RESEARCH ARTICLE

Suppressors of *trp1* Fluorescence Identify a New Arabidopsis Gene, *TRP4*, Encoding the Anthranilate Synthase β Subunit

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Suppressors of the blue fluorescence phenotype of the Arabidopsis *trp1-100* mutant can be used to identify mutations in genes involved in plant tryptophan biosynthesis. Two recessive suppressor mutations define a new gene, *TRP4*. The *trp4* mutant and the *trp1-100* mutant are morphologically normal and grow without tryptophan, whereas the *trp4; trp1-100* double mutant requires tryptophan for growth. The *trp4; trp1-100* double mutant does not segregate at expected frequencies in genetic crosses because of a female-specific defect in transmission of the double mutant genotype, suggesting a role for the tryptophan pathway in female gametophyte development. Genetic and biochemical evidence shows that *trp4* mutants are defective in a gene encoding the β subunit of anthranilate synthase (AS). Arabidopsis *AS* β subunit genes were isolated by complementation of an *Escherichia coli* anthranilate synthase mutation. The *trp4* mutation cosegregates with one of the genes, *ASB1*, located on chromosome 1. Sequence analysis of the *ASB1* gene from *trp4-1* and *trp4-2* plants revealed different single base pair substitutions relative to the wild type. Anthranilate synthase α and β subunit genes are regulated coordinately in response to bacterial pathogen infiltration.

INTRODUCTION

In plants, aromatic amino acids are precursors to proteins as well as a diverse array of important plant secondary metabolites involved in normal development and defense responses, including the phytohormone indole-3-acetic acid, the structural polymer lignin, antimicrobial phytoalexins, and alkaloids. Until recently, genetic analysis of aromatic amino acid metabolism in plants was hindered by the lack of biosynthetic mutants. Isolation of tryptophan auxotrophs in Arabidopsis and maize, however, has uncovered a variety of interesting developmental and biochemical phenotypes that underscore the importance of aromatic amino acid metabolism in plant development (Last and Fink, 1988; Last et al., 1991; Wright et al., 1992). These phenotypes include decreased apical dominance, increased indole-3-acetic acid production, seedling lethality, decreased fertility, altered leaf morphology, amino acid analog resistance, and blue fluorescence.

Extragenic suppressors or enhancers that alter the phenotype of a known mutation may be used to identify new genes in a pathway. Suppressor analysis has been a powerful method for identifying interacting genes in many organisms, including bacteriophage (Jarvik and Botstein, 1975), fungi (Novick et al., 1989), Drosophila (Mortin, 1990), *Caenorhabditis elegans* (Greenwald and Horvitz, 1980), and plants (Koornneef et al., 1982). For example, Arabidopsis abscisic acid–deficient mutants were isolated as suppressors of the germination defect of gibberellin mutants (Koornneef et al., 1982). This provided genetic confirmation of the role of abscisic acid in Arabidopsis seed germination.

To identify new genes involved in aromatic amino acid biosynthesis in Arabidopsis, we screened for suppressors of the blue fluorescence phenotype of the *trp1-100* mutation (Rose et al., 1992). Arabidopsis seedlings homozygous for *trp1-100* are deficient in the enzyme anthranilate phosphoribosyltransferase (PAT), encoded by the *TRP1* gene (Rose et al., 1992). As shown in Figure 1, PAT catalyzes the second step in the tryptophan branch of the aromatic pathway. Decreased PAT activity results in the accumulation of anthranilate compounds in the cotyledons and leaves of mutant plants, which consequently fluoresce blue under UV illumination (Last and Fink, 1988; Rose et al., 1992). The *trp1-100* strain was chosen for suppressor analysis, because, in addition to exhibiting the blue fluorescence phenotype, it is prototrophic and displays

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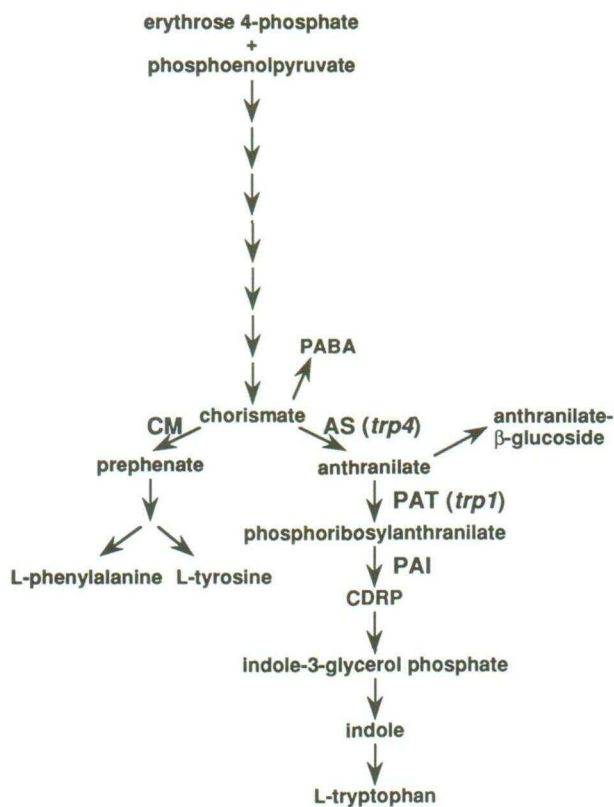
erythrose 4-phosphate
+
phosphoenolpyruvate

↓

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PABA

CM ↙ **chorismate** **AS (trp4)** ↘ anthranilate-
β-glucoside

prephenate   **anthranilate**

↙   ↘   ↓ **PAT (trp1)**

L-phenylalanine L-tyrosine   **phosphoribosylanthranilate**

↓ **PAI**

CDRP

↓

indole-3-glycerol phosphate

↓

indole

↓

L-tryptophan

Figure 1. The Plant Aromatic Amino Acid Biosynthetic Pathway.

Selected intermediates, products, and enzymes are shown. The enzymes defective in Arabidopsis *trp4* and *trp1* mutants are indicated. PABA, *para*-aminobenzoic acid; CM, chorismate mutase; AS, anthranilate synthase; PAT, anthranilate phosphoribosyltransferase; PAI, phosphoribosylanthranilate isomerase; and CDRP, 1-(*o*-carboxyphenyl-amino)-1-deoxyribulose 5-phosphate.

Figure 2. Phenotypes of *trp1-100* Suppressors.

Arabidopsis plants were grown for 8 days as described in Methods. The plants in rows are Col-0 wild type, *trp1-100*, and three independent suppressors, D1, E17, and A2.
(A) PNS medium supplemented with 10 μM each of Phe, Tyr, Trp, and PABA; photographed under white light.
(B) Same as shown in **(A)** but photographed under UV light.
(C) PNS medium; photographed under white light.

RESULTS

Two Suppressors of *trp1-100* Blue Fluorescence Are Conditional Tryptophan Auxotrophs

M$_2$ seeds (28,000) prepared from a homozygous *trp1-100* mutant strain (Rose et al., 1992) were germinated on synthetic

normal growth and fertility, despite greatly diminished levels of PAT activity. Furthermore, the blue fluorescence phenotype is easily scored in UV-illuminated cotyledons of *trp1-100* seedlings grown on agar medium.

Several different classes of *trp1-100* suppressors are expected from this genetic screen. Of particular interest are loss-of-function mutations that reduce the activity of enzymes acting earlier in the pathway than *TRP1*. These mutations would be expected to decrease the metabolic flow through the pathway, thereby reducing production of fluorescent anthranilate compounds. Other classes of suppressors might include mutations in *trans*-acting regulatory genes that alter metabolic flux in the aromatic pathway, intragenic revertants of the *trp1-100* mutant allele, and mutations that inactivate or mask the fluorescent anthranilate compounds. Here, we describe the isolation of suppressors of *trp1-100* blue fluorescence and present the genetic, biochemical, and molecular characterization of two extragenic suppressors, which identify a new gene, *TRP4*, encoding the β subunit of anthranilate synthase (AS).
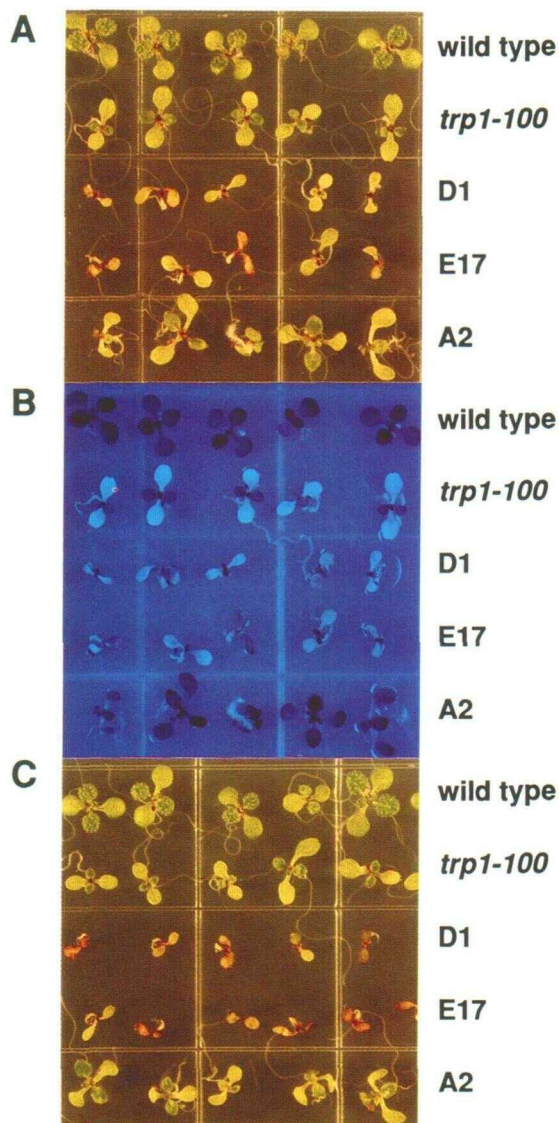
agar medium and screened for decreased blue fluorescence (see Methods). To improve the chances of recovering mutations conferring aromatic amino acid auxotrophy, the agar medium was supplemented with 10 μM each of phenylalanine, tyrosine, tryptophan, and *para*-aminobenzoic acid (PABA) (see Figure 1). M_2 plants with reduced fluorescence (165) were transferred to soil, and 28 produced less fluorescent M_3 progeny when self-pollinated. Blue fluorescence was completely suppressed in some mutant strains and only partially suppressed in others, as shown in Figure 2. Strain A2 is a representative mutant that was completely nonfluorescent (Figure 2B), but it did not require supplementation of the medium for growth (Figure 2C). Two weakly blue fluorescent strains, called D1 and E17, required the amino acid supplement for growth on synthetic medium (Figure 2C) and were chosen for further analysis.

Intermediates in the tryptophan branch of the aromatic pathway fulfilled the growth requirement of D1 and E17. Anthranilate, indole, or tryptophan at a concentration of 10 μM allowed growth of D1 and E17, as evidenced by root elongation and appearance of true leaves (data not shown). Consistent with a block in the trytophan pathway, chorismate, PABA, phenylalanine, tyrosine, or phenylalanine and tyrosine together failed to support growth of D1 or E17. As anthranilate supported growth, but chorismate did not, we inferred that the auxotrophy in D1 and E17 might be caused by a defect in AS activity (see Figure 1). Data from enzyme activity assays that confirmed this prediction, as well as the phenotypes of D1 and E17 grown in soil, are discussed subsequently.

The auxotrophic phenotype of D1 and E17 is light conditional. D1 and E17 seeds germinated on unsupplemented agar medium and grown under standard light intensities (125 μE m^{-2} sec^{-1}) displayed the auxotrophic phenotype (Figure 2C). When grown under "low" light conditions (40 μE m^{-2} sec^{-1}), D1 and E17 seedlings elaborated roots and produced several sets of true leaves (data not shown). All of the Arabidopsis tryptophan auxotrophs isolated to date display this light-conditional phenotype, the implications of which have been discussed previously (Last et al., 1991).

Genetic Characterization of Tryptophan-Requiring Suppressors

When D1 and E17 were backcrossed to the parent *trp1-100* strain, all F_1 progeny were prototrophic (Trp^+), as shown in Table 1. Moreover, the tryptophan auxotrophic (Trp^-) phenotype appeared in ~25% of the subsequent F_2 progeny. These data indicated that in the *trp1-100* genetic background, the Trp^- phenotype is caused by segregation of a single, recessive nuclear mutation. The Trp^+:Trp^- ratio observed in the F_2 populations from individual backcrosses was often closer to 4:1 and may be caused by decreased viability of mutant gametes (certation; see Dellaert, 1980) or by deleterious effects of other ethylmethane sulfonate–induced mutations in D1 or E17.

Crosses between D1 and E17 yielded only Trp^- F_1 progeny (Table 1). This lack of complementation suggests that D1 and E17 contain mutations in the same gene. D1 and E17 were isolated from separate pools of M_2 seeds and are therefore derived from independent mutagenic events.

Segregation of phenotypes in outcrosses between D1 and three different wild-type strains, shown in Table 2, suggests that the suppressor mutation is unlinked to *trp1-100* and that both the suppressor mutation and *trp1-100* are necessary for the Trp^- phenotype. Results of outcrosses of E17 were essentially identical (data not shown). The recovery of Trp^+, strongly blue fluorescent, homozygous *trp1-100* progeny in the F_2 populations shows that the suppressor mutation and *trp1-100* are not tightly linked. All Trp^- F_2 progeny were also weakly blue fluorescent, demonstrating that the Trp^- phenotype of D1 and E17 is due to interactions between at least two unlinked homozygous recessive mutations, one of which is *trp1-100*. The second (suppressor) mutation defines a new gene, *TRP4*. We have designated the mutant allele in D1 as *trp4-1* and the

Table 1. Results of Crosses between D1, E17, and *trp1-100* Strains[a]

Cross	Type	Total	Trp^+ Strongly Fluor.	Trp^- Weakly Fluor.	Trp^-/Total
trp4-1/trp4-1; *trp1-100/trp1-100* (D1)♂	F_1	30	30	0	
× *TRP4/TRP4*; *trp1-100/trp1-100* ♀	F_2[b]	600	466	134	22.3%
	F_2[c]	360	296	64	18%
trp4-2/trp4-2; *trp1-100/trp1-100* (E17) ♂	F_1	22	22	0	
× *TRP4/TRP4*; *trp1-100/trp1-100* ♀	F_2	379	313	66	17%
trp4-2/trp4-2; *trp1-100/trp1-100* (E17) ♂	F_1	85	0	85	
× *trp4-1/trp4-1*; *trp1-100/trp1-100* (D1) ♀					

[a] Pollen from the male parent (♂) was crossed onto the stigmas of the female parent (♀) to generate F_1 seeds. The heterozygous F_1 plants were allowed to self-pollinate to generate F_2 seeds. The phenotypes of F_1 and F_2 plants were scored after 1 to 2 weeks growth on PNS medium as described in Methods. Fluor., blue fluorescent.
[b] First backcross.
[c] Second backcross.

Table 2. Results of Crosses between D1 and Wild-Type Strains

Cross	Type	Total	Trp+ Nonfluor.	Trp+ Strongly Fluor.	Trp- Weakly Fluor.	Fluor./ Total	Trp-/Total
trp4-1/trp4-1; trp1-100/trp1-100 ♂	F₁	27	27	0	0		
× TRP4/TRP4; TRP1/TRP1 (Col-0) ♀	F₂	669	564	96	9	15.7%	1%
trp4-1/trp4-1; trp1-100/trp1-100 ♂	F₁	14	14	0	0		
× TRP4/TRP4; TRP1/TRP1 (Ler) ♀	F₂	1432	1180	234	18	17.6%	1.3%
trp4-1/trp4-1; trp1-100/trp1-100 ♂	F₁	6	6	0	0		
× TRP4/TRP4; TRP1/TRP1 (No-0) ♀	F₂	600	448	145	7	25.3%	1%

Pollen from the male parent (♂) was crossed onto the stigmas of the female parent (♀) to generate F₁ seeds. The heterozygous F₁ plants were allowed to self-pollinate to generate F₂ seeds. The phenotypes of F₁ and F₂ plants were scored after 1 to 2 weeks growth on PNS medium as described in Methods. Fluor., blue fluorescent.

mutant allele in E17 as trp4-2. The trp4-1 and trp4-2 mutations were indistinguishable in their interaction with trp1-100, and for simplicity we will refer only to the trp4-1 allele in the subsequent results.

Tryptophan-Requiring Double Mutants Are Underrepresented in Crosses Due to a Female-Specific Effect

The frequency of Trp⁻ F₂ progeny from outcrosses ranged between 1.0 and 1.3% in each of three independent crosses, (Table 2), lower than the expected value of 6.25% (15:1 Trp⁺:Trp⁻) for the segregation of two unlinked recessive mutations. Two unlinked recessive visible markers in the Landsberg erecta (Ler) strain, erecta (er) and transparent testa 4 (tt4), displayed standard Mendelian 9:3:3:1 segregation (of 129 F₂ plants scored, 76 were Er⁺, Tt4⁺; 20 were Er⁻, Tt4⁺; 25 were Er⁺, Tt4⁻; and eight were Er⁻, Tt4⁻ [χ^2 = 0.92, P > 0.05]), indicating that the low recovery of Trp⁻ F₂ plants was not due to some general distortion of segregation in these crosses.

The low percentage of Trp⁻ F₂ progeny suggested that the Trp⁻ phenotype may require the presence of additional unlinked recessive mutations segregating in the outcrosses. However, the testcross data shown in Table 3 demonstrated that two unlinked recessive mutations (trp4-1 and trp1-100) can account fully for the Trp⁻ phenotype. When pollen from a doubly heterozygous trp4-1/TRP4; trp1-100/TRP1 plant was crossed onto the stigmas of trp4-1/trp4-1; trp1-100/trp1-100 plants (Table 3), approximately one-quarter (22%) of the resulting F₁ progeny were Trp⁻ (trp4-1/trp4-1; trp1-100/trp1-100). An additional one-quarter of the F₁ progeny were Trp⁺ and strongly blue fluorescent (trp4-1/TRP4; trp1-100/trp1-100). These data are consistent with a genetic model in which unlinked homozygous trp4-1 and trp1-100 alleles are both required for the Trp⁻ phenotype. These data also predicted that plants homozygous for the trp4 mutant allele would not be tryptophan auxotrophs unless they are also homozygous for trp1-100 (see below).

The reciprocal testcross was performed in which pollen from homozygous trp4-1/trp4-1; trp1-100/trp1-100 plants was crossed onto stigmas of doubly heterozygous plants (Table 3). One-quarter of the F₁ progeny of this testcross were Trp⁺ and strongly fluorescent (trp4-1/TRP4; trp1-100/trp1-100), as

Table 3. Results of Reciprocal Testcrosses

Cross	Type	Total	Trp+ Nonfluor.	Trp+ Strongly Fluor.	Trp- Weakly Fluor.	Fluor./ Total	Trp-/Total
trp4-1/TRP4; trp1-100/TRP1 ♂ × trp4-1/trp4-1; trp1-100/trp1-100 ♀	testcross	98	51	28	22	48%	22%
trp4-1/trp4-1; trp1-100/trp1-100 ♂ × trp4-1/TRP4; trp1-100/TRP1 ♀	testcross	402	279	98	25	30.6%	6.2%
trp4-1/TRP4; trp1-100/trp1-100 ♂ × trp4-1/trp4-1; trp1-100/trp1-100 ♀	testcross	60	0	30	30	100%	50%
trp4-1/trp4-1; trp1-100/trp1-100 ♂ × trp4-1/TRP4; trp1-100/trp1-100 ♀	testcross	164	0	95	69	100%	42%

Pollen from the male parent (♂) was crossed onto the stigmas of the female parent (♀) to generate F₁ seeds. The phenotypes of testcross F₁ plants were scored after 1 to 2 weeks growth on PNS medium as described in Methods. Fluor., blue fluorescent.

expected, but the Trp⁻ homozygous double mutant phenotype appeared at an unexpectedly low frequency. If heterozygous plants produced *trp4-1*; *trp1-100* double mutant female gametes at the same frequency that they produced double mutant male gametes, we would have expected approximately 25% of the F_1 progeny from this testcross to be Trp⁻. Table 3 shows that only 6.2% of these F_1 plants were Trp⁻. These results suggest that transmission of the *trp4-1*; *trp1-100* double mutant genotype in female gametophytic tissue is severely depressed in plants heterozygous for both mutations. It is not known whether this female-specific defect disrupts the formation of viable gametes, zygotes, or embryos. The relative proportion of Trp⁻ F_1 progeny in these testcrosses was used to predict the percentage of Trp⁻ plants expected among the outcrossed F_2 population (specifically, $0.22 \times 0.062 = 0.014 = 1.4\%$ Trp⁻ F_2 plants). As shown in Table 2, the observed frequencies correspond closely to this expected percentage.

Testcrosses were also performed with strains heterozygous only for the *trp4-1* mutation. When pollen from a *trp4-1/TRP4*; *trp1-100/trp1-100* plant was crossed onto the double mutant *trp4-1/trp4-1*; *trp1-100/trp1-100* strain, 50% of the resulting F_1 progeny were Trp⁻ (Table 3). Because the *trp1-100* allele was not segregating in this cross, this result confirms that only one additional unlinked recessive mutation (*trp4-1*) was required for the Trp⁻ phenotype. Similar results were obtained in reciprocal testcrosses, although the number of Trp⁻ F_1 progeny was slightly lower (42%) when the heterozygous parent was female (Table 3). The proportion of Trp⁻ F_2 progeny predicted from a backcross between a *trp4-1/trp4-1*; *trp1-100/trp1-100* strain and a *TRP4/TRP4*; *trp1-100/trp1-100* strain would be $0.50 \times 0.42 = 0.21 = 21\%$. This is consistent with the ratio of ∼4:1 Trp⁺:Trp⁻ observed in backcrosses (Table 1).

Isolation of *trp4-1/trp4-1 TRP1/TRP1* Mutants

Because the data presented above suggest that *trp4-1/trp4-1*; *TRP1/TRP1* plants would show neither blue fluorescence nor tryptophan auxotrophy, we followed the procedure outlined in Figure 3 to obtain homozygous *trp4-1/trp4-1*; *TRP1/TRP1* strains. Given the absence of a visible *trp4* mutant phenotype, we first sought to identify a *trp4-1/trp4-1*; *trp1-100/TRP1* plant, which was predicted to be Trp⁺ and nonfluorescent. A further prediction was that self-pollination of a plant of this genotype would never yield Trp⁺, strongly fluorescent progeny but would yield two other phenotypic classes: Trp⁻ (*trp4-1/trp4-1*; *trp1-100/trp1-100*) and Trp⁺, nonfluorescent (*trp4-1/trp4-1*; *trp1-100/TRP1* and *trp4-1/trp4-1*; *TRP1/TRP1*) (Figure 3A). The desired *trp4-1/trp4-1*; *TRP1/TRP1* plants could be distinguished from their *trp4-1/trp4-1*; *trp1-100/TRP1* siblings by progeny testing (Figure 3B). The Trp⁺, nonfluorescent F_2 progeny from the outcross between the *trp4-1/trp4-1*; *trp1-100/trp1-100* strain (D1) and a wild-type strain (Table 2) were transferred to soil, and F_3 seeds were collected. Progeny from 56 different F_3 families were scored for the Trp⁻ and blue fluorescence phenotypes. Three F_2 plants, designated 12, 14, and 59, produced F_3 progeny in which the
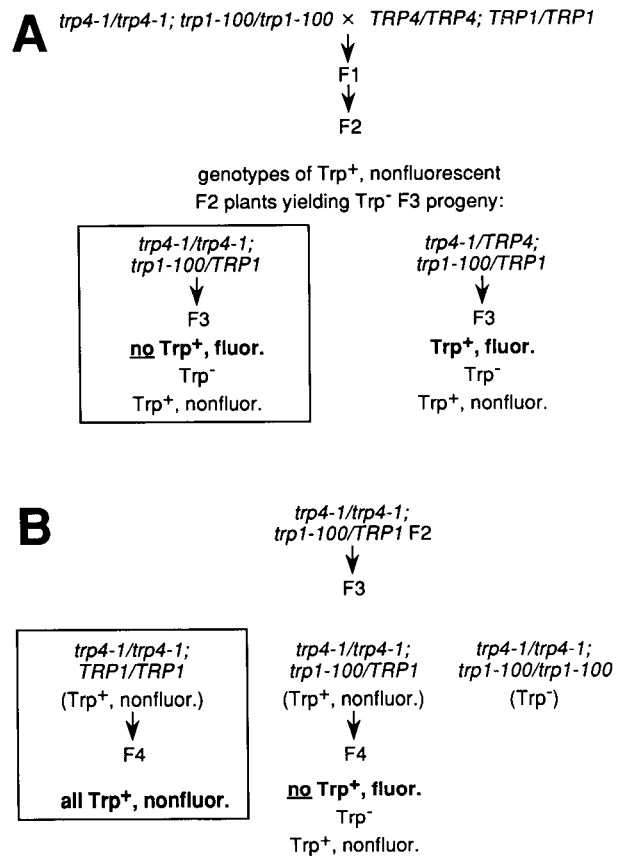

Figure 3. A Scheme for Identification of *trp4-1/trp4-1*; *TRP1/TRP1* Plants.

(A) Initial identification of *trp4-1/trp4-1*; *trp1-100/TRP1* F_2 plants. Individual nonfluorescent (nonfluor.), Trp⁺ F_2 progeny of a cross between D1 and wild type were self-pollinated, and F_3 seeds were collected as families. Nonfluorescent F_2 plants that produced Trp⁻ F_3 progeny must have been heterozygous for the recessive *trp1-100* mutation and either homozygous or heterozygous for the *trp4-1* mutation. F_2 plants with the *trp4-1/trp4-1*; *trp1-100/TRP1* genotype segregated no Trp⁺, strongly fluorescent F_3 progeny.

(B) Genotypes and phenotypes of F_3 progeny of *trp4-1/trp4-1*; *trp1-100/TRP1* F_2 plants. The desired *trp4-1/trp4-1*; *TRP1/TRP1* strain was Trp⁺ and nonfluorescent and yielded no Trp⁻ F_4 progeny after self-pollination. Additional genetic and biochemical tests to confirm this genotype are discussed in the text.

only fluorescent seedlings were Trp⁻ and weakly blue fluorescent, as expected for a *trp4-1/trp4-1*; *trp1-100/TRP1* F_2 plant. Eight Trp⁺, nonfluorescent F_3 siblings from plant 59 were transferred to soil, and those with the *trp4-1/trp4-1*; *TRP1/TRP1* genotype were identified by three genetic criteria: (1) they segregated neither Trp⁻ nor blue fluorescent progeny when self-pollinated, (2) when crossed to the original *TRP4/TRP4*; *trp1-100/trp1-100* strain, the resulting F_1 progeny were Trp⁺ and nonfluorescent, and (3) when these doubly heterozygous F_1 seeds were self-pollinated, blue fluorescent and Trp⁻ F_2

Table 4. Results of Crosses between *trp4-1* and *trp1-100* Strains

Cross	Type	Total	Trp+ Nonfluor.	Trp+ Strongly Fluor.	Trp- Weakly Fluor.	Fluor./ Total	Trp-/ Total
trp4-1/trp4-1; TRP1/TRP1 (59-A) ♂ × *trp4-1/trp4-1; TRP1/TRP1* (59-A) ♀	self	235	235	0	0		
trp4-1/trp4-1; TRP1/TRP1 (59-A) ♂ × *TRP4/TRP4; trp1-100/trp1-100* ♀	F₁ F₂	36 592	36 501	0 76	0 15	 15%	 2.5%
trp4-1/trp4-1; TRP1/TRP1 (59-B) ♂ × *trp4-1/trp4-1; TRP1/TRP1* (59-B) ♀	self	226	226	0	0		
trp4-1/trp4-1; TRP1/TRP1 (59-B) ♂ × *TRP4/TRP4; trp1-100/trp1-100* ♀	F₁ F₂	37 596	37 504	0 92	0 11	 17.3%	 1.8%

Plants were allowed to self-pollinate to generate self-progeny. Pollen from the male parent (♂) was crossed onto the stigmas of the female parent (♀) to generate F₁ seeds. The heterozygous F₁ plants were allowed to self-pollinate to generate F₂ seeds. The phenotypes of plants resulting from selfing or crossing were scored after 1 to 2 weeks growth on PNS medium as described in Methods. Fluor., blue fluorescent.

progeny appeared at frequencies similar to those seen in the outcrosses listed in Table 2. As shown in Table 4, two distinct F₃ plants, 59-A and 59-B, met these three criteria and were therefore *trp4-1/trp4-1; TRP1/TRP1* strains.

Figure 4 shows the phenotype of *trp4-1/trp4-1* plants, *trp1-100/trp1-100* plants, and *trp4-1/trp4-1; trp1-100/trp1-100* double mutant plants grown in soil. Both single mutant strains appeared phenotypically normal and flowered at approximately the same age as wild-type plants. The double mutant strain was not strictly auxotrophic on soil, although it grew slowly and produced small, dark purple leaves. When these double mutant seeds were first germinated on agar medium containing anthranilate, indole, or tryptophan and then transferred to soil, the rosette leaves were greener and somewhat larger, although they never attained the size of wild-type or single mutant leaves, even when watered with tryptophan (data not shown). The double mutant strain was fertile and produced viable seeds, although it flowered slightly later than either wild-type or single mutant strains. Similar results were observed for *trp4-2/trp4-2; trp1-100/trp1-100* double mutant strains (data not shown).

trp4-1 Mutants Are Deficient in AS Activity

The ability of D1 plants to grow in the presence of anthranilate, indole, or tryptophan suggested that the *trp4-1* mutation results in a defect in AS activity. Results from enzyme assays conducted in duplicate with whole plant extracts are shown in Table 5. The *trp4-1/trp4-1; TRP1/TRP1* mutant strains 59-A and 59-B, as well as the *trp4-1/trp4-1; trp1-100/trp1-100* double mutant strain (D1), had decreased AS activity when compared to two wild-type strains (Columbia [Col-0] and Ler) and the

**trp1-100   trp4-1   trp4-1
                              trp1-100**

Figure 4. Phenotypes of *trp1-100*, *trp4-1*, and Double Mutant Plants Grown in Soil.

Seeds from *trp1-100* and *trp4-1* single and double mutant strains were germinated on soil and grown under continuous light for 29 days. Phenotypes are discussed in detail in the text.

Table 5. Enzyme Specific Activities[a]

Strain	Background	AS	PAT	PAI[b]	CM[c]
TRP4/TRP4; TRP1/TRP1	Col-0	18.5 ± 1.5	1600 ± 200	1600 ± 500	690 ± 160
TRP4/TRP4; TRP1/TRP1	Ler	36.0 ± 10.0	1700 ± 100	2550 ± 550	720 ± 170
TRP4/TRP4; trp1-100/trp1-100	Col-0	29.5 ± 0.5	<100[d]	1700 ± 500	720 ± 170
trp4-1/trp4-1; TRP1/TRP1 (59-A)	Col-0 × Ler	7.1 ± 0.8	2100 ± 0	2400 ± 100	670 ± 140
trp4-1/trp4-1; TRP1/TRP1 (59-B)	Col-0 × Ler	3.0 ± 1.4	2850 ± 50	2750 ± 250	670 ± 140
trp4-1/trp4-1; trp1-100/trp1-100 (D1)	Col-0	<1[e]	<100[d]	2600 ± 100	645 ± 25

[a] The results (mean ± difference from the mean) of two independent experiments are reported in picomoles of product formed or substrate used per minute per milligram of protein.
[b] PAI, phosphoribosylanthranilate isomerase.
[c] CM, chorismate mutase.
[d] The limit of detection for the PAT assay was estimated to be 5% of the Col-0 wild-type value.
[e] The limit of detection for the glutamine-dependent AS assay was estimated to be 5% of the Col-0 wild-type value. Glutamine-dependent AS activity was detectable in *trp4-1/trp4-1; trp1-100/trp1-100* extracts in other experiments, but those experiments did not include all the strains listed in this table and were not presented.

trp1-100/trp1-100 strain. The level of AS activity from *trp4-1* strains was less than 40% of the activity from the Col-0 wild type. As expected, PAT activity, encoded by the *TRP1* gene (Rose et al., 1992), was not detected in extracts from either the *trp1-100/trp1-100* strain or the *trp4-1/trp4-1; trp1-100/trp1-100* double mutant strain. The activities of two other aromatic pathway enzymes, chorismate mutase and phosphoribosylanthranilate isomerase, were not significantly altered in either *trp4-1* or *trp1-100* mutant strains. These data suggest that *trp4* plants are defective in an AS structural gene or in a regulatory gene specific for AS.

TRP4 Maps to Chromosome 1

The *trp4-1* mutation was mapped relative to restriction fragment length polymorphism (RFLP) markers to determine whether the decreased AS activity in *trp4-1* strains was due to a mutation in either of the previously characterized Arabidopsis AS α subunit genes, *ASA1* and *ASA2* (Niyogi and Fink, 1992). Genomic DNA was prepared from plants in F_3 families derived from 33 Trp⁻ F_2 progeny of the cross between the *trp4-1/trp4-1; trp1-100/trp1-100; TT4/TT4; ER/ER* strain (Col-0 background) and the *TRP4/TRP4; TRP1/TRP1; tt4/tt4; er/er* strain (Ler background) (Table 2), and the segregation of RFLP markers was determined for three loci on each chromosome using a polymerase chain reaction (PCR)–based technique (Konieczny and Ausubel, 1993; also see Methods).

Because the Trp⁻ F_2 progeny were homozygous for both *trp4-1* and *trp1-100*, we expected to detect two Col-0 loci cosegregating with the Trp⁻ phenotype. One locus mapped approximately 8 centimorgans (cM) from the *ASA1* gene on chromosome 5, as shown in Figure 5. Because *TRP1* was previously mapped to this region of chromosome 5 (Rose et al., 1992), this result strongly supports the genetic evidence that

the Trp⁻ phenotype in these strains requires homozygous *trp1-100* alleles. In addition, no Trp⁻, Tt4⁻ mutants were recovered in the F_2 population, reflecting the close linkage between *trp1* and *tt4* (Rose et al., 1992). The second locus, *trp4*, mapped to chromosome 1 between the RFLP markers *GAPB* (glyceraldehyde-3-phosphate dehydrogenase B) and *CHL1* (chlorate resistance) approximately 15 cM from *GAPB* and 22 cM from *CHL1* (Figure 5), demonstrating that *trp4* is unlinked to *ASA1* and *ASA2*. No other Col-0 or Ler loci showed statistically significant linkage to the Trp⁻ or fluorescence phenotypes.

Isolation of Arabidopsis AS β Subunit Genes

Plant AS, like its microbial counterparts, appears to be composed of α and β subunits (Niyogi and Fink, 1992; Poulsen et al., 1993). The α subunit binds the substrate chorismate and catalyzes its aromatization, and the β subunit supplies the glutamine amidotransferase activity. Because the *trp4-1* mutation leads to a defect in AS activity (Table 5), yet is unlinked to the α subunit genes *ASA1* and *ASA2* (Figure 5), we investigated the possibility that *TRP4* encodes the AS β subunit.

Arabidopsis genes encoding the β subunit of AS were isolated by complementation in *Escherichia coli*. *E. coli trpΔED27 tnaA2* (Jackson and Yanofsky, 1974) is unable to grow on minimal medium lacking tryptophan, because it lacks AS α subunit (encoded by *trpE*) and β subunit (encoded by the amino-terminal third of *trpD*) activities. The α subunit deficiency of *trpΔED27* was covered by introducing an Arabidopsis AS α subunit gene on a plasmid, as shown in Figure 6A. The resulting *trpΔED27* strains containing the *ASA1* plasmid, pKN243A, or the *ASA2* plasmid, pKN132A, were unable to grow on low ammonia minimal medium lacking tryptophan, because AS β subunit activity is required under these conditions (Niyogi and Fink, 1992). These strains were transformed with an
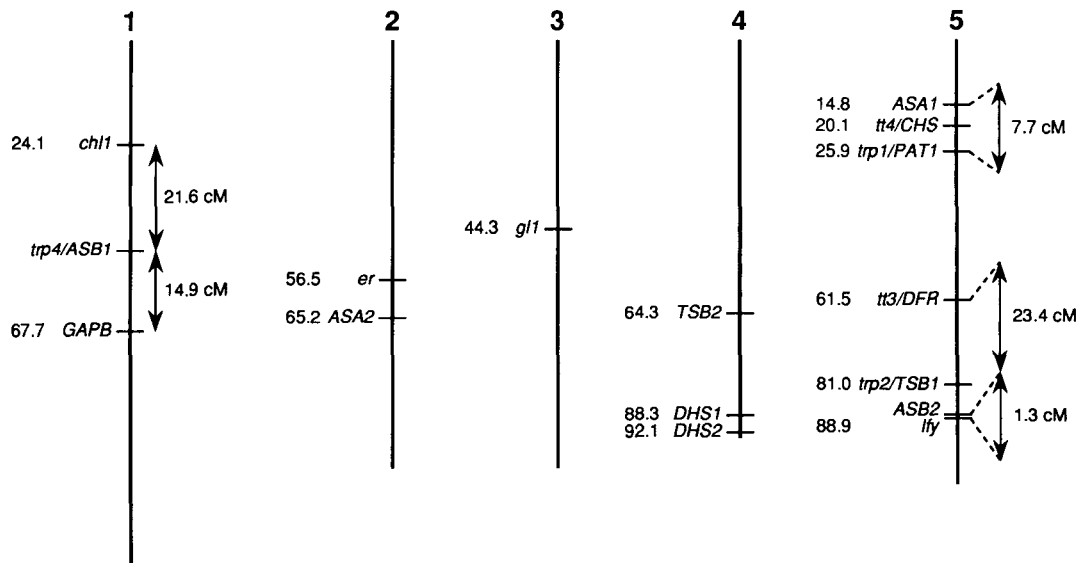
Figure 5. Map Positions of *TRP4*, *TRP1*, *ASB1*, and *ASB2* Relative to RFLP Markers.

Numbers next to markers are integrated map positions (Hauge et al., 1993) from AAtDB release 1-4 (Cherry et al., 1992). Numbers next to arrows represent map distances in centimorgans calculated from our data (see Methods).

Arabidopsis cDNA expression library (Elledge et al., 1991), and cDNAs that allow growth on low ammonia minimal medium without tryptophan were selected as diagrammed in Figure 6A.

The plasmids from 77 Trp⁺ colonies contained cDNAs derived from a single gene or closely related genes. Forty-eight cDNAs from the *ASA1*-containing *trpΔED27* strain and 29 cDNAs from the *ASA2*-containing strain were analyzed by restriction mapping using EcoRI and XhoI. Among library transformants of both strains, Trp⁺ clones arose at a frequency of approximately 10^{-5}. The complementation phenotypes of three representative clones in the *ASA1*-containing *trpΔED27* strain are shown in Figure 6B. Partial DNA sequence analysis showed that these three cDNAs represented the same gene, designated *ASB1*.

Analysis of *ASB1* DNA and Deduced Protein Sequences

The complete sequence of the *ASB1* cDNA is presented in Figure 7. The *ASB1* cDNA contains an 828-nucleotide open reading frame, capable of encoding a protein 276 amino acids in length, with a calculated molecular mass of 30,463 D.

The deduced protein encoded by the *ASB1* cDNA was aligned with the AS β subunits from yeast and *E. coli* along with the PABA synthase β subunit from *E. coli*, as shown in Figure 8. Table 6 shows that the ASB1 protein is 34 to 45% identical to its microbial counterparts. The ASB1 amino acid sequence also exhibited 84% identity over 63 amino acids to the predicted product of a partial open reading frame from a

randomly selected maize cDNA that apparently encodes a monocot AS β subunit (Keith et al., 1993). Arabidopsis ASB1, like the ASA1 and ASA2 predicted proteins, has an amino-terminal extension with characteristics of chloroplast transit peptides (Figure 8).

Multiple, Closely Related *ASB* Genes in Arabidopsis

Genomic DNA gel blot analysis, shown in Figure 9A, revealed the existence of multiple *ASB* genes in Arabidopsis. Approximately two extra sets of hybridizing bands were detected in addition to those corresponding to *ASB1*.

Genomic DNA clones for three *ASB* genes were isolated. The three genes shared numerous restriction sites, including an EcoRI site near the end of the putative transit peptide-encoding region. Partial DNA sequences from the ends of the 5′ EcoRI fragments revealed very high identity among the three Arabidopsis genes (Figure 9B). *ASB1* and the second gene, designated *ASB2*, showed significant sequence identity to each other even within the first intron, whereas the sequence of the third gene, *ASB3*, diverged in this region of the genomic clone.

Additional cDNA clones isolated by complementation or hybridization were analyzed by DNA sequencing. Of 21 clones isolated by complementation, 19 represented the *ASB1* gene, and two represented the *ASB2* gene. Of six clones isolated by hybridization, four were from *ASB1*, and two were from *ASB2*. No cDNAs representing *ASB3* were found by either screen. The relative abundance of each cDNA suggests that *ASB1* is the predominantly expressed *ASB* gene in Arabidopsis.
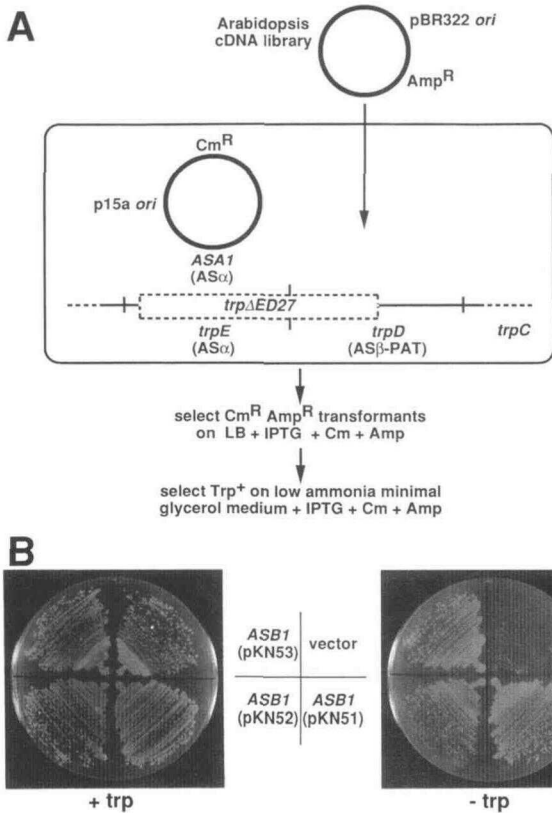
A

Arabidopsis cDNA library

pBR322 *ori*

Amp^R

Cm^R

p15a *ori*

ASA1 (ASα)

trpΔED27

trpE (ASα)   trpD (ASβ-PAT)   trpC

select Cm^R Amp^R transformants
on LB + IPTG + Cm + Amp

select Trp+ on low ammonia minimal
glycerol medium + IPTG + Cm + Amp

B

ASB1 (pKN53) | vector

ASB1 (pKN52) | ASB1 (pKN51)

+ trp   − trp

Figure 6. Isolation of *ASB* Genes by Complementation in *E. coli.*

(A) Schematic diagram of complementation strategy. The *E. coli trpΔED27* lacks AS α and AS β subunit activities due to a deletion mutation extending from near the amino-terminal part of the *trpE* coding region to one-third of the way through *trpD*, but it retains PAT activity encoded by the carboxyl-terminal two-thirds of *trpD*, because that domain is fused to the remaining amino-terminal part of the *trpE* coding region (Jackson and Yanofsky, 1974). The *trpΔED27* strain containing an Arabidopsis *ASA* gene (*ASA1* in the diagram) was transformed with an Arabidopsis cDNA library, and Trp+ clones were selected on low ammonia (1 mM NH₄Cl) M9 minimal medium lacking tryptophan. Cm^r, chloramphenicol resistant; Amp^r, ampicillin resistant; LB, Luria-Bertani medium; IPTG, isopropylthiogalactoside.

(B) Complementation by Arabidopsis *ASB1*. *E. coli trpΔED27* containing *ASA1* on pKN243A was transformed with pSE936* (vector) or one of three independent *ASB1* plasmids (pKN51, pKN52, or pKN53), and ampicillin-resistant and chloramphenicol-resistant transformants were grown to saturation at 37°C in liquid Luria-Bertani medium containing 100 μg/mL of ampicillin and 30 μg/mL of chloramphenicol and then streaked onto agar plates containing M9 minimal medium plus 0.2% glycerol, 100 μg/mL of ampicillin, 30 μg/mL of chloramphenicol, 200 μM of L-tryptophan (+ trp) or M9 minimal medium plus 0.2% glycerol, 100 μg/mL of ampicillin, 30 μg/mL of chloramphenicol, and 0.1 mM of isopropylthiogalactoside (− trp). Growth after 3 days at 37°C is shown. After several more days of incubation at 37°C, some growth of the vector transformant was detected, because expression of ammonia-dependent AS activity from the *ASA1* gene allows slow growth on standard ammonia (19 mM NH₄Cl) M9 medium (Niyogi and Fink, 1992).

Figure 7. Nucleotide and Deduced Amino Acid Sequences of the *ASB1* cDNA.

The DNA sequence of the *ASB1* open reading frame is shown in uppercase letters, and the 5′ and 3′ untranslated regions are shown in lowercase letters. The positions of seven introns in the *ASB1* genomic DNA are designated by arrows. The locations of the mutations in the *ASB1* gene from *trp4-1* and *trp4-2* plants are shown by † and ‡ symbols, respectively. Asterisk indicates stop codon. The GenBank accession number of *ASB1* is L22585.

ASB1 Is the Defective Gene in Arabidopsis *trp4* Mutants

Because the *trp4-1* mutation resulted in the reduction of AS activity in Arabidopsis extracts by more than 60% (Table 5),
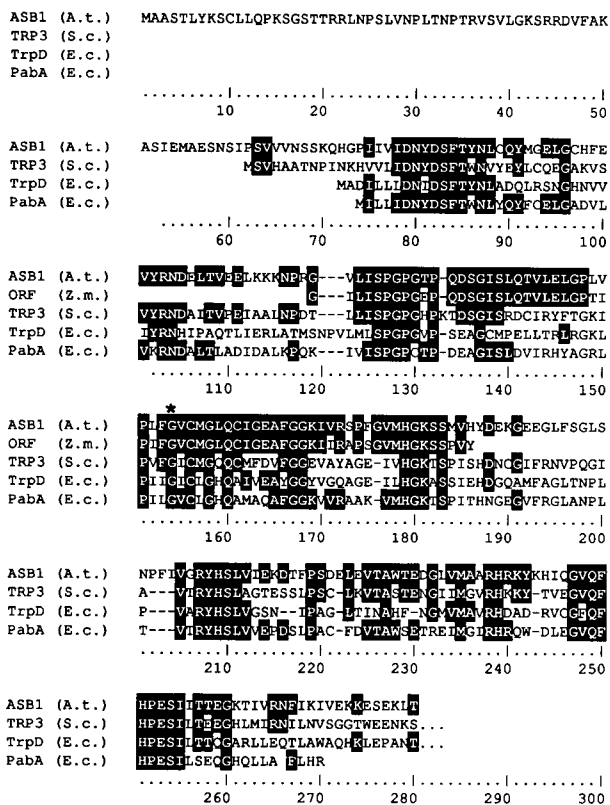
```
ASB1 (A.t.)    MAASTLYKSCLLQPKSGSTTRRLNPSLVNPLTNPTRVSVLGKSRRDVFAK
TRP3 (S.c.)
TrpD (E.c.)
PabA (E.c.)
               .........|.........|.........|.........|.........|
                        10        20        30        40        50

ASB1 (A.t.)    ASIEMAESNSIF SVVVNSSKQHGF IIV IDNYDSFTYNL QY MG ELG CHFE
TRP3 (S.c.)        M SV HAATNPINKHVV I IDNYDSFT NVYE LCQE GAKVS
TrpD (E.c.)        MAD ILLL DN DSFTYNL ADQLRSN GHNVV
PabA (E.c.)        M ILL I IDNYDSFT NL QY FC ELG ADVL
               .........|.........|.........|.........|.........|
                        60        70        80        90        100

ASB1 (A.t.)    VYRND LTV LKKK NP G---V LISPGPGTP QDSGISLQTVLELGP LV
ORF  (Z.m.)                        G---I LISPGPG P-QDSGISLQTVLELGP TI
TRP3 (S.c.)    VYRND ITVP IAAI NP DT---L LISPGPG PKTDSGIS RDCIRYFTGKI
TrpD (E.c.)    IYRN HIPAQTLIERLATMSNPVLM SPGPG VP-SEA CMPELLTR RGKL
PabA (E.c.)    V RND LTLADIDALK QK---IV ISPGP TP-DEA GISL DVIRHYAGRL
               .........|.........|.........|.........|.........|
                        110       120       130       140       150

                                *
ASB1 (A.t.)    P FGVCMGLQCIGEAFGGKIVR P GVMHGKSS VHY DEK EEGLFSGLS
ORF  (Z.m.)    P FGVCMGLQCIGEAFGGKI RA P GVMHGKSS VY
TRP3 (S.c.)    P FG CMG QOCMFDVFGGEVAYAGE-IVHGK SP ISH DNC IFRNVPQGI
TrpD (E.c.)    PI IC C C QA VEA GG YVGQAGE-ILHGKA SIEH GQAMFAGLTNPL
PabA (E.c.)    PI IGVC G QAMA AFGGKV RAAK-VMHGK SP ITHNGE VFRGLANPL
               .........|.........|.........|.........|.........|
                        160       170       180       190       200

ASB1 (A.t.)    NPFI RYHSLV EK T PS DEL VTAWTE G VMA RHRKY KHI GVQF
TRP3 (S.c.)    A---V RYHSL AGTESSI PSC-L VTA TE G I MCVRH KKY-TVE GVQF
TrpD (E.c.)    P---V RYHSLVGSN-I AG L TIN HF-N VMA RH DAD-RVC Q F
PabA (E.c.)    T---V RYHSLV E DSI AC-FD VTAWS TRE I RHRQW-DLE GVQF
               .........|.........|.........|.........|.........|
                        210       220       230       240       250

ASB1 (A.t.)    HPESI TTEG KTIV RN IKIVEK ESEKL
TRP3 (S.c.)    HPESI T EGHLM RN LNVSGGTWEENKS...
TrpD (E.c.)    HPESI TT GARLLEQTLAWAQH LEPAN ...
PabA (E.c.)    HPESI LSEC HQLLA LHR
               .........|.........|.........|.........|.........|
                        260       270       280       290       300
```

Figure 8. Alignment of Amino Acid Sequences of Glutamine Amido-transferases.

The sequences shown are Arabidopsis ASB1 [ASB1 (A.t.)], a predicted partial open reading frame from a random maize cDNA [ORF (Z.m.)] (Keith et al., 1993), the AS β domain of S. cerevisiae TRP3 [TRP3 (S.c.)] (Zalkin et al., 1984), the AS β domain of E. coli TrpD [TrpD (E.c.)] (Yanofsky et al., 1981), and E. coli PabA [PabA (E.c.)] (Kaplan and Nichols, 1983). Amino acid positions showing identity to the ASB1 sequence are shaded. The position of the conserved glycine affected in trp4-1 mutants is designated with an asterisk.

the predominant ASB1 gene was the most likely candidate for the mutant gene in trp4 plants. DNA sequencing revealed different single base pair changes in the ASB1 gene from homozygous trp4-1 plants and homozygous trp4-2 plants as compared with TRP4 plants. The trp4-1 allele of ASB1 had a G-to-A transition mutation at position 465 (Figure 7), resulting in the substitution of glutamic acid for glycine in the predicted amino acid sequence at a position that is absolutely conserved in all known glutamine amidotransferase sequences (see Figure 8). The trp4-2 allele of ASB1 had a G-to-A transition mutation that changes the conserved AG dinucleotide at the 3' splice site of the second to last intron in the ASB1 gene (Figure 7).

The ASB1 mutation found in trp4-1 mutants cosegregated with the trp4-1 mutant phenotype in a genetic cross. The G-to-A mutation at position 465 eliminated a site for the restriction

enzyme BsII, which enabled the use of RFLP analysis to follow the mutant ASB1 allele in the cross of the trp4-1/trp4-1; trp1-100/trp1-100 mutant plant by a TRP4/TRP4; TRP1/TRP1 (Ler) wild-type plant (Table 2). As shown in Figure 10, DNA from trp4-1/trp4-1; trp1-100/trp1-100 F$_3$ families derived from single Trp⁻ F$_2$ plants contained only the mutant allele of ASB1 (lanes 10 through 19). The cosegregation of the ASB1 mutation and the Trp⁻ phenotype reflected cosegregation of the ASB1 mutation and the trp4-1 mutation rather than the trp1-100 mutation, because DNA from a trp4-1/trp4-1; TRP1/TRP1 family (59-A) was homozygous for the ASB1 mutation (Figure 10, lane 9). Similarly, Trp⁺ F$_2$ progeny that were either fluorescent (trp1-100/trp1-100 [Figure 10, lanes 7 and 8]) or nonfluorescent (trp1-100/TRP1 or TRP1/TRP1 [Figure 10, lanes 5 and 6]) were all either homozygous wild-type ASB1 or heterozygous for the ASB1 mutation. Together, these data suggested that ASB1 maps to the position of the trp4 mutation on chromosome 1, as shown in Figure 5.

The ASB2 gene is unlinked to TRP4. A HinfI RFLP between the Col-0 and Ler ecotypes of Arabidopsis was identified in the 5' flanking region of the ASB2 gene. RFLP linkage analysis placed the ASB2 gene very close to LEAFY3 on chromosome 5 (Figure 5).

Pathogen-Induced Expression of ASB mRNA Levels

ASB mRNA expression was induced by infiltration of Arabidopsis leaves with avirulent or virulent strains of Pseudomonas syringae. Figure 11 shows that infiltration of the avirulent strain P. s. pv tomato MM1065, which elicits a resistance response in the Col-0 ecotype of Arabidopsis (Dong et al., 1991), caused a transient induction of ASB mRNA peaking at 6 hr. Infiltration of the virulent strain P. s. pv maculicola ES4326 (Dong et al., 1991) resulted in a later induction of ASB mRNA, with a maximum at 24 to 48 hr after inoculation. ASA1 mRNA levels increased with the same timing as ASB mRNA levels in response to bacterial pathogen infiltration (Figure 11; Niyogi and Fink, 1992). Mock-inoculated leaves did not show increased levels of ASB or ASA1 mRNAs. The levels of RNA transcripts from the ASA2 gene and the Arabidopsis HIS4 gene (P. Grisafi

Table 6. Pairwise Amino Acid Identity between Arabidopsis AS β Subunit and Microbial AS β and PABA Synthase β Subunit Sequences[a]

Gene	TRP3[b] (%)	TrpD[c] (%)	PabB[c] (%)
ASB1	42	34	45
TRP3[b]		39	52
TrpD[c]			44

[a] Percent identity was calculated for the sequences as aligned in Figure 8.
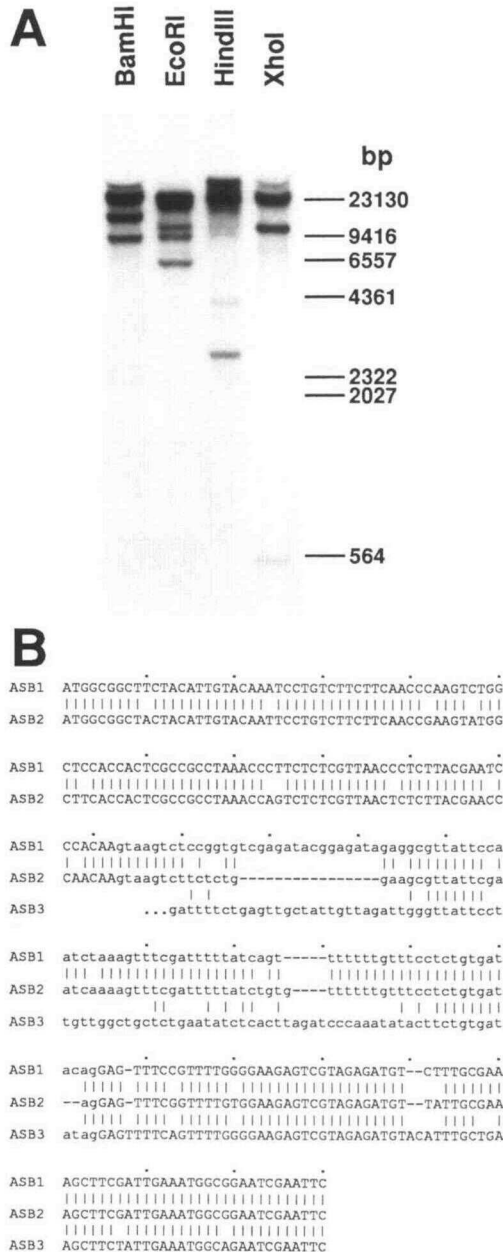[b] From S. cerevisiae.
[c] From E. coli.

Figure 9. A Family of *ASB* Genes in Arabidopsis.

(A) Genomic DNA gel blot analysis. Two micrograms of Arabidopsis genomic DNA were digested with the indicated restriction enzymes, fractionated by electrophoresis on a 0.9% agarose gel, transferred to a MagnaGraph membrane (Micron Separations Inc., Westboro, MA), and hybridized with the ^{32}P-labeled 0.67-kb XhoI fragment of pKN58C. Lengths of DNA markers (HindIII-digested λ DNA) are shown in base pairs.

(B) Partial DNA sequences of *ASB* genomic clones. The sequences shown are from the putative chloroplast transit peptide–encoding regions at the ends of the genomic clones pKN89 (ASB1), pKN80 (ASB2), and pKN91 (ASB3). The presumed intron sequences are in lowercase letters, and the coding sequences are in uppercase letters; dashes optimize alignments.

and G. R. Fink, unpublished results) were unaffected by these treatments (Figure 11). Because the sequences of the three *ASB* genes are so similar, it is not known which *ASB* gene(s) was induced by bacterial pathogen infiltration.

DISCUSSION

A Female-Specific Defect in Transmission of the *trp4*; *trp1-100* Double Mutant Genotype

The F$_2$ progeny of the cross between the *trp4-1/trp4-1*; *trp1-100/trp1-100* double mutant (Trp$^-$) strain and the wild type was expected to show a ratio of 15:1 Trp$^+$:Trp$^-$, yet the observed ratio was ~80:1 Trp$^+$:Trp$^-$ (Table 2). This distorted segregation could be caused by zygotic lethality or by reduced transmission of the mutant genotype through male or female gametophytes. The latter phenomenon (certation) has been described in many plant species and is usually attributable to defective mutant pollen (Dellaert, 1980). The testcross data in Table 3 demonstrate, however, that doubly heterozygous male parents transmit the *trp4-1*; *trp1-100* genotype at a frequency (22%) close to the expected value (25%). In contrast, reciprocal testcrosses showed that transmission of the *trp4-1*; *trp1-100* double mutant genotype is severely depressed in female
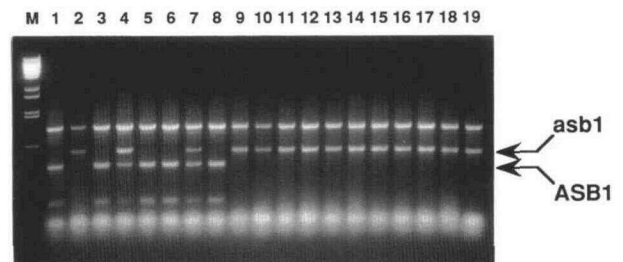

Figure 10. Cosegregation of *ASB1* and *trp4-1*.

ASB1 genomic DNA was amplified by PCR from F$_3$ families derived from individual F$_2$ progeny of a cross between a *trp4-1/trp4-1*; *trp1-100/trp1-100* mutant plant and a *TRP4/TRP4*; *TRP1/TRP1* wild-type plant. The PCR products were digested with BsII to distinguish the mutant (asb1) and wild-type (ASB1) alleles of *ASB1* that were segregating in the F$_2$ population, and the resulting fragments were separated on a 1.5% agarose gel stained with ethidium bromide. Lanes 1 to 4 show the results of the RFLP analysis for the *TRP4/TRP4*; *TRP1/TRP1* Col-0 wild type (lane 1), the *trp4-1/trp4-1*; *trp1-100/trp1-100* mutant parent (lane 2), the *TRP4/TRP4*; *TRP1/TRP1* Ler wild-type parent (lane 3), and the *trp4-1/TRP4*; *trp1-100/TRP1* F$_1$ (lane 4). Trp$^+$ F$_2$ progeny that were either nonfluorescent (*trp1-100/TRP1* or *TRP1/TRP1* [lanes 5 and 6]) or fluorescent (*trp1-100/trp1-100* [lanes 7 and 8]) were either homozygous wild-type *ASB1* or heterozygous for the *ASB1* mutation. In contrast, the *trp4-1/trp4-1*; *TRP1/TRP1* Trp$^+$ mutant 59-A (lane 9) and *trp4-1/trp4-1*; *trp1-100/trp1-100* Trp$^-$ F$_2$ progeny (lanes 10 through 19) were all homozygous for the *ASB1* mutation. The DNA size markers are BstEII-digested λ DNA (lane M).
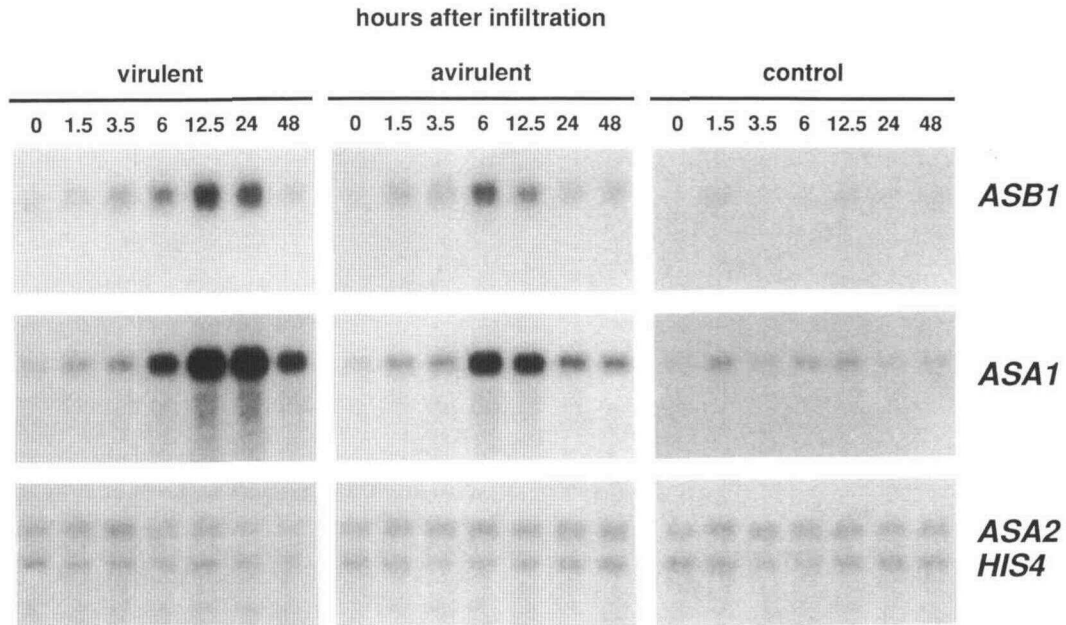
hours after infiltration

Figure 11. Induction of *ASB* RNA Levels by Bacterial Pathogens.

Ten micrograms of total RNA per lane from leaves of 3-week-old Arabidopsis plants inoculated as described previously (Dong et al., 1991) with *P. s.* pv *maculicola* ES4326 (virulent), *P. s.* pv *tomato* MM1065 (avirulent), and 10 mM MgSO₄ (control) were subjected to RNA gel blot analysis. The probes were a 0.67-kb XhoI fragment of pKN58C (*ASB1*), a 2-kb XhoI fragment of pKN41 (*ASA1*), a 1.8-kb BamHI fragment of pKN108A (*ASA2*), and the 2- and 1-kb products of digestion of pG-HisBΔS with KpnI and SacI (*HIS4*). The titer of the inoculated bacterial strains was 1 × 10⁷ colony-forming units per mL.

parents heterozygous for both mutations, producing only 6.2% Trp⁻ F₁ seeds instead of the expected value of 25%. The frequency of total fluorescent progeny of some outcrosses (15.7% and 17.6%; Table 2) and testcrosses in which the double heterozygote was the female parent (30.6%; Table 3) also appeared to be lower than expected, but the decreases were entirely attributable to the decreases in the Trp⁻, weakly fluorescent class. The testcross ratios predicted that self-pollination of a doubly heterozygous mutant F₁ plant would produce only 1.4% Trp⁻ F₂ progeny (0.22 × 0.062 = 1.4%). This value is in close agreement with the outcross data in Table 2, and indicates that cosegregation of *trp4-1* and *trp1-100* mutations alone account fully for the Trp⁻ phenotype. Therefore, although female gametes with the *trp4-1*; *trp1-100* double mutant genotype are clearly capable of producing viable progeny, they appear to be at a selective disadvantage in plants heterozygous for both mutations.

At least two different genetic models might account for the unexpectedly low number of *trp4/trp4*; *trp1-100/trp1-100* progeny derived from doubly heterozygous females. The first is that ovules containing double mutant gametes develop at the expected frequencies, but that a majority of these fail to develop normally, because (1) the double mutant ovules are immature at the time of pollination, (2) the double mutant ovules fail to produce sufficient metabolites from the tryptophan pathway

that are critical for fertilization, or (3) the developing embryos derived from *trp4*; *trp1-100* double mutant female gametes are less viable than wild type, and a large percentage arrest or develop into inviable seeds. Any of these events should lead to the production of siliques that display a frequency of unfertilized ovules, arrested embryos, or inviable seed large enough to account for the "missing" class of Trp⁻ progeny. Examination of developing siliques from wild-type and heterozygous mutant plants does not reveal sufficient numbers of aborted seeds to explain the ratios (K. K. Niyogi, unpublished data), but no direct test of this general model has been performed.

Alternatively, ovules containing double mutant gametes might not be produced at the expected frequency. Specifically, selection against the double mutant genotype may occur postmeiotically and prior to embryo sac formation. During ovule development in Arabidopsis and other monosporic species, the haploid meiotic products (megaspores) form a linear tetrad. The megaspore nearest the chalaza in this tetrad divides to produce all the cells of the megagametophyte (embryo sac), including the egg cell, whereas the other three megaspores degenerate. It is possible that the *trp4-1*; *trp1-100* genotype compromises the development of the expected chalazal megaspore into the megagametophyte when wild-type haploid megaspores are also present. Therefore, megaspores containing wild-type *TRP1* or *TRP4* alleles might have a competitive advantage over

the double mutant megaspore and develop preferentially into the megagametophyte. This "gametic competition" model could account for the unexpectedly low frequency of outcrossed Trp⁻ F_2 progeny without invoking decreased viability of double mutant gametes or embryos. Megaspore competition between genetically distinct meiotic products has been well documented in different species of Oenothera (Swanson et al., 1981).

Genetic analysis of ovule development in general has been limited by the small number of available mutants. Two recently described female-sterile mutants of Arabidopsis, *short integuments (sin1)* and *bell (bel1)*, develop characteristically aberrant integuments and fail to produce embryo sacs (Robinson-Beers et al., 1992). A third Arabidopsis mutation, *Gf*, is also female sterile and segregates in the male parent in a non-Mendelian fashion (Redei, 1965). Other examples of female-specific defects in genetic transmission have been observed in plants: in maize, preferential segregation of an "abnormal" heterochromatic chromosome 10 into the megagametophyte produces non-Mendelian segregation of linked loci (Rhoades, 1942).

A Family of *ASB* Genes in Arabidopsis

Arabidopsis appears to have three very similar *ASB* genes encoding the β subunit of AS. Accumulating molecular and biochemical evidence suggests that the subunit structure of plant AS is analogous to microbial AS (Niyogi and Fink, 1992; Poulsen et al., 1993), which is composed of nonidentical α and β subunits. In Arabidopsis the α subunit is encoded by two genes, *ASA1* and *ASA2* (Niyogi and Fink, 1992). *ASB1*, the most thoroughly characterized Arabidopsis *ASB* gene, encodes a β subunit that can interact with either ASA1 or ASA2 in *E. coli* to provide functional glutamine-dependent AS activity (Figure 6 and data not shown). The predicted ASB1 protein shows significant amino acid sequence identity to bacterial and fungal AS β subunits (Figure 8 and Table 6). *ASB1* encodes a monofunctional polypeptide like the *trpG* genes from many bacteria, but unlike the fused β subunit genes from other organisms such as *E. coli* (Yanofsky et al., 1981), *Rhizobium meliloti* (Bae et al., 1989), yeast (Zalkin et al., 1984), and *Neurospora crassa* (Schechtman and Yanofsky, 1983). The predicted protein from the *ASB1* cDNA sequence has an amino-terminal putative chloroplast transit peptide, supporting the idea that plastids are sites of tryptophan biosynthesis in plants (Bagge and Larson, 1986).

Arabidopsis *ASA* and *ASB* genes are coordinately regulated in response to bacterial pathogen infiltration. The level of RNA hybridizing to the *ASB1* cDNA was induced along with *ASA1* by infiltration of leaves with avirulent and virulent strains of *P. syringae* (Figure 11). Overexpression of *ASA* genes in transgenic Arabidopsis plants suggests that the AS β subunit is limiting for AS activity when *ASA* expression is increased (K. K. Niyogi and G. R. Fink, manuscript in preparation), leading us to expect coordination of *ASA* and *ASB* gene induction when increased AS activity is necessary. It will be interesting to

determine which *ASB* gene(s) is induced by pathogens. Characterization of the response of *trp4* mutants to bacterial pathogen infiltration may be useful in analyzing the role of increased AS expression during the Arabidopsis defense response.

The biochemical characterization of *trp4* mutants (Table 5) suggested that *TRP4* is an AS structural gene or encodes a protein necessary for AS activity, but genetic mapping data showed that *TRP4* is unlinked to *ASA1* and *ASA2* (Figure 5). The isolation of Arabidopsis *ASB* genes allowed the identification of *ASB1* as the defective gene in *trp4* mutants. The mutations in the *ASB1* gene from *trp4-1* and *trp4-2* plants are likely null mutations. The *trp4-1* mutation changes an absolutely conserved amino acid near a conserved cysteine, which has been shown to be at the active site of *Serratia marcescens* ASB (Kawamura et al., 1978; Paluh et al., 1985). The *trp4-2* mutation alters the conserved dinucleotide at the 3' splice site of the sixth intron of *ASB1*, resulting in a probable splicing defect.

Isolation of Amino Acid Auxotrophs in Plants

How does the molecular genetic characterization of Arabidopsis *trp4* influence our thinking about the scarcity of tryptophan mutants, and amino acid auxotrophs in general, in higher plants? At least two explanations for the lack of tryptophan mutants in plants have been proposed (see, for example, Last and Fink, 1988; Last et al., 1991). One possibility is that the existence of multiple genes encoding tryptophan pathway enzymes makes isolation of auxotrophs difficult. A second possibility is that null mutations in tryptophan biosynthetic genes are lethal, and only specific missense mutations are viable.

The determination of the molecular basis of the *trp4* mutations directly supports a model in which genetic redundancy explains the paucity of tryptophan mutants in higher plants. *ASB1* appears to be an example of a tryptophan biosynthetic gene that is functionally redundant for normal growth of wild-type Arabidopsis plants. Although *trp4* plants carry probable null mutations in *ASB1*, they do not require tryptophan or have any obvious morphological defects (Figure 4). Without the screen for loss of fluorescence, it seems unlikely that the *trp4* mutants would have been isolated without resorting to direct screening for reduced AS enzyme activity.

Genetic redundancy is a prevalent theme, but not an absolute rule, in plant aromatic amino acid biosynthesis. So far, all the enzymes in the Arabidopsis aromatic pathway are encoded by multiple genes (Klee et al., 1987; Berlyn et al., 1989; Keith et al., 1991; Last et al., 1991; Li et al., 1992; Niyogi and Fink, 1992), with the exception of PAT, encoded by the single-copy *PAT1* gene (Rose et al., 1992). The similarity of *ASB1*, *ASB2*, and *ASB3* includes the regions encoding the putative chloroplast transit peptides (Figure 9B), which are usually the most divergent parts of duplicated aromatic biosynthetic genes (Keith et al., 1991; Last et al., 1991; Niyogi and Fink, 1992). The *ASB* genes are an example of a very closely related small gene

family encoding tryptophan biosynthetic enzymes in Arabidopsis, in contrast to the *ASA* genes, which comprise the most divergent of these families characterized thus far.

The genetic characterization of the *trp4-1/trp4-1; trp1-100/trp1-100* double mutant suggests that gametophyte lethality may explain the lack of some tryptophan mutants. The reduced transmission of the *trp4-1; trp1-100* genotype through female gametophytes produced by plants heterozygous for both mutations is consistent with the idea that certain recessive mutations that arise during the diploid generation may be eliminated during the haploid gametophyte phase of the plant life cycle. Analysis of the specific mechanism resulting in decreased transmission of the *trp4-1; trp1-100* genotype may provide further insight into the scarcity of amino acid auxotrophs and may also reveal novel roles for the tryptophan pathway in female gametophyte development or fertilization in plants.

METHODS

Plant Strains and Growth Conditions

Arabidopsis thaliana ecotypes Columbia (Col-0), Landsberg *erecta* (Ler), and Nossen (No-0) were grown on Grace Sierra Metro-Mix 200 or Fafard Growing Mix No. 2 (Griffin Greenhouse and Nursery Supplies, Inc., Tewksbury, MA) under continuous illumination (75 to 150 μE m^{-2} sec^{-1}) at 22 to 25°C as described by Last and Fink (1988).

Seeds (50,000) of a *trp1-100/trp1-100; gl1-1/gl1-1; pgm/pgm* (Col-0) strain were mutagenized with 0.24% (v/v) ethylmethane sulfonate (Sigma) for 16 hr at room temperature and then washed five times with H_2O. The M$_1$ plants derived from these seeds were grown as 10 separate pools and allowed to self-pollinate. The resulting M$_2$ seeds were surface sterilized (Last and Fink, 1988) and suspended in 0.1% agar at 2000 seeds per mL. Aliquots of 1000 seeds were mixed with 7.5 mL of molten 0.75% agar and poured onto the surface of 150 × 25 mm Petri plates, containing 75 mL of plant nutrient medium with 0.5% sucrose (PNS; Haughn and Somerville, 1986) solidified with 0.75% agar and supplemented with 10 μM each of L-phenylalanine, L-tyrosine, L-tryptophan, and *para*-aminobenzoic acid (PABA). Plates were wrapped with Parafilm (American National Can, Greenwich, CT) and incubated at 22°C under continuous illumination (150 μE m^{-2} sec^{-1}). Seedlings were screened for blue fluorescence under short-wavelength UV illumination at 7 days and again at 14 days after germination. For rescreening of putative mutants and testing the phenotypes of progeny of genetic crosses, seeds were surface sterilized, suspended in 0.1% agar, and planted in rows on 100 × 100 × 15 mm plates containing 45 mL of PNS agar medium, with or without supplementation as noted. Genetic crosses were performed as described by Somerville and Ogren (1982), except that plastic film was not used.

Inoculation of 3-week-old plants with *Pseudomonas syringae* pv *tomato* MM1065 and *P. s.* pv *maculicola* ES4326 was performed as described by Dong et al. (1991).

Enzyme Assays

Tryptophan biosynthetic enzymes were assayed as described by Last and Fink (1988) with the following modifications. Plant extracts were prepared by grinding 0.5 g of frozen 3-week-old plants in liquid nitrogen in a mortar and pestle. The frozen powder was added to 0.8 mL of grinding buffer plus 25 mg of polyvinylpolypyrrolidone (Sigma), and particulate material was removed by centrifugation for 10 min in a microcentrifuge (Eppendorf). The supernatant (0.5 mL) was loaded onto a Sephadex G-25 column (NAP-5; Pharmacia, Piscataway, NJ), equilibrated with column buffer, and eluted in 1.0 mL of column buffer. Glutamine-dependent anthranilate synthase (AS) activity was assayed in 1.0-mL reactions (instead of 2.0-mL) containing 20 mM of L-glutamine (instead of 10 mM). Anthranilate phosphoribosyltransferase (PAT) and phosphoribosylanthranilate isomerase reactions contained the same amounts of components as described previously (Last and Fink, 1988) in a total volume of 1.0 mL instead of 1.1 mL. Reactions were terminated by the addition of 0.1 mL of 1 M HCl. Heat-inactivated (100°C for 5 min) extracts were used as negative controls.

Chorismate mutase activity was measured essentially as described previously (Gilchrist and Connelly, 1987; Kuroki and Conn, 1988) with minor modifications. Fifty microliters of the desalted extract described above was diluted with 50 μL of column buffer and incubated with 150 μL of reaction mix (50 mM Tris-HCl, pH 8.0, 1 mM chorismic acid [barium salt; Sigma]) for 15 min at 25°C. Reactions were stopped by the addition of 250 μL of 2 M HCl. After incubation at room temperature for 10 min, 500 μL of 3.2 M NaOH was added and the A_{320} was measured immediately against a 1 M NaOH blank. Activity was linear with respect to amount of extract added and reaction time under these conditions.

Protein concentrations of plant extracts were determined by the dye binding method (Bradford, 1976) using a kit from Bio-Rad with BSA standards.

DNA and RNA Methods

Isolation of total DNA and RNA from rosette leaves of 3-week-old Arabidopsis (ecotype Col-0) plants grown in soil was done as described previously (Niyogi and Fink, 1992).

Standard techniques of DNA analysis and cloning were performed as described by Ausubel et al. (1989). DNA fragments were purified from agarose gels using QIAEX (Qiagen, Chatsworth, CA). DNA hybridization probes were labeled using α-^{32}P-dCTP (DuPont–New England Nuclear Research Products) and random hexamer primers (Prime Time; International Biotechnologies, New Haven, CT). For DNA gel blot analysis of genomic DNA, total DNA was digested with restriction enzymes (New England Biolabs, Beverly, MA), separated on agarose gels, and transferred to MagnaGraph (Micron Separations Inc., Westboro, MA) in 20 × SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate). RNA gel blot analysis was done as described by Ausubel et al. (1989). Blots were hybridized at 65°C overnight according to the method of Church and Gilbert (1984), except that 0.25 M of sodium phosphate was used instead of 0.5 M. DNA blots were washed in 0.25 × SSC, 0.1% SDS at room temperature. RNA blots were washed in 0.1 × SSC, 0.1% SDS at 65°C.

Escherichia coli DH5α was used as the host strain for construction of the plasmids described in Table 7. Oligonucleotides were synthesized by the Massachusetts Institute of Technology Biopolymers Laboratory.

E. coli Complementation

E. coli media were prepared as described by Ausubel et al. (1989). *E. coli trpΔED27 tnaA2* (Jackson and Yanofsky, 1974) containing

Table 7. Plasmids Used in This Study

Name	Description	Reference
pACYC184	*E. coli* cloning vector compatible with ColE1 origin	Chang and Cohen (1978)
Bluescript KS –	*E. coli* cloning vector	Stratagene
pG-HisBΔS	The 3.2-kb BglII-SalI fragment of the Arabidopsis *HIS4* gene ligated into pBluescript KS – cut with BamHI and SalI	P. Grisafi and G.R. Fink, unpublished data
pKN41	*ASA1* cDNA used as hybridization probe	Niyogi and Fink (1992)
pKN51	*ASB1* cDNA in vector looped out of λ-YES-R	This work
pKN52	*ASB1* cDNA in vector looped out of λ-YES-R	This work
pKN53	*ASB1* cDNA in vector looped out of λ-YES-R	This work
pKN58A	The 1.1-kb EcoRI (partial) fragment of pKN53 ligated into the EcoRI site of pUC118	This work
pKN58C	Same as pKN58A, except in the opposite orientation	This work
pKN80	The 6-kb EcoRI fragment of *ASB2* genomic clone λKN58 ligated into the EcoRI site of pUC118	This work
pKN83	The 2-kb BglII-BamHI fragment of pKN80 ligated into the BamHI site of pBluescript KS –	This work
pKN89	The 6.7-kb EcoRI fragment of *ASB1* genomic clone λKN66 ligated into the EcoRI site of pBluescript KS –	This work
pKN91	The 4-kb EcoRI fragment of *ASB3* genomic clone λKN61 ligated into the EcoRI site of pBluescript KS –	This work
pKN108A	*ASA2* cDNA used as hybridization probe	Niyogi and Fink (1992)
pKN109A-1	Truncated *ASA2* cDNA with in-frame ATG	Niyogi and Fink (1992)
pKN132A	The 1.8-kb BamHI fragment of pKN109A-1 ligated into the BamHI site of pACYC184 in the sense orientation relative to the Tet promoter	This work ·
pKN235	Truncated *ASA1* cDNA with engineered in-frame ATG	K.K. Niyogi and G.R. Fink, manuscript in preparation
pKN243A	The 1.8-kb BamHI fragment of pKN235 ligated into the BamHI site of pACYC184 in the sense orientation relative to the Tet promoter	This work
pSE936*	Vector looped out of λ-YES-R	Elledge et al. (1991); Niyogi and Fink (1992)
pUC118	*E. coli* cloning vector	Vieira and Messing (1987)

pKN243A or pKN132A was transformed by electroporation with the plasmid form of an Arabidopsis cDNA library constructed in λ-YES-R (Elledge et al., 1991). Transformants were grown overnight at 37°C on Luria-Bertani medium solidified with 1.5% agar containing 10 mM MgSO₄, 100 μg/mL ampicillin, 30 μg/mL chloramphenicol, and 0.1 mM isopropylthiogalactoside. Lawns were replica plated to low-ammonia (1 mM NH₄Cl) M9 minimal agar medium containing 0.2% glycerol, 10 mM MgSO₄, 100 μg/mL ampicillin, 30 μg/mL chloramphenicol, and 0.1 mM isopropylthiogalactoside. Replica plates were incubated for 4 days at 37°C. Library plasmids from Trp⁺ colonies were purified by transformation into *E. coli* DH5α and screening for ampicillin-resistant, chloramphenicol-sensitive clones. The ability of cDNA clones to complement *trpΔED27* (pKN243A) was confirmed by retransformation.

Isolation of Genomic Clones and Additional cDNA Clones by Hybridization

An Arabidopsis genomic DNA library (from J. T. Mulligan and R. W. Davis) and the λ-YES-R cDNA library (Elledge et al., 1991) were grown in *E. coli* host LE392 and screened by plaque hybridization with a ³²P-labeled 0.8-kb EcoRI fragment of pKN52. Biotrans nylon filters (ICN Biomedicals, Irvine, CA) were hybridized in the same buffer used for gel blots (see above) at 60°C overnight, and filters were washed in 0.25 × SSC, 0.1% SDS at room temperature.

DNA Sequencing

DNA sequencing of cDNA and genomic clones was performed as described previously (Niyogi and Fink, 1992). Genomic DNA fragments of *trp4* mutant and wild-type alleles of *ASB1* were amplified by polymerase chain reaction (PCR) using oligonucleotides KN56 (5′-GCGGATCCCAAAAGAGGTTTCTAATC-3′) and KN62 (5′-CGGGAT-CCAACTGAAAGGCCCTCGG-3′) and *Taq* DNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, IN). Double-stranded PCR fragments were sequenced according to the method of Ausubel et al. (1989) using oligonucleotide primers.

Genetic Mapping

Recombination mapping of *trp4-1* and *trp1-100* relative to restriction fragment length polymorphism (RFLP) markers between the Col-0 and Ler ecotypes of Arabidopsis was done using PCR as described by Konieczny and Ausubel (1993). Genomic DNA was isolated as described

by Dellaporta et al. (1983) from families of F₃ plants derived from single F₂ plants of a cross between trp4-1/trp4-1; trp1-100/trp1-100 (Col-0) and tt4/tt4; erfer (Ler). Taq DNA polymerase was from Boehringer Mannheim Biochemicals, and restriction enzymes were from New England Biolabs.

Recombination fractions between trp4-1 (or trp1-100) and linked RFLP markers were calculated as (number of crossovers)/(total number of chromosomes scored), and map distances were calculated using the Kosambi mapping function (Kosambi, 1944). Between trp4 and CHL1, 13 crossovers were detected among 64 chromosomes scored, and nine crossovers among 62 chromosomes were found for the trp4-GAPB interval.

Mapping of ASB1 and ASB2 relative to RFLP markers was done using PCR. A BslI RFLP in the trp4-1 allele of ASB1 was used to show linkage of ASB1 to trp4. A genomic DNA fragment containing the BslI RFLP was amplified by PCR using oligonucleotides KN66 (5'-GGAAGA-GTCGTAGAGATGTCTTTGC-3') and KN69 (5'-GACCATCTTCTGTCC-ATGCTGTAAC-3') at an annealing temperature of 65°C. No recombinants were detected among 68 chromosomes scored. A HinfI RFLP linked to ASB2 was identified in the genomic DNA fragment amplified by PCR using oligonucleotides KN44 (5'-GAAGATCTTGTTGTAACATAGC-3') and KN45 (5'-GGTATCGAATTCGATTCC-3') at an annealing temperature of 50°C. The best-fit map of ASB2 and the linked RFLP markers LEAFY3 and DFR (dihydroflavonol 4-reductase) was calculated using MapMaker (Lander et al., 1987) with the Kosambi mapping function (Kosambi, 1944) based on data for 80 chromosomes.

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