *trp2-100* have reproducibly higher concentrations of both the TSA and TSB proteins. These results are consistent with the idea that the wild-type TSB protein stabilizes the plant TSA subunit *in vivo*. This is a plausible hypothesis because it is known that interactions between the bacterial subunits influence the conformation of the alpha subunit (MILES 1991). Data from this laboratory indicate

TABLE 6

Results of complementation crosses

			Tryptophan auxotrophy			5-fluoroindole resistance		
Cross[a]	Type	Total	Trp^+	Trp^-	$\%Trp^-$	FIS[b]	FIR[c]	%FIR
trp2-1/trp2-1 × *trp2-2/trp2-2*	F_1	20	0	6	100	0	14	100
	F_2	300	0	200	100	0	100	100
trp2-1/trp2-1 × *trp2-3/trp2-3*	F_1	10				0	10	100
	F_2	300	0	200	100	0	100	100
trp2-2/trp2-2 × *trp2-6/trp2-6*	F_1	15	0	2	100	0	13	100
	F_2	959	0	266		0	693	100
trp2-6/trp2-6 × *trp2-4/trp2-4*	F_1	8				0	8	100
	F_2	752				0	752	100
trp2-1/trp2-1 × *trp2-5/trp2-5*	F_1	5	0	2	100	0	3	100
	F_2	203				0	203	100
trp2-3/trp2-3 × *trp2-7/trp2-7*	F_1	13	0	7	100	0	6	100
	F_2	881				0	881	100
trp2-6/trp2-6 × *trp2-8/trp2-8*	F_1	19	0	7	100	0	12	100
	F_2	1013				0	1013	100
trp2-6/trp2-6 × *trp2-9/trp2-9*	F_1	5				0	5	100
	F_2	966				0	966	100
trp2-3/trp2-3 × *trp2-10/trp2-10*	F_1	15	0	8	100	0	7	100
	F_2	360				0	360	100
trp2-3/trp2-3 × *trp2-11/trp2-11*	F_1	10				0	10	100
	F_2	73				0	73	100
trp2-3/trp2-3 × *trp2-100/trp2-100*	F_1	21	10	0	0	0	11	100
	F_2	191				0	191	100
trp2-6/trp2-6 × *trp2-101/trp2-101*	F_1	14	4	0	0	0	10	100
	F_2	81				0	81	100
trp2-6/trp2-6 × *trp2-102/trp2-102*	F_1	30	10	0	0	0	20	100
	F_2	1285				0	1285	100

[a] Pollen from the M_3 5-fluoroindole resistant mutant was crossed with a plant of the indicated genotype to generate F_1 seeds. The heterozygous F_1 plants were allowed to self-pollinate to yield F_2 progeny.
[b] 5-Fluoroindole sensitive.
[c] 5-Fluoroindole resistant.

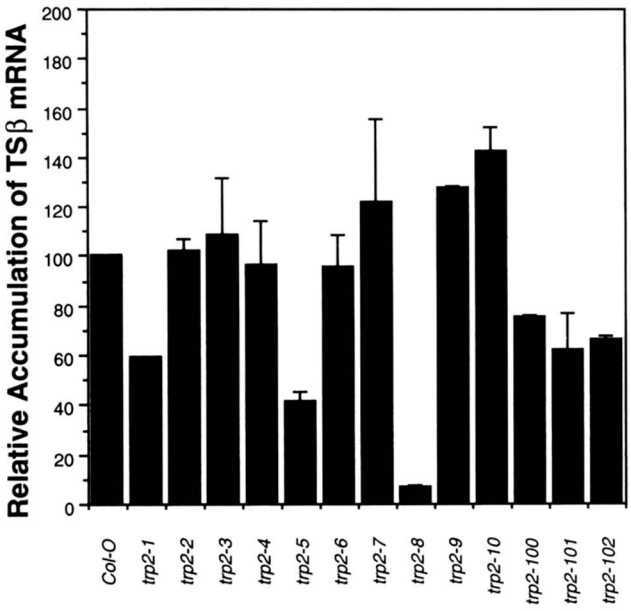

FIGURE 2.—TSB mRNA accumulation in *trp2* mutants. All values are corrected for rRNA hybridization, and the mutant results are normalized to the wild type ("100"). Each value represents the mean of triplicate blots from a single experiment, and error bars are standard error of the mean.

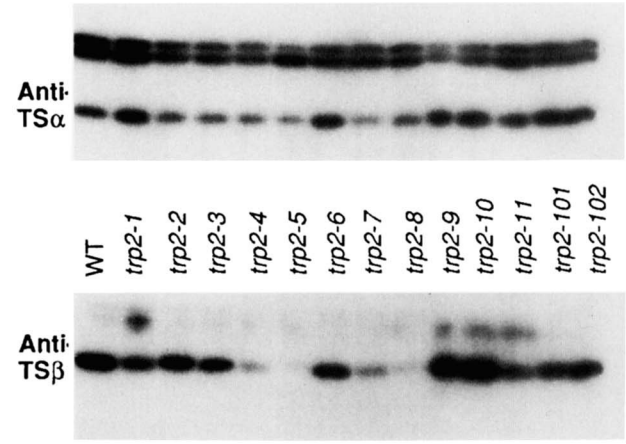

FIGURE 3.—Immunoblot analysis of tryptophan synthase alpha and beta subunit accumulation in *trp2* mutants. Duplicate protein blots were probed with antiserum against TSA (top) or TSB subunit (bottom) followed by ^{125}I-protein A and then subjected to autoradiography. The doublet above the alpha subunit signals is nonspecific: it is reduced in intensity when affinity purified antibody is used and is present in extracts from *trp3–2* mutant, which accumulates undetectable levels of TSA mRNA and protein (E. R. RADWANSKI, J. ZHAO and R. L. LAST, unpublished data).

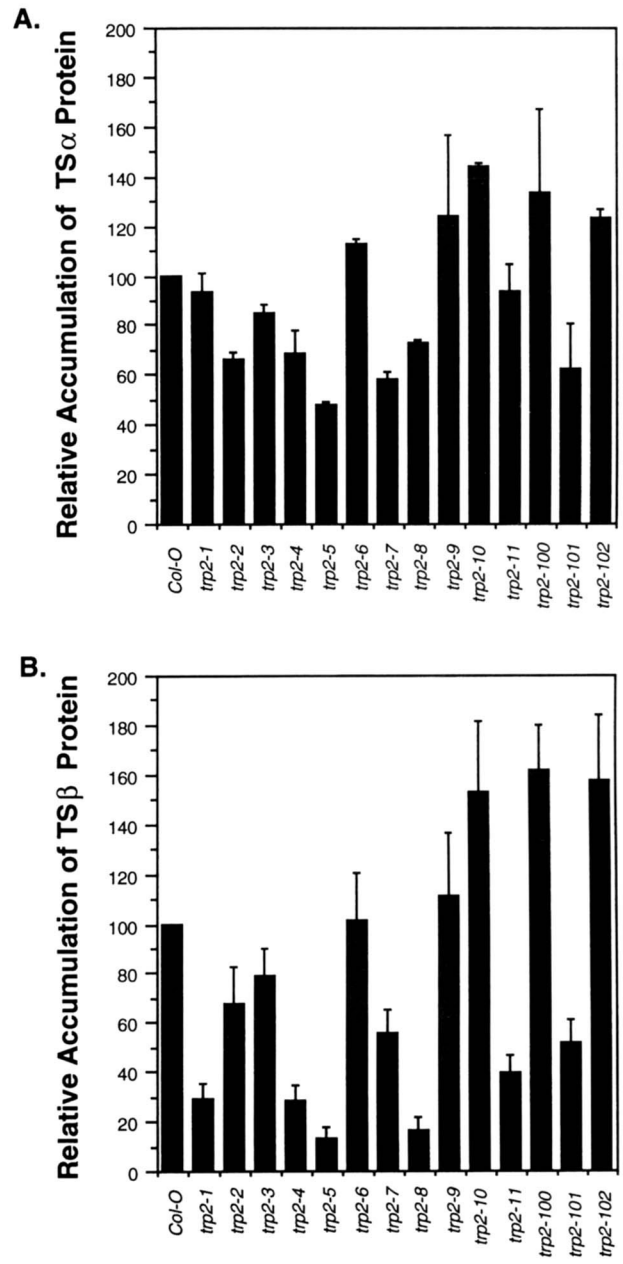

FIGURE 4.—Quantitation of tryptophan synthase subunit levels in *trp2* mutants. A and B show data for TSA and TSB results, respectively. The data plotted represent the cross-reactive material that migrated with the band of wild-type mobility. The mutant values are normalized to the wild-type signal for each blot ("100"). Each datum represents the mean of results from eight immunoblots of extracts derived from three independent sets of plants. Standard errors of the mean are indicated.

that the Arabidopsis TSA and TSB subunits co-purify during immunoaffinity chromatography, consistent with the existence of stable subunit interactions (E. R. RADWANSKI, J. ZHAO and R. L. LAST, unpublished results). It is interesting to note that there are exceptions to the correlation between TSA and TSB. For example, *trp2-1* and *trp2-11* have nearly wild-type levels of TSA protein despite decreased mutant TSB subunit accumulation.

Increased accumulation of anthranilate synthase alpha subunit: Previous results demonstrated ~50% increased anthranilate synthase enzyme activity in both the *trp1–100* (Table 5 in NIYOGI *et al.* 1993) and *trp2-1* (Table 3 in LAST *et al.* 1991) mutants. We extended these results by examining whether the concentration of immunologically detectable ASA protein was similarly affected in leaf tissue of the thirteen *trp2* mutants

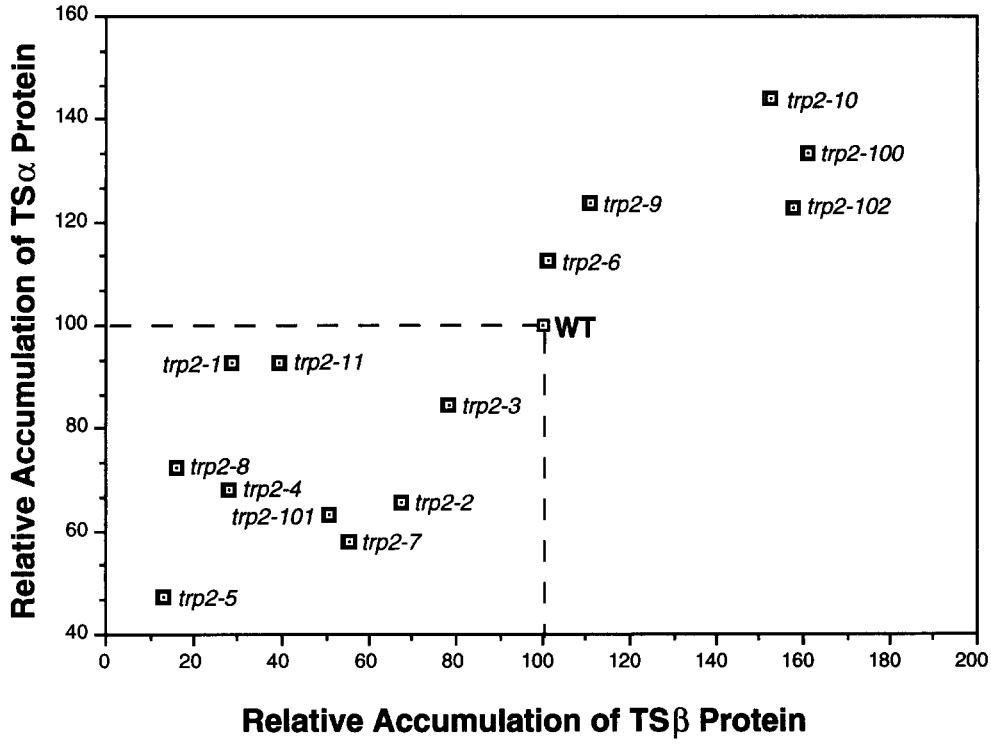

FIGURE 5.—Comparison of tryptophan synthase subunit levels in *trp2* mutants. The data plotted are from Figure 4, and the coefficient of linear regression (R^2) = 0.75.

tested. The results summarized in Figure 6 indicate that these mutants have reproducibly increased levels of ASA antigen, ranging from two- to sixfold higher than the wild type. Although further experiments will be necessary to assess the significance of the allele-specific differences and to elucidate the mechanism(s) responsible for this increase, it is clear that a defect in the last step in tryptophan biosynthesis alters the regulation of the commiting enzyme ASA protein.

Molecular biological analysis of the *trp2* mutations: We tested the hypothesis that *trp2* mutations might be associated with deletions or other rearrangements at the *TSB1* locus that would be detected by genomic Southern blot hybridization. When genomic DNA of the mutants was digested with restriction enzyme *Eco*RI, *trp2-10* was the only allele that displayed an aberrant pattern (A. J. BARCZAK, unpublished results). This mutant yielded *Eco*RI fragments of 1.3 and 2.9 kb, instead of the wild-type 4.2 kb fragment. This result is consistent with a mutation that creates a new *Eco*RI recognition site within the *TSB1* gene. As shown in Figure 7, DNA sequence analysis of *trp2-10* revealed a transition mutation at position 2859 of the *TSB1* gene that creates an *Eco*RI site and converts a phylogenetically conserved glycine (at amino acid 266 in Figure 2 of BERLYN *et al.* 1989) to a glutamate residue.

To understand the molecular defects that led to dramatically reduced protein accumulation, the *TSB1* genomic DNA sequence was also obtained for *trp2-5* and *trp2-8* (Figure 7). The EMS-induced allele *trp2-5* is a

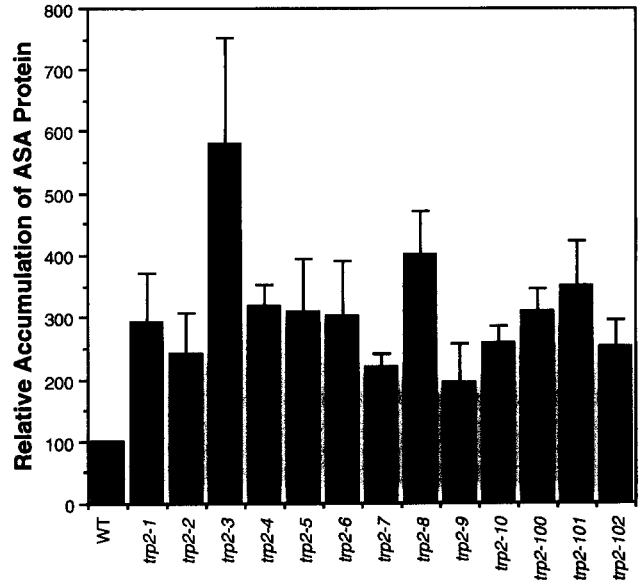

FIGURE 6.—Anthranilate synthase levels in *trp2* mutants. The mutant values are normalized to the wild-type for each blot ("100"). Each datum represents the mean of data from triplicate independent extracts, each assayed on duplicate immunoblots, for a total of six experiments. Standard errors of the mean are indicated. Extracts from *trp2-11* are not included in this set of data because multiple extracts were not available.

transition mutation that results in conversion of a phylogenetically conserved glycine to arginine at amino acid 351 (BERLYN *et al.* 1989). In contrast, the gamma ray-induced allele *trp2-8* has a more complex structure:

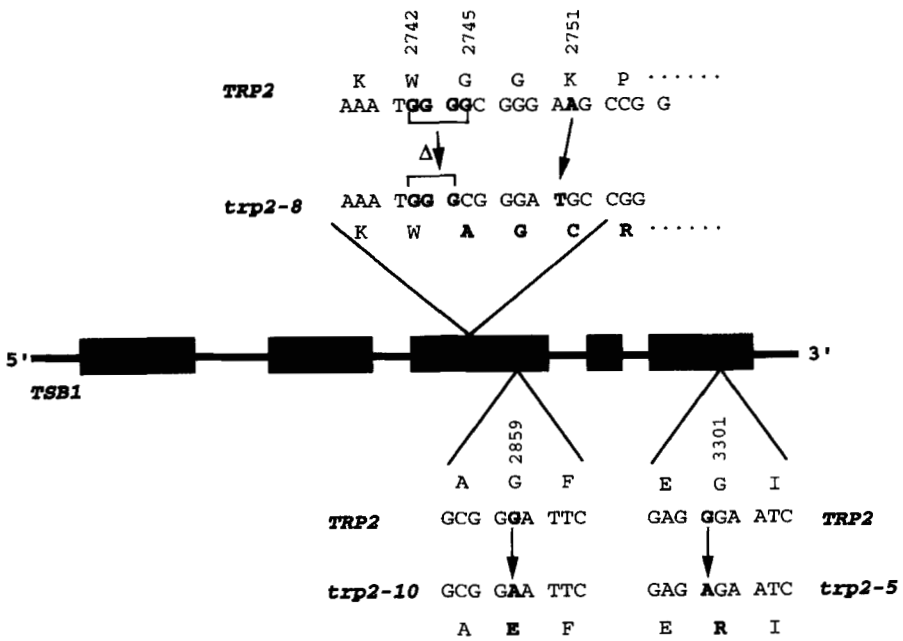

FIGURE 7.—DNA sequence changes in the *trp2-5* and *trp2-8* and *trp2-10* alleles. The *TSB1* gene is represented schematically, with exons as filled rectangles and introns as thinner lines. The wild-type and mutant sequences are indicated with the altered bases in boldface type.

there is a single base pair deleted from a cluster of four guanine residues (at positions 2742–2745 of the wild-type sequence) and a transversion mutation six nucleotides downstream (at position 2751 of the wild-type sequence). Translation of this mutant open reading frame is expected to yield a truncated protein of 251 amino acids (the inferred wild-type protein is 470 long), which would contain 23 abberant amino acids at the new COOH terminus.

DISCUSSION

Selection for resistance to 5-fluoroindole was used to identify 13 new mutations at the Arabidopsis *TRP2* locus, which encodes *TSB1*, the more highly expressed of duplicate tryptophan synthase beta subunit genes. The selection also produced several other classes of mutants, including four lines that contain recessive alleles that complement *trp2* and define at least two other complementation groups (A. J. BARCZAK, unpublished results). Interestingly, one of these complementing recessive alleles (isolate 13H4) causes accumulation of a variant TSA subunit that migrates unusually slowly on SDS-PAGE gels (data not shown). This result suggests that changes in the TSA protein can affect the efficiency of the beta subunit-mediated conversion of 5-fluoroindole to 5-fluorotryptopan. This observation is consistent with results in *Salmonella typhimurium*, demonstrating that changes in the conformation of either subunit can affect the catalytic activity of the other (MILES 1991). Thus, the selection for 5-fluoroindole resistance in Arabidopsis is versatile because it not only yields *trp2* alleles but also identifies plants with other types of defects. It is likely that the 5-fluoroindole resistance selection could also be sucessfully used to identify TS-deficient mutants of other organisms, including agronomically important plants. Because such mutants might have altered indolic secondary metabolism, these would be particularly useful to obtain in plants that produce pharmacologically important indolic alkaloids.

We analyzed the DNA sequence of the *TSB1* locus for three mutants, two of which were found to contain missense mutations. The *trp2-10* mutation creates an *Eco*RI polymorphism as a result of a G to A transition. This mutation is expected to produce an altered protein with a negatively charged amino acid (glutamate) in place of an absolutely phylogenetically conserved glycine (BERLYN *et al.* 1989). The role of this specific site was not defined from mutational or structural analysis of the *S. typhimurium* enzyme. However, it is in a region that interacts with the alpha subunit and is involved in producing the hydrophobic tunnel (HYDE *et al.* 1988). Presumably, the change from a small neutral amino acid to a charged residue in this important region affects the catalytic activity of the enzyme, leading to a tryptophan auxotrophy. *trp2-5* also contains a G to A transition mutation that changes a phylogenetically conserved glycine to a positively charged arginine residue. This amino acid falls in a random coil domain between helices 11 and 12 (HYDE *et al.* 1988). Surprisingly, these two missense mutations both affect TSB mRNA accumulation but in opposite directions. Whereas *trp2-10* causes enhanced mRNA and protein levels, *trp2-5* mutant plants accumulate only 34% of wild-type mRNA and an even lower amount of protein. It is unclear why these mutations alter TSB mRNA and protein accumulation. Further biochemical analysis of these missense mutant proteins and molecular analysis of the remaining alleles are necessary to understand the reasons for differences in accumulation of various mutant beta subunits.

The *trp2-8* mutant, which has the lowest concentra-

tions of TSB mRNA and protein, has both a single nucleotide deletion and a transversion clustered within a 10-nucleotide region of the third exon of *TSB1* (Figure 7). This complex mutation is expected to cause a translational frameshift and premature termination of translation following amino acid 251. Despite the expectation that the mutant *tsb1* gene would encode a truncated protein, the TSB protein that accumulates in *trp2-8* plants is the same size as that in the wild type (Figure 3, bottom). This strongly suggests that the residual TSB protein that accumulates in the *trp2-8* mutant is produced by the duplicate *TSB2* gene. The reduced accumulation of TSB mRNA is consistent with the hypothesis that the *trp2-8* mutation causes decreased stability of the mutant tsb1 mRNA. Analogous frameshift mutations were demonstrated to cause dramatically reduced mRNA accumulation for both a *Glycine max* (soybean) Kunitz trypsin inhibitor (JOFUKU *et al.* 1989) and *Phaseolus vulgaris* (pinto bean) phytohemagglutinin (VOELKER *et al.* 1990). In both cases, the reduced transcript accumulation was due to mRNA destabilization (JOFUKU *et al.* 1989; SULLIVAN and GREEN 1993).

The two clustered single nucleotide changes in the gamma ray-induced *trp2-8* allele contrast with the relatively large changes found in other characterized ionizing radiation-induced Arabidopsis mutations. For example, deletions were observed by Southern blot hybridization for all three gamma ray-induced *chl3* alleles in the major nitrate reductase gene *NIA2* (WILKINSON and CRAWFORD 1991) and for *chl1-5*, one of three gamma-induced mutations in a nitrate transporter gene (TSAY *et al.* 1993). Similarly, fast-neutron mutagenesis induced an inversion of the chalcone isomerase *TT5* gene and x-irradiation caused a complex *tt3* dihydroflavonol 4-reductase allele consisting of two adjacent deletions with an inversion of the large intervening region (SHIRLEY *et al.* 1992). Although our Southern blot hybridization data do not rule out the possibility of large rearrangements with breakpoints beyond the 4.2-kb *Eco*RI fragment that was analyzed, it seems likely that the *trp2-8* reduction in TSB mRNA is due to the two point mutations that were identified. This is because it was previously demonstrated that the 4.2-kb fragment was sufficient to complement the *trp2-1* mutation and restore wild-type TSB enzyme activity in transgenic plants (LAST *et al.* 1991).

Although mutations in the *TRP2* gene were shown to affect levels of three different tryptophan biosynthetic proteins (TSA, TSB and ASA), it seems likely that these altered protein levels are caused by different mechanisms. The differences in TSB protein accumulation are presumably the direct result of the mutations causing altered synthesis or stability of the mutant protein. In contrast, the positive correlation between TSA and TSB subunit accumulation (Figure 5) suggests that the TSA protein is less stable in the absence of a normal interaction with the TSB subunit. An alternative hypothesis to account for the apparent influence of beta subunit on alpha protein accumulation is that the plant uses a genetic regulatory mechanism to maintain the 1:1 subunit stoichiometry required for maximal catalytic activity. In this scenario, the cell would respond to accumulation of unusually low or high amounts of TSB protein concentration by reducing or stimulating the synthesis of TSA. This model is complicated by mutants such as *trp2-1* and *trp2-11*, which deviate from the general correlation between beta and alpha protein accumulation in the *trp2* mutants.

The increased ASA protein in *trp2* mutants (Figure 6) and enzyme activity in *trp1* and *trp2* mutants (LAST *et al.* 1991; NIYOGI *et al.* 1993) might also reflect a genetic regulatory mechanism. In one scenario, decreased tryptophan biosynthetic capacity would directly trigger an increase in synthesis or stability of the committing enzyme anthranilate synthase. An alternate model is suggested by the previous observation that mRNAs for both subunits of this enzyme are induced in response to stress induced by mechnical wounding and bacterial pathogenesis (NIYOGI and FINK 1992; NIYOGI *et al.* 1993). It is possible that synthesis of anthranilate synthase is induced by a wider variety of stress agents, and the observed increase in anthranilate synthase in *trp2* mutants is the result of a tryptophan limitation-provoked stress response.

The increased activity of this regulated enzyme might be expected to increase the flux of chorismate into indolic biosynthesis. This may account for the observation that the *trp2-1* mutant accumulates unusually high levels of indolic compounds such as the ester and amide conjugates of indole-3-acetic acid and free indole-3-acetonitrile (NORMANLY *et al.* 1993). Further characterization of this regulatory response should provide new insights into the regulation of the Arabidopsis tryptophan pathway in general and into the committing enzyme in particular. These studies may also suggest novel approaches to manipulating indolic secondary metabolism in plants.

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