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Purification and cDNA cloning of anthranilate synthase from *Ruta graveolens*: modes of expression and properties of native and recombinant enzymes

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Summary

***Ruta graveolens* utilizes anthranilate synthase (AS) for the synthesis both of tryptophan in primary metabolism and acridone alkaloids in secondary metabolism. AS has been purified from plants and cell cultures of *R. graveolens* 670- and 1700-fold, respectively. Glutamine- and ammonia-dependent AS activities were strictly co-purified in all steps. Through cDNA cloning and complementation of *Escherichia coli* deletion mutants defective for AS, it is shown that young *Ruta* plants express two genes for functional ASα subunits, *ASα1* and *ASα2*. The data indicate that ASα from *Ruta* requires an ASβ subunit with a native molecular weight of 60–65 kDa for the glutamine-dependent reaction. Protein synthesized *in vitro* from cloned cDNA is processed upon import into isolated chloroplasts, indicating that mature ASα subunits are active in plastids *in vivo*. *ASα1* and *ASα2* are constitutively expressed in *Ruta* cell cultures, but *ASα1* steady-state mRNA levels are increased 100-fold 6 h subsequent to elicitation whereas *ASα2* expression remains constitutive. Increased *ASα1* transcription corresponds to elicitor-induced alkaloid accumulation. The data indicate that *Ruta* regulates anthranilate flux into primary and secondary metabolism through differential regulation of AS genes specific to these pathways.**

Introduction

Anthranilate synthase (AS) (E.C. 4.1.3.27) catalyzes the synthesis of anthranilic acid from chorismic acid and is the key regulatory enzyme of tryptophan biosynthesis in bacteria and fungi (Crawford and Milkman, 1990; Hütter *et al.*, 1986; Zalkin, 1980). In micro-organisms and higher plants, numerous metabolic pathways leading to secondary compounds, hormones and co-factors radiate from both intermediates and the end product of tryptophan biosynthesis (Bentley, 1990; Collins *et al.*, 1991; DeLuca, 1993; Dewick, 1994; Gray, 1993; Niemann, 1993; Ninomiya and Kiguchi, 1990; Roos, 1990; Tillequin *et al.*, 1993). In bacteria, fungi and plants, the synthesis of secondary metabolites can branch from the tryptophan pathway at the level of anthranilic acid. In such cases, AS appears to be rate limiting and to have a regulatory role, as in the biosynthesis of benzodiazepine alkaloids in *Penicillium cyclopium* (Roos, 1990) or acridone alkaloids in *Ruta graveolens* (Bohlmann and Eilert, 1994). The dual function of AS in primary and secondary metabolism is summarized in Figure 1.

Relatively little is known about plant AS, but the complexity of this enzyme is evident from extensive genetic and biochemical studies in bacterial and fungal systems (Crawford, 1989; Crawford and Milkman, 1990; Hütter *et al.*, 1986; Zalkin, 1980). In accordance with the nomenclature of Crawford (1989), genes for the enzymatic functions of the tryptophan pathway are designated *trpA-G* as originally assigned in *Escherichia coli*. Bacterial AS is composed of two non-identical subunits, designated as ASα (AS component I) and ASβ (AS component II). ASα has a molecular weight of 60–80 kDa and is encoded by *trpE*. ASα catalyzes the conversion of chorismic acid to anthranilic acid with ammonia as amino donor via a two-step reaction. Aminodeoxyisochorismate synthase activity of ASα converts chorismate into the enzyme-bound intermediate aminodeoxyisochorismate (ADIC), which eliminates pyruvate through ADIC lyase activity of ASα (Morollo and Bauerle, 1993). *trpE* is related at the level of function and sequence homology to *pabB* which encodes aminodeoxychorismate (ADC) synthase of *p*-aminobenzoate biosynthesis; ADC lyase of *p*-aminobenzoate biosynthesis is found in the *pabC* gene product which has no similarity to ASα (Crawford and Milkman, 1990; Essar *et al.*, 1990).

ASβ contains a glutamine amidotransferase domain that donates ammonia from glutamine to ASα. Prokaryotic ASβ can be either monofunctional or bifunctional. In monofunctional ASβ, glutamine amidotransferase, encoded by *trpG*, is the sole enzyme activity. Monofunctional ASβ has a molecular weight of 15–24 kDa; in several bacteria ASβ is

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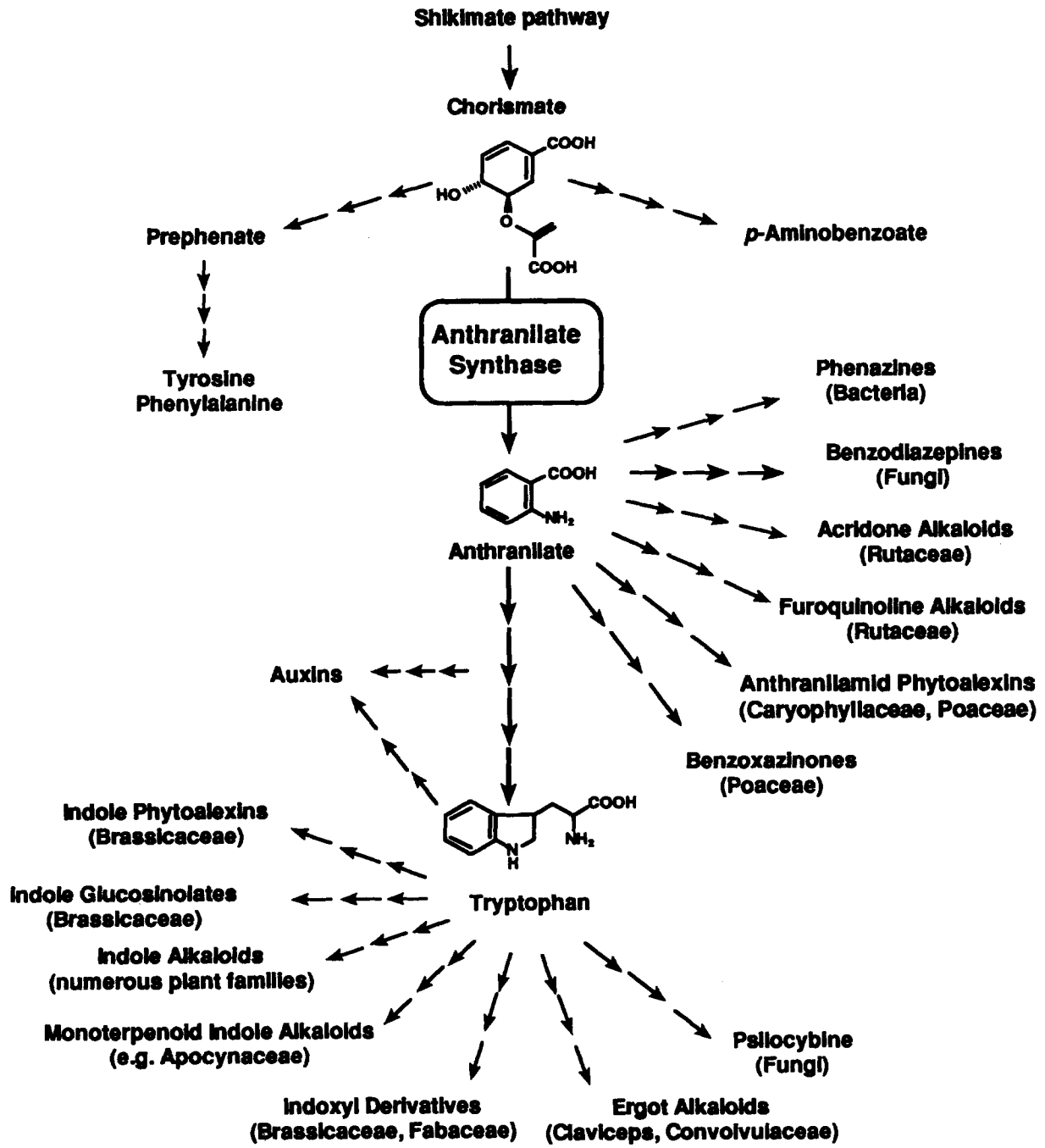

Figure 1. Overview of the role of anthranilate synthase in primary and secondary metabolism of bacteria, fungi and plants.

an amphibolic (shared) glutamine amidotransferase which forms a functional complex with either ASα or the α-subunit of *p*-aminobenzoate synthase (Kaplan *et al.*, 1985). In bifunctional ASβ, the glutamine amidotransferase domain is fused to other enzymes of tryptophan biosynthesis. Bifunctional ASβ in *E. coli* and many other bacteria studied is the result of *trpG–trpD* gene fusion (Crawford, 1989). In *Rhizobium meliloti*, *trpG* is fused to the C-terminus of *trpE*, resulting in a single 729 amino acid AS peptide (Bae *et al.*, 1989). Various fusions of ASβ are observed in fungi. In *Saccharomyces*, *trpG* and *trpC* are fused (Zalkin *et al.*, 1984), whereas in *Neurospora*, *Aspergillus* and *Schizosaccharomyces* a *trpG–trpC–trpF* fusion is found (Hütter *et al.*, 1986).

Although *Arabidopsis thaliana* cDNAs which can complement *trpE* (Niyogi and Fink, 1992) and *trpG* (Niyogi *et al.*,

1993) mutants of *E. coli* have been described, the structure and biochemistry of the plant enzymes themselves are still obscure. Studies with crude extracts from corn and pea (Hankins *et al.*, 1976) as well as with the partially purified enzyme from *Catharanthus roseus* (Poulsen *et al.*, 1993) have led to speculation that AS in these species possesses a monofunctional β-subunit. But AS from plants is difficult to purify and, as a consequence, no direct evidence has yet been marshalled which conclusively demonstrates the function and size of ASβ in plants.

Anthranilic acid is the immediate precursor for acridone and furoquinoline alkaloids, which occur only in representatives of the Rutaceae (Gray, 1993) and some of which are strongly antimicrobial compounds. In cell cultures of *R. graveolens*, the biosyntheses of these alkaloids are elicitor-inducible (Eilert, 1989) and elicitor-induced acridone alkaloid accumulation correlates with an increase of AS activity (Bohlmann and Eilert, 1994). The increase of AS activity could be due to elicitor-induction of an AS enzyme which functions specifically in alkaloid metabolism. *Ruta* is thus a highly suitable system for investigations of chorismate partitioning into primary and secondary metabolism, and particularly for understanding AS biochemistry and gene regulation. Here we report the purification and properties of AS holoenzyme from *Ruta* plants and cell cultures. We also describe cloning and expression in *E. coli* of full size cDNAs encoding two different functional *Ruta* ASα subunits. Differential expression of the corresponding genes in response to elicitation of *Ruta* cell cultures indicates that AS plays a central role in regulation of metabolite flux to tryptophan and alkaloid biosyntheses.

Results

Purification of anthranilate synthase

A protocol was developed for the purification of AS from *Ruta* cell cultures and whole plants which requires no desalting or buffer exchange steps (PEG fractionation, Q-Sepharose, hydroxyapatite, Orange A, Mono Q). Use of HEPES buffers supplemented with 1 mM DTT, 10% (v/v) glycerol and 20 mM L-glutamine improved enzyme stability and activity.

AS from 250 g of young *R. graveolens* shoots was purified 670-fold with a recovery of 0.5%. The final preparation had a specific activity of 21.8 nkat mg^{-1} protein. AS from *R. graveolens* cell cultures was purified 1700-fold with a recovery of 0.23% and a specific activity of 15.3 nkat mg^{-1} protein. All enzyme assays were performed in parallel both for glutamine-dependent activity of AS holoenzyme (AS-Gln) and for ammonia-dependent activity of ASα (AS-NH_3) in order to monitor potential loss of ASβ (glutamine amidotransferase) during purification. We emphasize the fact that both activities strictly co-purified in all steps and

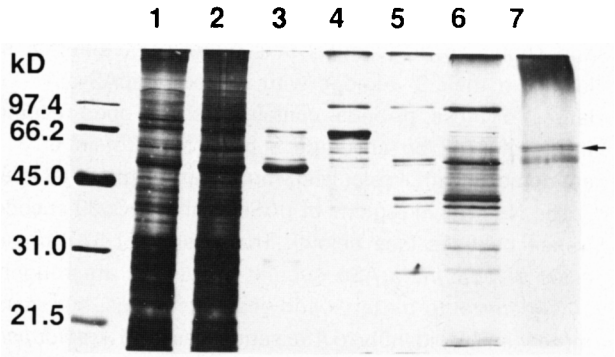

Figure 2. Silver staining pattern obtained at different stages of AS purification from cell cultures of *R. graveolens*.
Fractions exhibiting AS-Gln activity were applied to SDS–PAGE and proteins were visualized by silver staining. Sizes of molecular mass markers are indicated. Lane 1, crude extract (~1.5 μg); lane 2, polyethylene glycol precipitation (~1.5 μg); lane 3, Q-Sepharose (~0.5 μg); lane 4, hydroxyapatite (~0.5 μg); lane 5, Orange A (~0.5 μg); lane 6, Mono Q (~0.5 μg); lane 7, Superose 12 (<0.5 μg). An arrow indicates protein bands of 61 and 62 kDa, respectively, which co-purify with AS-Gln activity (see text).

that no loss of AS-Gln occurred, e.g. the ratio of AS-Gln to AS-NH_3 in crude extracts from cell cultures was 1.37 whereas that of the 1700-fold purified enzyme was 1.32. In addition, no separation of *Ruta* AS isozymes was observed from either source during purification. The final Superose 12-purified preparation contained two major bands with molecular weights of 61 and 62 kDa, respectively (Figure 2, lane 7), in addition to a smear at approximately 55 kDa.

Aliquots of individual fractions from the penultimate Mono Q step which contained AS activity (pooled in Figure 2, lane 6) were examined by SDS- PAGE and silver staining. The result clearly showed that the elution profile of the 61 and 62 kDa proteins (arrow in Figure 2) strictly corresponded to the identical AS-Gln and AS-NH_3 elution profiles and that no other bands from Mono Q fractions co-eluted with AS activity (data not shown).

cDNA cloning of ASα

We constructed a cDNA library from mRNA isolated from young shoots which contained AS activity and acridone alkaloids. We screened the library with a fragment of pAS4 (Zalkin *et al.*, 1984) containing the highly conserved C-terminal region of ASα in the yeast *TRP2* gene. From 30 000 recombinants we identified 19 clones which hybridized to the yeast probe. These fell into two classes of identical sequences represented by the clones pASα27 and pASα12.

pASα12 contains an open reading frame of 1827 bp encoding a 68 kDa protein. pASα27 contains an open reading frame of 1665 bp but lacks a start codon. To obtain full size clones corresponding to pASα27, 60 000 new recombinants were screened by plaque hybridization with a synthetic 16 base oligomer designed against the 5′ prime end of pASα27. Out of 76 positives obtained, three

contained inserts larger than 2 kb. These were subcloned and shown to be identical in sequence yet different in length in their 3′ regions with respect to pASα27. The largest of these, pASα39, contains a single open reading frame of 1842 bp encoding a predicted protein of 614 amino acids with a calculated molecular weight of 68 kDa.

The N-terminal regions of pASα12 and pASα39 encode transit peptides (see below). The C-terminal 520 amino acids of both *Ruta* ASα subunit sequences are roughly 34% identical to bacterial and yeast homologs, but share more than 70% identity to ASα sequences from *Arabidopsis* (Niyogi and Fink, 1992) (Figure 3). We designate the pASα39 encoded protein as ASα1 since it is more similar to the wound- and pathogen-inducible ASA1 of *Arabidopsis* (78% amino acid identity) than to ASA2 (73%). The pASα12 encoded protein shows 82% identity to ASA2 but only 78% to ASA1 and is designated ASα2. The term α instead of A was used for consistency with the literature on bacterial AS.

pASα12 and pASα39 encode functional AS

To determine whether ASα1 and ASα2 encode functional AS enzymes we complemented *E. coli* mutants defective in AS activity. The *Not*I insert of pASα39 encodes ASα1 in the sense orientation behind the β-galactosidase promoter. It was transformed into the *E. coli trpE* deletion mutant Δ*trpE5*, which does not produce endogenous ASα subunit, and into the double mutant Δ*trpED27* which additionally lacks glutamine amidotransferase activity in the ASβ subunit (Jackson and Yanofsky, 1974). These strains were designated Δ*trpE5*/pASα39 and Δ*trpED27*/pASα39. For testing ASα2, the 1.7 kb *Sal*I-*Not*I fragment of pASα12 was ligated into *Sal*I-*Not*I-digested pGEX-4T-3 (Pharmacia) to yield pGASα12 which was transformed into the same mutants to yield strains Δ*trpE5*/pGASα12 and Δ*trpED27*/pGASα12, respectively. Amp^r colonies were streaked from LB on to M9IA100 agar (M9 minimal medium with IPTG, ampicillin and 100 mM NH_4Cl) and incubated for 3 days at 37°C. Replica streaks from M9IA100 were grown at 37°C on M9IA1, M9IA20 and M9IA100 containing 1, 20 and 100 mM NH_4Cl, respectively.

The *Ruta* cDNAs complement *E. coli trpE* mutants. Δ*trpE5*/pGASα12 and Δ*trpED27*/pGASα12 grew well after 3 days on 20 and 100 mM NH_4Cl, indicating that ASα2 encoded by pGASα12 is a functional ASα subunit. Δ*trpE5*/pASα39 grew on 20 and 100 mM NH_4Cl, indicating that ASα1 encoded by pASα39 is also functional. Δ*trpED27*/pASα39 grew well on 100 mM NH_4Cl but not at all on M9IA20. This result indicates that glutamine amidotransferase activity provided by the ASβ subunit of *E. coli* present in Δ*trpE5* is required for AS-Gln activity of the pASα39-encoded protein and furthermore suggests that ASα1 interacts effectively with *E. coli* ASβ. Even after 5 days of incubation, vector controls were unable to grow in the absence of tryptophan and all strains expressing *Ruta* ASα were unable to grow on 1 mM NH_4Cl plates.

The fact that ΔtrpED27/pGASα12 grows well on M9IA20 whereas ΔtrpED27/pASα39 does not can be due to several factors. One explanation could be that ASα2 may not require an ASβ subunit for glutamine-dependent activity. As an alternative, ASα2 may interact much more efficiently than ASα1 expressed in these experiments with other endogenous glutamine amidotransferases of *E. coli*, such as the *pabA* gene product, which has homology to the glutamine amidotransferase domain (TrpG) of TrpD (Crawford and Milkman, 1990). This prompted us to test the relative amounts of AS-Gln and AS-NH_3 activity in the complemented mutants and compare these with the values for the native *Ruta* enzyme.

The results of these experiments are shown in Table 1. In contrast to the *E. coli* wild-type enzyme, native *Ruta* AS has roughly equal levels of AS-Gln and AS-NH_3 activity. The enzyme from Δ*trpE5*/pASα39 has fivefold less AS-Gln than AS-NH_3 activity whereas the enzyme from Δ*trpE5*/pGASα12 shows the same activity with both ammonia sources, similar to the native *Ruta* enzyme. This suggests that the pGASα12-encoded protein interacts more efficiently with *E. coli* ASβ present in the Δ*trpE5* mutant than does the pASα39-encoded product.

The enzymes from Δ*trpED27*/pASα39 and Δ*trpED27*/pGASα12 have fivefold less AS-Gln than AS-NH_3 activity (Table 1). That AS-Gln activity was observed at all for Δ*trpED27*/pASα39 and Δ*trpED27*/pGASα12, which lack endogenous *E. coli* ASβ, suggested that pASα39- and pGASα12-encoded proteins either do not require a glutamine amidotransferase for AS-Gln activity, or that they can interact to some extent with glutamine amidotransferases present in *E. coli* other than ASβ, e.g. PabA. The fivefold drop in AS-Gln relative to AS-NH_3 activity for pGASα12 in Δ*trpED27* as compared with Δ*trpE5* (Table 1) favored the latter view and suggested that such interaction is weaker than that with *E. coli* ASβ. To see if the AS-NH_3 and AS-Gln activities of Δ*trpED27*/pGASα12 were separable, we purified the protein from 1 l of that culture by affinity chromatography over glutathion Sepharose. The ratio of AS-Gln to AS-NH_3 decreased from 0.20 in the desalted crude extract to 0.03 in the affinity chromatography eluate. This confirms that *Ruta* AS requires a β-subunit for the glutamine-dependent reaction, and that expressed ASα2 interacts with an *E. coli* glutamine amidotransferase which is not encoded by *trpD* and which is selectively but not quantitatively removed by affinity chromatography. These findings are consistent with the differential growth observed for Δ*trpED27*/pASα39 and Δ*trpED27*/pGASα12 on M9IA20 and M9IA100, respectively.

Molecular mass of native AS

The apparent native molecular weight of AS from plants and from *E. coli* mutants expressing ASα was estimated

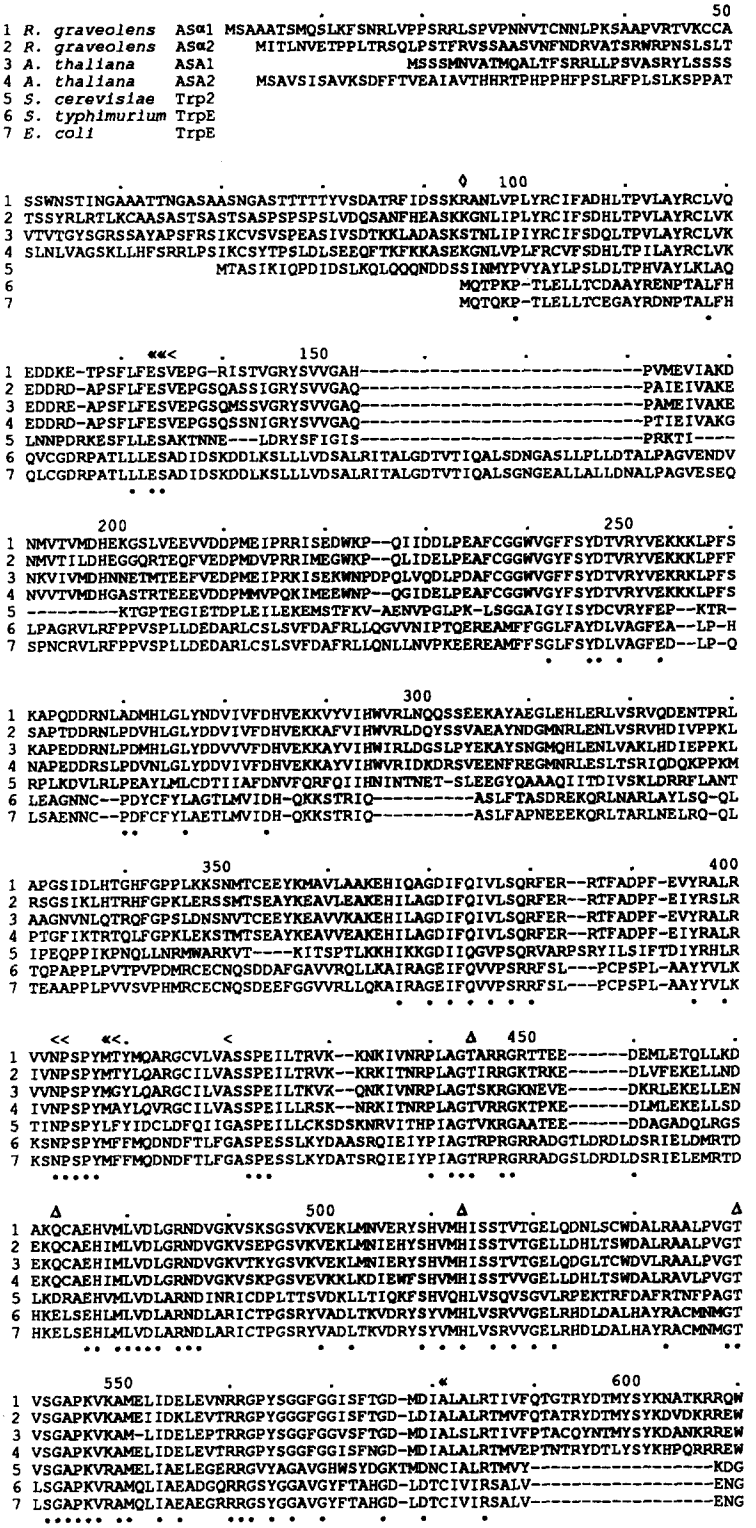

Figure 3. Alignment of ASα amino acid sequences.

Deletions are indicated with dashes, the putative processing site for the transit peptide is indicated with a diamond, strictly conserved residues in the alignment are indicated by dots below the *E. coli* sequence. The N-terminus of the *R. graveolens* ASα1 precursor was arbitrarily chosen as the first amino acid of the numbering scheme. Sources of sequences are: *Arabidopsis thaliana,* Niyogi and Fink (1992); *Saccharomyces cerevisiae,* Zalkin *et al.* (1984), corrected as suggested by Crawford (1989) from positions 615 to 629; *Salmonella typhimurium,* Caliguri and Bauerle (1991); *Escherichia coli,* Yanofsky *et al.* (1981). The conserved sequence motif L(L/F)ES (131–134) is critical for allosteric inhibition by tryptophan (Matsui *et al.*, 1987). Other residues known to affect function in the *Salmonella* enzyme (Caliguri and Bauerle, 1991) are designated above the alignment: '<' indicates residues at which mutation reduces allosteric inhibition by tryptophan, '≪' indicates residues at which mutation abolishes allosteric inhibition by tryptophan, 'Δ' indicates residues at which mutation results in loss of enzyme function. His to Met mutation at His-513 of the *Salmonella* sequence results in ASα which retains aminodeoxyisochorismate (ADIC) synthase activity, but lacks ADIC lyase activity (Morollo and Bauerle, 1993).

Table 1. Glutamine- and ammonia-dependent anthranilate synthase activity in desalted crude extracts of *R. graveolens*, *E. coli* Trp^+ cells and *E. coli trp* deletion mutants complemented with cDNAs for *Ruta* ASα1 (pASα39) and ASα2 (pASα12)

Enzyme source	Specific activity (pkat mg^{-1} protein)	
	AS-Gln	*AS-NH3*
R. graveolens[a]	6.6	6.5
E. coli nm522 (Trp^+)	22.7	64.5
E. coli Δ*trpE5*/pASα39	6.6	34.3
E. coli Δ*trpE5*/pGASα12	6.9	4.4
E. coli Δ*trpED27*/pASα39	0.8	4.7
E. coli Δ*trpED27*/pGASα12	2.4	11.8

[a]Filtered supernatant prior to PEG fractionation. Fifty microliters of desalted *E. coli* crude extracts were assayed.

by chromatography on Superdex-75 and Superose-12. For 1700-fold purified AS from *R. graveolens*, a native molecular weight of 64–67 kDa was determined for the AS-Gln activity. Very similar values were determined for enzyme extracts from Δ*trpE5*/pASα39 (56–64 kDa, respectively). By comparison, 119 kDa was found for AS from *E. coli* trp^+, which is very close to the value expected for the stable *E. coli* αβ dimer (120 kDa; Zalkin, 1980). These results indicate that ASα and ASβ of purified AS from *Ruta* are of a similar molecular weight in the range of 60–65 kDa, but do not remain associated during gel filtration, probably due to the necessary ommission of glycerol from the buffer. That Δ*trpE5*/pASα39 AS-Gln activity also elutes with a molecular weight of approximately 60 kDa indicates that *Ruta* ASα1, in contrast to *E. coli* TrpE, does not remain associated with the 57 kDa *E. coli trp*D gene product (Horowitz *et al.*, 1982) under these conditions. This is consistent with the results of complementation experiments which suggest an ASβ requirement for ASα1 yet poor interaction between ASα1 of Δ*trpE5*/pASα39 and *E. coli* endogenous TrpD.

The precursor of Ruta *ASα is imported into plastids*

The molecular weights of proteins encoded by pASα39 and pASα12 are both 68 kDa and these contain serine-rich regions which extend 90–93 amino acids beyond the N-terminus of ASα from *E. coli* (Figure 3). AS, like other cloned enzymes of aromatic amino acid biosynthesis, is thought to be a chloroplast protein (Eberhard *et al.*, 1993; Görlach *et al.*, 1993a, 1993b; Last, 1993). We synthesized capped mRNA from *Xba*I-linearized pASα39 with T_3 RNA polymerase and translated it *in vitro* in the presence of ^{35}S-methionine. The translation products were incubated with isolated pea chloroplasts which were then purified and separated into stromal and thylakoid fractions (Figure 4). The translation product of pASα39 is efficiently

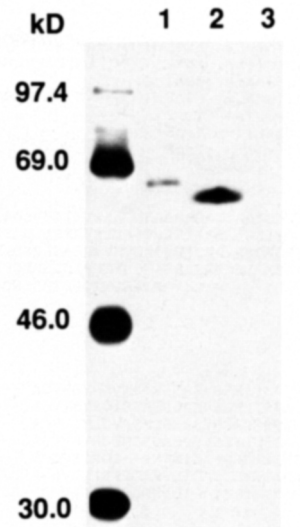

Figure 4. *In vitro* synthesized ASα1 precursor imported into pea chloroplasts. Molecular weights of standards are indicated. Lane 1, precursor synthesized *in vitro* from *in vitro* transcribed mRNA before transport, 0.5% of a translation mix was loaded in each slot. Lane 2, stroma proteins after transport corresponding to 20% of the total stroma fraction per lane. Lane 3, thylakoid proteins after transport corresponding to 20% of the total thylakoid fraction per lane. The gel was exposed for 7 days at −80°C.

imported by pea chloroplasts. Since the stromal protein has a molecular weight of roughly 60 kDa it is processed upon import and pASα39 encodes a functional transit peptide. These data indicate that ASα in *Ruta* is synthesized as a cytosolic precursor and that the active subunit is localized in the stroma of plastids.

Differential expression of Ruta *ASα genes*

It was previously shown that AS activity increases in cell cultures of *R. graveolens* upon elicitation with *Rhodotorula* extract to a maximum of fourfold 12 h after induction (Bohlmann and Eilert, 1994). We performed Northern hybridization with probes for pASα12 and pASα39 against total RNA isolated from elicited and non-elicited cell cultures in order to determine whether the induction is due to an increase in the steady-state mRNA level and if so, whether both genes respond to elicitor induction. The results of these experiments show that *ASα1* and *ASα2* are constitutively expressed in non-elicited *Ruta* cell cultures at low levels detectable only after long exposure times (Figure 5). Upon elicitation steady-state mRNA levels emanating from the *ASα1* gene are dramatically induced whereas *ASα2* expression remains constant. The increase in steady-state mRNA levels for *ASα1* is roughly 30-fold 3 h after elicitation and at least 100-fold after 6 h, falling off to roughly 60-fold after 9 and 12 h, respectively (data not shown). The maximum induction in AS enzyme activity observed in *Ruta* cell cultures correlates with *ASα1* mRNA induction kinetics (Bohlmann and Eilert, 1994). The marked

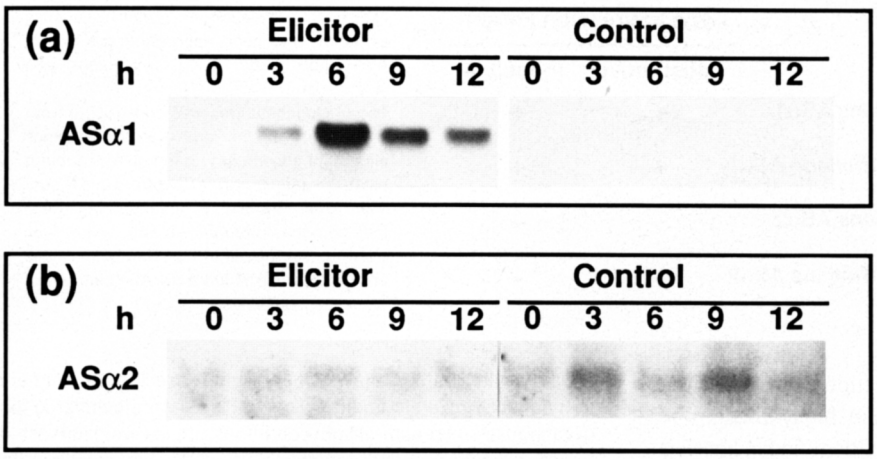

Figure 5. Northern blot of total RNA from cell cultures of *R. graveolens*.
(a) Signal for *ASα1* after 9 h exposure.
(b) Signal for *ASα2* after 288 h exposure.
Cell cultures of *R graveolens* were treated with *Rhodotorula rubra* extract (elicitor) or sterile water (controls), harvested in liquid nitrogen at the times indicated and stored for RNA extraction. Twenty micrograms of total RNA per lane was electrophoresed in a formaldehyde-agarose gel, transferred to a nylon membrane and probed with ^{32}P-labeled cDNA inserts of pASα39 (*ASα1*) and pASα12 (*ASα2*) (see Experimental procedures). Note that panel (b) was exposed 30 times longer than panel (a). After 288 h of exposure, control lanes of panel (a) revealed constitutive expression levels virtually identical to those observed in the control lanes of panel (b).

discrepancy between the degree of induction of mRNA accumulation and enzyme activity after elicitation suggests that post-transcriptional or enzymatic regulatory mechanisms (allosteric or feedback inhibition) are involved in the modulation of ASα1 activity in *Ruta*.

Discussion

AS has not been purified to complete homogeneity from any plant. As a consequence, relatively little is known about either the enzyme's subunit structure or the properties of the β-subunit. We purified AS from cell cultures and shoots of *R. graveolens* 1700- and 670-fold, respectively. In contrast to previous studies of this plant enzyme, we monitored NH_3- and glutamine-dependent activities through all purification procedures. We found that both activities strictly co-purified and that the ratio of AS-Gln to AS-NH_3 activities did not vary during purification, indicating that glutamine amidotransferase activity contributed by ASβ was not selectively lost during any step. That *Ruta* ASα requires a β-subunit for the AS-Gln reaction was shown by expression of cloned ASα1 and ASα2 in *E. coli* mutants lacking both ASα (TrpE) and ASβ (TrpG-domain of TrpD).

The purified *Ruta* enzyme consists of α- and β-subunits of approximately 60–65 kDa each. This is in contrast to recent findings for AS from *Catharanthus roseus*, which was reported to have a native molecular weight of 145 kDa and to consist of two 67 kDa α-subunits and two 25 kDa β-subunits (Poulsen *et al.*, 1993). A third study of plant AS suggests that the partially purified *Dianthus caryophyllus* enzyme has a native molecular weight of 140 kDa and may be composed of two subunits of 68 and 72 kDa, respectively (Matern, 1994), which would be congruent with our data. The existence of 25 kD ASβ subunits in *A. thaliana* was deduced from *E. coli* functional complementation approaches and from *Arabidopsis* mutant analyses (Niyogi *et al.*, 1993), although ASβ proteins from *Arabidopsis* have not been isolated. Thus, the molecular weight of the β-subunit may vary markedly across plants, as it does across bacteria and fungi (Hütter *et al.*, 1986; Zalkin, 1980; Zalkin *et al.*, 1984).

Young *Ruta* shoots express two genes for ASα subunits, *ASα1* and *ASα2*. As judged by the frequency of positive clones in our cDNA library, these genes are expressed at equal levels in young plants and each contributes roughly 0.05 % to the clonable poly(A)$^+$ fraction. The *E. coli* complementation experiments demonstrate conclusively that both genes encode active and functional ASα subunits which are able to interact with *E. coli* glutamine amidotransferases. Similar to *Ruta*, *Arabidopsis* also expresses two genes for ASα (Niyogi and Fink, 1992), one of which is inducible in response to environmental stimuli, and one of which is constitutively expressed. In *Ruta* cell cultures, *ASα2* mRNA is constitutively expressed whereas *ASα1* mRNA is induced at least 100-fold in response to elicitation (Figure 5). Although *Arabidopsis* synthesizes indole phytoalexins from tryptophan in defense response (Tsuji *et al.*, 1992) whereas *Ruta* synthesizes acridone alkaloids from anthranilate as antimicrobial compounds (Figure 1), the AS genes implicated in secondary metabolism share a common origin, as do those which are constitutively expressed under various conditions and therefore likely involved in tryptophan biosynthesis for translation (Figure 6). The evolutionary history of inducible and constitutively expressed ASα genes in *Arabidopsis* and *Ruta* suggests duplication in their common ancestor and conservation of the expression regime.

A conserved expression pattern for duplicated ASα genes specific to primary and secondary metabolism, respectively, may be a general principle in those plants which synthesize defense-related compounds derived from anthranilic acid, such as representatives of the Caryophyllaceae or Poaceae (Figure 1). Notably, constitutively expressed and elicitor-inducible gene pairs of two other enzymes of the shikimate pathway, DAHP synthase and chorismate synthase, have recently been characterized in

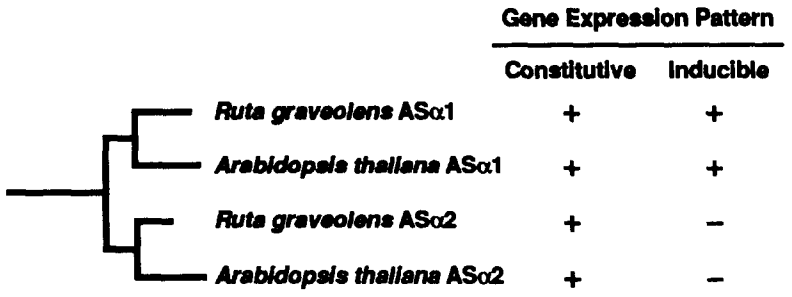

Figure 6. Correlation of protein sequence evolution and gene expression pattern for ASα1 and ASα2 of *R. graveolens* and *A. thaliana*.
The phylogenetic tree was constructed from the region of the amino acid sequence alignment encompassing the mature subunit using the paup program (Swofford and Olson, 1990) after removal of invariant positions. *Pseudomonas aeruginosa* and yeast ASα were used as the outgroups. Bootstrap values are 77/100 and 61/100 for the ASα1 and ASα2 branches, respectively.

tomato (Schmid, personal communication). Duplicated AS gene pairs participating in tryptophan biosynthesis and secondary metabolism have also been identified in bacteria (Essar *et al.*, 1990).

Radioactively labeled ASα precursor is imported into the stroma of isolated chloroplasts (Figure 4), indicating plastid localization of ASα in *Ruta*. In accordance with this, only plastid-specific forms have been identified at the molecular level for other enzymes of the shikimate pathway (Eberhard *et al.*, 1993; Görlach *et al.*, 1993a, 1993b) as well as for other enzymes involved in the biosynthesis of tryptophan (Last, 1993). Thus, the hypothesis (Jensen *et al.*, 1989) that the independent regulation of primary and secondary shikimate metabolism is accomplished by differential chloroplast/cytosol compartmentation may require re-evaluation.

Differential expression of ASα genes in *Ruta* indicates that regulatory separation of tryptophan and alkaloid biosynthetic pathways within plant cells occurs at the level of anthranilate synthase. The anthranilate utilizing enzyme of the acridone alkaloid branch, SAM: anthranilic acid N-methyltransferase (NMT), is co-induced with AS and may channel anthranilic acid out of the tryptophan branch (Bohlmann and Eilert, 1994; Eilert and Wolters, 1989). Other putative mechanisms involved in partitioning of anthranilic acid between the tryptophan and the alkaloid branch require further attention, such as the possible release of the aminodeoxyisochorismate intermediate of AS catalysis and its specific conversion by the downstream enzyme of secondary metabolism (Morollo and Bauerle, 1993), or the potential role of multifunctional ASβ subunits.

Experimental procedures

Plant material

Cell cultures of *Ruta graveolens* (R-MS) were grown on Eriksson medium (Scharlemann, 1972) containing 4% sucrose, 0.02 mg ml^{-1} kinetin and 0.1 mg ml^{-1} NAA on a gyratory shaker (120 r.p.m.) at 24°C under a 16/8 h light/dark photoperiod. Cells were subcultured in 8-day intervals, harvested by filtration, frozen in liquid nitrogen and stored at −80°C. Elicitation of 8-day-old cultures for Northern blots was performed by addition of 1% (v/v) *Rhodotorula* extract as described (Bohlmann and Eilert, 1994; Eilert *et al.*, 1984); 30 g of elicited cell culture was harvested for each time point and immediately frozen in liquid nitrogen. Plants were cultivated in a greenhouse under natural light conditions, shoots were harvested, frozen in liquid nitrogen and stored at −80°C. Pea seedlings for chloroplast isolation were grown at 24°C under a 16/8 h light/dark photoperiod.

E. coli *strains and media*

The *E. coli* trpE deletion mutant Δ*trpE5* (*W3110 tnaA2 trpΔE5*) contains a deletion in the ASα subunit but produces glutamine amidotransferase activity of ASβ. The *trpED* double deletion mutant Δ*trpED27* (*W3110 trpR⁻trpΔED27*; Jackson and Yanofsky, 1974) is defective in both ASα and ASβ activity. Prior to transformation by the $CaCl_2$ method, Trp^- strains were maintained on M9 medium, pH 7.5 (Ausubel *et al.*, 1989) containing 200 μM tryptophan. Basis for all media involving transformed Trp^- strains was M9 medium containing 100 μg ml^{-1} ampicillin and 0.1 mM IPTG but lacking NH_4Cl. NH_4Cl was added to a final concentration of either 1 mM (M9IA1 medium), 20 mM (M9IA20 medium), or 100 mM (M9IA100 medium). *E. coli* nm522 was obtained from Stratagene.

Enzyme assay

AS activity was assayed as described (Bohlmann and Eilert, 1994) with a slight modification of the assay buffers. AS-Gln assays were incubated for 1 h at 30°C in 100 μl containing 12.5 mM HEPES, 30 mM L-glutamine, 2.5% (v/v) glycerol, 250 μM DTT, 10 mM $MgCl_2$, 1.15 mM chorismic acid, pH 8.0 and 50 μl enzyme sample. AS-NH_3 assays were incubated for 1 h at 30°C in 100 μl containing 12.5 mM Tris, 1.25% (v/v) glycerol, 250 μM DTT, 250 μM EDTA, 10 mM $MgCl_2$, 1.15 mM chorismic acid, 100 mM NH_4Cl, pH 8.5 and 50 μl enzyme sample. Reactions were stopped by the addition of 10 μl of 5 M H_3PO_4. Controls for each assay were run by addition of acid prior to incubation. Crude extracts and PEG-fractionated proteins were assayed after desalting on PD10 columns (Pharmacia) equilibrated with the appropriate assay buffer lacking substrates.

Protein purification from cell cultures

All extraction and purification procedures were carried out at 4°C. Frozen cells (1 kg) were homogenized with 1000 ml extraction buffer containing 100 mM HEPES, 20 mM L-glutamine, 10% (v/v) glycerol, 1 mM DTT, pH 8.0 in a Waring blender. Fifty grams of polyvinylpolypyrrolidone were added, the homogenate was gently stirred for 30 min and centrifuged at 12 000 ***g*** for 15 min. The supernatant was filtered through miracloth, 50% (w/v) PEG 8000

was added to a final concentration of 5% (w/v) and the suspension was stirred for 20 min. Precipitated protein was removed by centrifugation at 12 000 ***g*** for 30 min. The supernatant was adjusted to a final concentration of 15% (w/v) PEG 8000 and proteins were precipitated as above. The pellet was resuspended to a final volume of 80 ml with 50 mM HEPES, 20 mM L-glutamine, 10% (v/v) glycerol, 1 mM DTT, pH 8.0 and loaded on to a 30 ml (1.6 × 15 cm) Q-Sepharose column. The column was washed with 90 ml of the same buffer at 1.33 ml min^{-1}. Proteins were eluted with a 300 ml linear gradient of 0–600 mM KCl. Active fractions were pooled and loaded at 1 ml min^{-1} on to a 90 ml (2 × 29 cm) hydroxyapatite column equilibrated with 10 mM potassium phosphate, pH 7.0, 20 mM L-glutamine, 10% (v/v) glycerol, 1 mM DTT, 1 mM EDTA. The column was washed with 100 ml of the same buffer, proteins were eluted with a 400 ml linear gradient of 10–200 mM potassium phosphate. Activity assays for the eluate from hydroxyapatite chromatography were carried out with 5 µl sample for AS-NH_3 to avoid interference with the assay pH. The active fractions were pooled and concentrated to 15 ml by ultrafiltration (Amicon YM-10). The retentate was loaded on to a 30 ml (1.6 × 15 cm) Matrex Gel Orange A (Amicon) column equilibrated with buffer A (100 mM potassium phosphate, 20 mM L-glutamine, 10% (v/v) glycerol, 1 mM DTT, 1 mM EDTA, pH 7.0). The column was washed with 60 ml at 0.35 ml min^{-1}. Proteins were eluted with a linear gradient (60 ml) of 0–100% buffer A to buffer B (50 mM HEPES, 20 mM L-glutamine, 10% (v/v) glycerol, 1 mM DTT, pH 8.0) and an additional 90 ml of buffer B. Active fractions were pooled and concentrated to 3 ml by ultrafiltration. Two milliliters of the retentate were applied to a Mono Q HR 5/5 column equilibrated with 50 mM HEPES, 20 mM L-glutamine, 1 mM DTT, pH 8.0. The column was washed with 13 ml and eluted with a 25 ml 0–600 mM KCl linear gradient at 0.5 ml min^{-1}. Peak fractions were pooled and chromatographed at 0.4 ml min^{-1} on a Superose-12 HR 10/30 column equilibrated with 50 mM HEPES, 20 mM L-glutamine, 1 mM DTT, 150 mM KCl, pH 8.0. Fractions of 0.25 ml were assayed for AS activity.

Purification of anthranilate synthase from plants

Purification of AS from plants was the same as for the purification from cell cultures, with some minor modifications as follows. Frozen plant material (250 g) was homogenized with 500 ml of extraction buffer, and 25 g of polyvinylpolypyrrolidone were added to the homogenate. The pellet after PEG fractionation was resuspended to a final volume of 40 ml, frozen in liquid nitrogen and stored at −80°C for 4 days; prior to the Q-Sepharose step the volume of the sample was increased to 80 ml.

Native molecular mass determination

The native molecular weight of proteins was estimated by FPLC gel filtration at 0.4 ml min^{-1} on Superose-12 and Superdex-75. Columns were equilibrated in 50 mM HEPES, pH 8.0, 20 mM L-glutamine, 1 mM DTT, 150 mM KCl. Samples were applied in a volume of 200 µl, fractions of 0.25 ml were collected and assayed. Standards for Superdex-75 were bovine serum albumin (67 kDa), ovalbumin (44 kDa), chymotrypsinogen A (25 kDa), and ribonuclease (13.5 kDa). For Superose-12 chromatography, the commercial standard from Bio-Rad was used with the addition of bovine serum albumin. Elution volumes of proteins and blue dextran were calculated from peak retention time and flow rate.

AS from wild-type *E. coli* for gel filtration was prepared from *E. coli* nm522 grown in M9 medium containing 20 mM NH_4Cl at 37°C for 6 h. Cells were harvested by centrifugation and resuspended with 20 ml of 100 mM HEPES, 20 mM L-glutamine, 10% (v/v) glycerol, 1 mM DTT, pH 8.0 and sonified. The lysate was cleared by centrifugation for 45 min at 20 000 ***g*** and 4°C. Ammonium sulfate was added to the supernatant to 60% saturation. The protein pellet was collected by centrifugation and resuspended in 1 ml of 50 mM HEPES, 20 mM L-glutamine, 10% (v/v) glycerol, 1 mM DTT, pH 8.0.

Crude extracts from *E. coli* complemented mutants for enzyme assays were prepared from a 100 ml culture grown in M9IA20 at 37°C for 6 h. *ΔtrpED27*/ASα39 was grown in M9IA100. Cells were harvested by centrifugation and resuspended in 2 ml of 50 mM Tris-HCl, 5% (v/v) glycerol, 1 mM DTT, 1 mM EDTA, 2 mM $MgCl_2$, pH 8.5 and sonified. The lysate was cleared by centrifugation for 15 minutes at 20 000 ***g*** and 4°C. For AS-NH_3 assay 1 ml was desalted on a PD10 column (Pharmacia) in the same buffer, 1 ml for AS-Gln assay was desalted on a PD10 column in 50 mM HEPES, 20 mM L-glutamine, 10% (v/v) glycerol, 1 mM DTT, 2 mM $MgCl_2$, pH 8.0.

Protein determination and SDS-PAGE

Protein was estimated by the method of Bradford (1976) using the Bio-Rad reagent dye concentrate according to the manufacturer's instructions with bovine serum albumin as a standard. For SDS-PAGE, samples from the PEG fractionation, Q-Sepharose, Hydroxyapatite, and Orange A steps containing 5 µg protein were diluted with water and desalted on 1 ml Sephadex-G25 spun columns. The eluate was precipitated with acetone and centrifuged. Dried pellets were dissolved in 30 µl of Laemmli buffer and boiled: 10 µl were loaded per lane. SDS–PAGE was performed as described by Maniatis *et al.* (1989).

Overproduction of ASα in E. coli

For overexpression of ASα2, the 1.7 kb *NotI-SalI* fragment of pASα12 was ligated into pGEX-4T-3 (Pharmacia) to yield pGASα12 which was transformed into *E. coli ΔtrpED27.* Amp^r colonies were streaked on to M9IA20 agar, from which liquid M9IA20 was inoculated. Harvested cells from 2000 ml culture grown according to the Pharmacia pGEX protocol were resuspended with 25 ml PBS, lysed by sonification and incubated for 30 min on ice after addition of 1 ml of 20% (v/v) Triton X-100. The extract was centrifuged as described for gel filtration above. Proteins from 2000 ml culture were purified over a 500 µl glutathion-Sepharose column (Pharmacia) according to the manufacturer's protocol. ASα was eluted after cleavage of the affinity-bound fusion protein with 25 units thrombin in 500 µl PBS for 6 h at 25°C.

Molecular methods

Total RNA from 20 g of 4-week-old *Ruta* seedlings was isolated by the method of Logemann *et al.* (1987) from which mRNA was purified (Martin *et al.*, 1993) for cDNA cloning as described (Martin *et al.*, 1990). Recombinants were screened by plaque hybridization at 55°C in 3 × SSPE with 10 ng ml^{-1} of random-labeled 0.9 kb *EcoRV* fragment from pAS4 (Zalkin *et al.*, 1984). *NotI* inserts of 10 positively hybridizing cDNAs were subcloned into pBluescriptSK plasmids (Stratagene). Rescreening for full size homologs of pASα27 was performed at 39°C in 6 × SSPE, with 0.4 nM of the end-labeled oligo 5′-ATCTGGTTGCATCACT-3′. Sequence analysis was performed with the GCG-Package (Devereux *et al.*, 1984). Transcription of *XbaI* linearized pASα39 with T_3 Polymerase

(Pharmacia), *in vitro* translation and import of precursors was performed as described (Clausmeyer *et al.*, 1993; Meyer-Gauen *et al.*, 1994).

For Northern blots, 20 μg of total RNA per lane were transferred to nylon membranes (Hybond-N, Amersham) according to the manufacturer's protocol and hybridized at 68°C in 3 × SSPE with 10 ng ml^{-1} of the 2.0 kb *Not*I insert of pASα12 or the 1.7 kb *Not*I fragment of pASα39 random-labeled to 3 × 10^8 c.p.m. μg^{-1}. Filters were washed for 45 min in 0.2 × SSPE, 0.1% (w/v) sodium dodecyl sulfate at 68°C and autoradiographed at −80°C for 9 h (elicited cultures with the pASα39 probe) or 288 h (all other exposures). Other molecular techniques were performed according to Maniatis *et al.* (1989).

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