Anthranilate Synthase from *Ruta graveolens*[1]

Duplicated *ASα* Genes Encode Tryptophan-Sensitive and Tryptophan-Insensitive Isoenzymes Specific to Amino Acid and Alkaloid Biosynthesis

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Anthranilate synthase (AS, EC 4.1.3.27) catalyzes the conversion of chorismate into anthranilate, the biosynthetic precursor of both tryptophan and numerous secondary metabolites, including inducible plant defense compounds. The higher plant *Ruta graveolens* produces tryptophan and elicitor-inducible, anthranilate-derived alkaloids by means of two differentially expressed nuclear genes for chloroplast-localized ASα subunits, *ASα1* and *ASα2*. Mechanisms that partition chorismate between tryptophan and inducible alkaloids thus do not entail chloroplast/cytosol separation of AS isoenzymes and yet might involve differential feedback regulation of pathway-specific ASα subunits. The two ASα isoenzymes of *R. graveolens* were expressed as glutathione S-transferase fusion proteins in *Escherichia coli* deletion mutants defective in AS activity and were purified to homogeneity. Differential sensitivity of the transformed *E. coli* strains toward 5-methyltryptophan, a false-feedback inhibitor of AS, was demonstrated. Characterization of affinity-purified ASα isoenzymes revealed that the noninducible ASα2 of *R. graveolens* is strongly feedback inhibited by 10 μM tryptophan. In contrast, the elicitor-inducible ASα1 isoenzyme is only slightly affected even by tryptophan concentrations 10-fold higher than those observed in planta. These results are consistent with the hypothesis that chorismate flux into biosynthesis of tryptophan and defense-related alkaloid biosynthesis in *R. graveolens* is regulated at the site of ASα isoenzymes at both genetic and enzymatic levels.

The shikimic acid pathway in bacteria, fungi, and plants generates the aromatic amino acids Phe, Tyr, and Trp and leads to the biosynthesis of numerous secondary metabolites (Poulsen and Verpoorte, 1991; Dewick, 1995). Secondary metabolites can be formed from common intermediates of the core shikimic acid pathway, e.g. anthraquinones derived from chorismate, or by conversion of aromatic amino acids, e.g. Phe-derived phenylpropanoids (Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995). The Trp branch of the shikimic acid pathway provides precursors for secondary metabolites of considerable structural diversity, e.g. indole alkaloids (Tillequin et al., 1993), monoterpenoid indole alkaloids (De Luca, 1993; Kutchan, 1995), ergot alkaloids (Ninomiya and Kiguchi, 1990), and anthranilate-derived phenazines (Essar et al., 1990) and benzodiazepines (Roos, 1990). Many of the Trp pathway-derived plant secondary metabolites have chemo-ecological functions as constitutive or preformed defense compounds, such as benzoxazinones in Poaceae (Niemann, 1993) and indole glucosinolates in Brassicaceae (Chavadej et al., 1994). Moreover, environmental stimuli trigger the biosynthesis of plant defense compounds derived from the Trp pathway. Pathogen attack induces the accumulation of an indolic phytoalexin in *Arabidopsis thaliana* (Tsuji et al., 1992) as well as the biosyntheses of anthranilate-derived phytoalexins in Caryophyllaceae and Poaceae (Niemann, 1993). In cultured cells of the medicinal plant *Ruta graveolens*, biosynthesis of antimicrobial anthranilate-derived acridone alkaloids and furoquinoline alkaloids is inducible by fungal elicitors (Eilert and Wolters, 1989; Bohlmann and Eilert, 1994; Bohlmann et al., 1995). Inducible AS (EC 4.1.3.27) has a key regulatory function for elicitor-inducible alkaloid accumulation in *R. graveolens* (Bohlmann et al., 1995). Although plant AS is extremely unstable and difficult to characterize, previous studies suggested that AS is the rate-limiting enzyme of plant Trp biosynthesis (Belser et al., 1971; Widholm, 1972; Radwanski and Last, 1995; Li and Last, 1996) and interest has focused on the possible regulatory role of feedback inhibition by Trp, in analogy to the allosteric regulation of bacterial AS (Zalkin, 1980). Plant AS has only recently been studied at the molecular genetic and biochemical levels. Enzyme purification (Poulsen et al., 1993; Bohlmann et al., 1995) and cDNA cloning (Niyogi and Fink, 1992; Niyogi et al., 1993; Bohlmann et al., 1995)

Abbreviations: Amp^r, ampicillin resistant; AS, anthranilate synthase; AS-NH₃, ammonia-dependent AS; GST, glutathione S-transferase, IPTG, isopropylthiogalactoside; 5-MT, 5-methyltryptophan.

revealed that plant AS, like its bacterial homologs (Zalkin, 1980), is composed of two nonidentical subunits, ASα and ASβ.

ASα catalyzes the conversion of chorismate to anthranilate with ammonia as the amino donor. The α subunit of both bacterial and yeast AS contains specific residues that were shown to be involved in Trp-dependent feedback inhibition of AS activity (Matsui et al., 1987; Caliguri and Bauerle, 1991; Graf et al., 1993). ASβ confers on the holoenzyme the capacity to utilize Gln in addition to ammonia as a nitrogen donor. ASα and ASβ from R. graveolens constitute a heterodimeric ASαASβ holoenzyme (Bohlmann et al., 1995). Expression of two nonallelic nuclear ASα genes, ASα1 and ASα2, which are differentially regulated at the transcriptional level, was demonstrated for R. graveolens (Bohlmann et al., 1995). Elicitation of R. graveolens cell cultures induces a transient 100-fold increase of the ASα1 steady-state mRNA level preceding elicitor-induced AS enzyme activity and acridone alkaloid accumulation. In contrast, the low constitutive expression of ASα2 is not affected by elicitation, suggesting different metabolic roles of ASα1 and ASα2 in primary and secondary metabolism. Because formation of anthranilate for Trp biosynthesis is strictly feedback inhibited at the site of ASα (Radwanski and Last, 1995), regulatory mechanisms are required for Trp-independent formation of anthranilate for alkaloid biosynthesis. Chloroplast/cytosol separation of AS isoenzymes has been suggested as such a mechanism for substrate partitioning between primary and secondary metabolism (Hrazdina and Jensen, 1992). However, as is the case for all cloned genes of aromatic amino acid biosynthetic enzymes (Herrmann, 1995; Radwanski and Last, 1995; Schmid and Amrhein, 1995), both R. graveolens ASα1 and ASα2 encode cytosolic precursors with N-terminal transit peptides for chloroplast import of active ASα enzymes (Bohlmann et al., 1995). Therefore, partitioning of chorismate toward Trp or inducible alkaloids, respectively, could involve differential feedback regulation of pathway-specific ASα isoenzymes rather than chloroplast/cytosol separation. Testing this hypothesis requires investigation of pure AS isoenzymes, which has previously not been possible because of the instability of native plant AS (Poulsen et al., 1993; Bohlmann et al., 1995). Here we describe purification of the two R. graveolens ASα isoenzymes to homogeneity after expressing ASα1 and ASα2 in Escherichia coli. The catalytic and differential regulatory properties of recombinant ASα isoenzymes are compared with the differential regulation of ASα isoenzymes isolated from alkaloid-producing cell cultures of R. graveolens. The results support the hypothesis of distinct functions of ASα isoenzymes specific to primary and secondary metabolism.

MATERIALS AND METHODS

Plant Material

Cell cultures of Ruta graveolens (Rutaceae) were grown, elicitor treated with an autoclaved extract of Rhodotorula rubra, harvested, and extracted for AS-NH$_3$ enzyme assays as described previously (Bohlmann and Eilert, 1994; Bohlmann et al., 1995).

Escherichia coli Strain and Growth Conditions

The trpED double-deletion mutant W3110 trpR⁻ trpΔED27 (Jackson and Yanofsky, 1974) is defective in both ASα and ASβ. Prior to transformation by the CaCl$_2$ method, the Trp⁻ strain was maintained on M9 medium, pH 7.5 (Ausubel et al., 1989) containing 200 μM L-Trp. Transformed bacteria were grown on M9 medium containing 100 μg/mL ampicillin, 0.1 mM IPTG, and 100 mM NH$_4$Cl (M9IA100 medium) at 37°C. For inhibition studies, filter-sterilized 5-MT (Sigma) was added to M9IA100 agar to a final concentration of 0.01, 1, or 100 μM.

Expression of GST-ASα Fusion Proteins in E. coli

Plasmids pASα39 and pASα12 have previously been described (Bohlmann et al., 1995). For expression of $^{Δ1-61}$ASα1, the 1.7-kb NotI fragment of pASα39 was ligated into pGEX-4T-3 (Pharmacia) to yield pGASα1-1. For expression of $^{Δ1-78}$ASα2, the 1.7-kb NotI-SalI fragment of pASα12 was ligated into pGEX-4T-3 to yield pGASα2-1 (Fig. 1). Plasmids pGASα1-1 and pGASα2-1 were transformed into E. coli ΔtrpED27 yielding bacterial strains ΔtrpED27/pGASα1-1 and ΔtrpED27/pGASα2-1. Amp^r colonies were selected on M9IA100 agar.

Purification of $^{Δ1-61}$ASα1 and $^{Δ1-78}$ASα2

For enzyme purification, ΔtrpED27/pGASα1-1 was grown in 1 L of Luria broth medium (Ausubel et al., 1989) supplemented with 100 μg/mL ampicillin and 2% (w/v) Glc at 37°C. IPTG was added to a final concentration of 0.1 mM at an A_{600} of 0.6, and bacteria were grown for another 3 h. ΔtrpED27/pGASα2-1 was grown in 1 L of M9IA100 medium at 37°C to an A_{600} of 0.6. Cells were harvested according to the Pharmacia pGEX protocol, resuspended in 25 mL of PBS, lysed by sonication, and incubated for 30 min on ice after addition of 1 mL of 20% (v/v) Triton X-100. The lysate was cleared by centrifugation for 15 min at 20,000g and 4°C. One milliliter of the cleared lysate was desalted on a PD10 column (Pharmacia) in assay buffer lacking substrates. Fusion proteins GST-$^{Δ1-61}$ASα1 and GST-$^{Δ1-78}$ASα2 were purified from the cleared lysate by affinity chromatography on 500 μL of glutathione-Sepharose (Pharmacia) according to the manufacturer's protocol. ASα isoenzymes, $^{Δ1-61}$ASα1 and $^{Δ1-78}$ASα2, were eluted after cleavage of the affinity-bound fusion protein with 25 units of thrombin in 500 μL of PBS for 2 h at 25°C. SDS-PAGE was performed as described by Ausubel et al. (1989).

Enzyme Assay

AS-NH$_3$ activity during protein purification was assayed by monitoring the formation of anthranilate using the HPLC/fluorescence-spectrophotometric system described by Bohlmann and Eilert (1994). The standard AS-NH$_3$ reaction mixture contained in a total volume of 100 μL: 12.5 mM Tris, 1.25% (v/v) glycerol, 250 μM DTT, 250 μM EDTA, 10 mM MgCl$_2$, 1.15 mM chorismate, 100 mM NH$_4$Cl, pH 8.5, and 5 μL of enzyme sample. Assays were incubated for 1 h at 30°C. Reactions were stopped by addition of 10 μL of 5

M H_3PO_4. Crude extracts were assayed after desalting on PD10 columns (Pharmacia). The reaction mixture for kinetic studies contained equal amounts of either affinity-purified $^{\Delta 1-61}AS\alpha 1$ or $^{\Delta 1-78}AS\alpha 2$ and variable concentrations of chorismate and L-Trp. Formation of anthranilate in the AS-NH_3 reaction mixture was continuously monitored for 5 min at 25°C using an F-3000 fluorescence-spectrophotometer (Kontron, Zurich, Switzerland) at 340 nm excitation and 400 nm emission. For monitoring induction of Trp-insensitive AS activity for native enzymes, desalted extracts of *R. graveolens* cell cultures were assayed as above but in the presence or absensce of 10 μM L-Trp. Protein was estimated by the method of Bradford (1976).

RESULTS

Expression of GST-ASα Fusion Proteins and Purification of ASα Isoenzymes

Two ASα isoenzymes, ASα1 and ASα2, are expressed in *R. graveolens* as nuclear-encoded proteins with N-terminal transit peptides for import of the cytosolic precursor into the stroma of plastids (Bohlmann et al., 1995). Deletion of the transit peptides was desirable for expression of ASα isoenzymes in *E. coli*. Processing sites of mature plant ASα subunits have been predicted for Arabidopsis and *R. graveolens* based on sequence homologies (Niyogi and Fink, 1992; Bohlmann et al., 1995). For expression of ASα1 and ASα2 as GST fusions, we introduced the thrombin cleavage site into Ala_{62} of ASα1 and Val_{79} of ASα2 (Bohlmann et al., 1995), i.e. within the transit peptides 32 and 12 amino acids, respectively, upstream of the putative in vivo N termini as predicted by alignment to bacterial homologs (Bohlmann et al., 1995), to preclude deletions of functionally important residues in the mature subunits of ASα1 and ASα2. The resulting constructs lack 61 (pGASα1–1) and 78 (pGASα2–1) residues of the transit peptides (Bohlmann et al., 1995). pGASα1–1 encodes the fusion protein GST-$^{\Delta 1-61}AS\alpha 1$, and pGASα2–1 encodes the fusion protein GST-$^{\Delta 1-78}AS\alpha 2$ (Fig. 1). pGASα1–1 and pGASα2–1 were transformed into the *E. coli* deletion mutant $\Delta trpED27$ (Jackson and Yanofsky, 1974), which lacks both ASα and ASβ subunits. Heterologously expressed plant ASα proteins tend to associate with bacterial AS proteins (Bohlmann et al., 1995). The use of the double-mutant $\Delta trpED27$ precludes co-purification of *E. coli* AS, which might interfere with the intended isoenzyme characterization. Expression in the *E. coli* Trp$^-$ strain and growth on M9IA100 medium lacking L-Trp allowed direct selection for transformants expressing GST-ASα fusion proteins with AS-NH_3 activity. Transformed *E. coli* strains $\Delta trpED27$/pGASα1–1 and $\Delta trpED27$/pGASα2–1 express fusion proteins GST-$^{\Delta 1-61}AS\alpha 1$ and GST-$^{\Delta 1-78}AS\alpha 2$, respectively, which exhibit the expected molecular mass of 90 kD (not shown). Both GST fusions are enzymatically active, since the expression of plasmids pGASα1–1 and pGASα2–1 complement *E. coli* $\Delta trpED27$ to grow on minimal medium lacking L-Trp (Fig. 2). Specific in vitro AS-NH_3 activities were 10.1 pkat/mg protein for crude extracts from $\Delta trpED27$/pGASα1–1 and 1.62 pkat/mg protein for crude extracts from $\Delta trpED27$/pGASα2–1.

For expression of active *R. graveolens* ASα fusion proteins, bacterial strains $\Delta trpED27$/pGASα1–1 and $\Delta trpED27$/

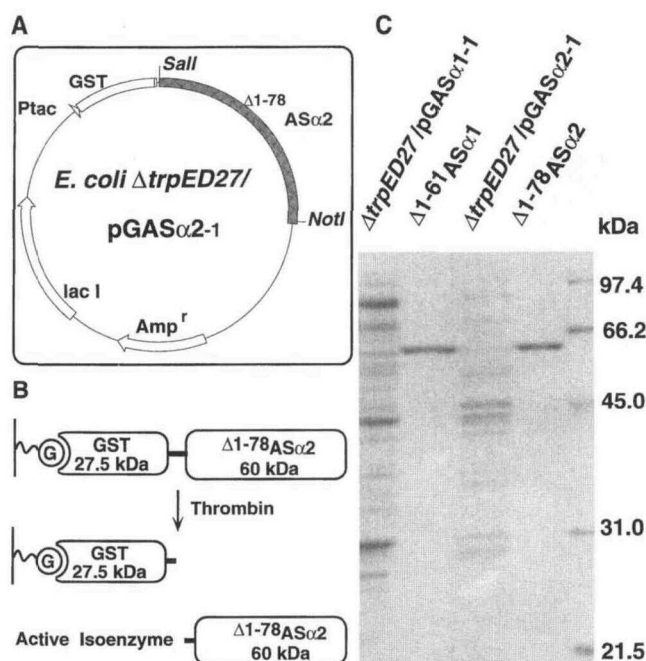**Figure 1.** Bacterial expression of GST-ASα fusion proteins and purification of ASα isoenzymes. A, Plasmid construction and expression of GST-ASα fusion proteins. cDNA fragments encoding $^{\Delta 1-61}AS\alpha 1$ and $^{\Delta 1-78}AS\alpha 2$ from *R. graveolens* were ligated into pGEX-4T-3 to yield plasmids pGASα1–1 and pGASα2–1. Plasmids were transformed into *E. coli* $\Delta trpED27$, yielding bacterial strains $\Delta trpED27$/pGASα1–1 and $\Delta trpED27$/pGASα2–1. B, Scheme of ASα isoenzyme purification. Crude extracts were prepared from $\Delta trpED27$/pGASα1–1 expressing GST-$^{\Delta 1-61}AS\alpha 1$ and from $\Delta trpED27$/pGASα2–1 expressing GST-$^{\Delta 1-78}AS\alpha 2$. Isoenzymes $^{\Delta 1-61}AS\alpha 1$ and $^{\Delta 1-78}AS\alpha 2$ were purified by affinity binding of GST-$^{\Delta 1-61}AS\alpha 1$ and GST-$^{\Delta 1-78}AS\alpha 2$ to glutathione (G)-Sepharose and thrombin cleavage of the affinity-bound fusion proteins to elute the 60-kD ASα isoenzymes. C, SDS-PAGE of crude extracts and purified ASα isoenzymes. Aliquots of 5 to 10 μg of protein of crude extracts of $\Delta trpED27$/pGASα -1 and $\Delta trpED27$/pGASα2–1 and purified $^{\Delta 1-61}AS\alpha 1$ and $^{\Delta 1-78}AS\alpha 2$ were subjected to SDS-PAGE and visualized by Coomassie blue staining.

pGASα2–1 require different culture conditions. $\Delta trpED27$/pGASα1–1 produces large amounts of active fusion protein when cultivated on Luria broth medium supplemented with 2% Glc and accumulation is induced by addition of IPTG. In extracts of $\Delta trpED27$/pGASα2–1, AS activity is detectable only when grown on M9IA100, which, however, limits the amount of protein due to slow growth on minimal medium. Active 60-kD isoenzymes $^{\Delta 1-61}AS\alpha 1$ and $^{\Delta 1-78}AS\alpha 2$ were purified by affinity chromatography of GST-$^{\Delta 1-61}AS\alpha 1$ and GST-$^{\Delta 1-78}AS\alpha 2$ on glutathione-Sepharose (Fig. 1). Affinity purification and thrombin cleavage of the GST-ASα fusion proteins resulted in a 5-fold increase of the total activities of both ASα isoenzymes. Then, 3.8 mg of processed ASα1 isoenzyme with a specific AS-NH_3 activity of 3006 pkat/mg protein was purified from 1 L of culture of $\Delta trpED27$/pGASα1–1, and 56 μg of processed ASα2 with a specific AS-NH_3 activity of 2430 pkat/mg protein was purified from 1 L of $\Delta trpED27$/pGASα2–1. Purified ASα isoenzymes were stable for at least
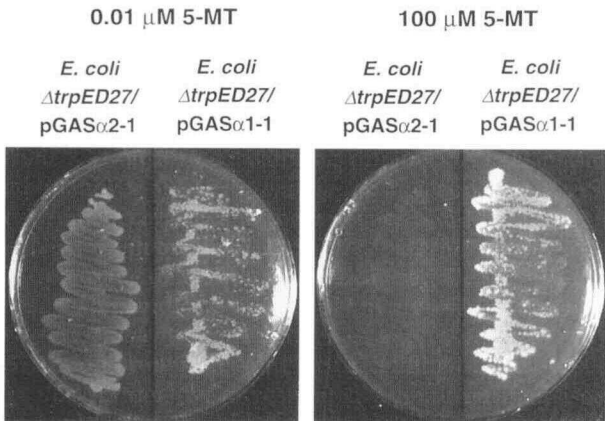
Figure 2. Differential in vivo sensitivity of ASα isoenzymes expressed in *E. coli* toward 5-MT. Expression of pGASα1–1 and pGASα2–1 complements *E. coli* ΔtrpED27 to grow on minimal medium lacking Trp and results in differential growth inhibition of ΔtrpED27/pGASα1–1 and ΔtrpED27/pGASα2–1 by 5-MT. Growth of transformants is shown after 3 d at 37°C on M9IA100 in the presence of 0.01 μM and 100 μM 5-MT.

3 months when stored with 50% glycerol at either −20 or −80°C.

Differential Growth Inhibition of ΔtrpED27/pGASα1–1 and ΔtrpED27/pGASα2–1 by 5-MT

Both transformed *E. coli* strains, ΔtrpED27/pGASα1–1 and ΔtrpED27/pGASα2–1, show the same growth rate on M9IA100 agar lacking Trp. To test the *R. graveolens* ASα isoenzymes in vivo for Trp-dependent feedback inhibition, ΔtrpED27/pGASα1–1 and ΔtrpED27/pGASα2–1 were streaked on minimal medium supplemented with different concentrations of 5-MT. Unlike exogenously applied L-Trp, the antimetabolite 5-MT cannot be used in the biosynthesis of functional proteins; however, 5-MT possesses the inhibitory effect of Trp on plant AS and causes Trp starvation (Moyed, 1960; Widholm, 1972). Both ΔtrpED27/pGASα1–1 and ΔtrpED27/pGASα2–1 grow on minimal medium in the presence of 0.01 μM 5-MT (Fig. 2), but 5-MT at a concentration higher than 1 μM totally prevents the growth of ΔtrpED27/pGASα2–1 expressing the GST-$^{\Delta1-78}$ASα2 fusion protein. In contrast, ΔtrpED27/pGASα1–1 expressing GST-$^{\Delta1-61}$ASα1 still grows at 100 μM 5-MT (Fig. 2). ΔtrpED27 transformed with the original pASα39 plasmid (Bohlmann et al., 1995) expressing the nondeleted ASα1 isoenzyme also grows in the presence of 5-MT. Therefore, the lack of inhibition of ΔtrpED27/pGASα1–1 by 5-MT is not due to the deletion of the transit peptide in $^{\Delta1-61}$ASα1. These results indicate differential in vivo sensitivity of the two *R. graveolens* ASα isoenzymes expressed in *E. coli* as GST fusion proteins toward feedback inhibition by Trp. To test this further, we investigated affinity-purified isoenzymes $^{\Delta1-61}$ASα1 and $^{\Delta1-78}$ASα2 in vitro.

Differential Kinetic Properties of Purified ASα Isoenzymes

$^{\Delta1-61}$ASα1 and $^{\Delta1-78}$ASα2 exhibit Michaelis-Menten kinetics in the ammonia-dependent conversion of chorismate

to anthranilate (Fig. 3). The apparent K_m values for chorismate in the presence of 100 mM NH_4Cl are 17 μM for $^{\Delta1-61}$ASα1 and 29 μM for $^{\Delta1-78}$ASα2. In accordance with the 5-MT-dependent growth inhibition of ΔtrpED27/pGASα2–1, the AS-NH_3 activity of $^{\Delta1-78}$ASα2 is strongly inhibited in vitro by L-Trp. In the presence of 50 μM chorismate the AS-NH_3 activity of $^{\Delta1-78}$ASα2 is reduced to less than 1% by 10 μM L-Trp compared to the reaction without L-Trp. An apparent K_i value of 2.8 μM L-Trp was determined for $^{\Delta1-78}$ASα2. In contrast, the in vitro AS-NH_3 activity of $^{\Delta1-61}$ASα1 is not affected by 10 μM L-Trp, and even at 100 μM L-Trp $^{\Delta1-61}$ASα1 still exhibits 80% AS-NH_3 activity in the presence of 50 μM chorismate (Fig. 3). Thus, differential inhibition of in vitro activities of $^{\Delta1-61}$ASα1 and $^{\Delta1-78}$ASα2 by L-Trp demonstrates differential enzymatic regulation of the two *R. graveolens* ASα isoenzymes.
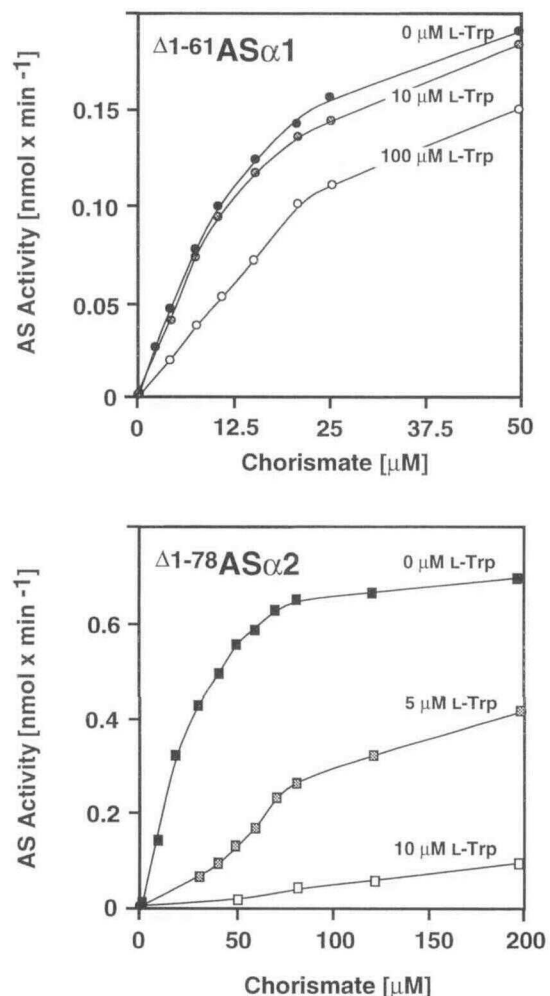

Figure 3. Differential effect of L-Trp on in vitro activity of purified ASα isoenzymes $^{\Delta1-61}$ASα1 and $^{\Delta1-78}$ASα2. AS-NH_3 activity of $^{\Delta1-61}$ASα1 is only slightly affected by 10 and 100 μM L-Trp. In contrast, $^{\Delta1-78}$ASα2 is strongly inhibited by physiological Trp concentrations with an apparent K_i value of 2.8 μM. Apparent K_m values for chorismate of 17 μM for $^{\Delta1-61}$ASα1 and 29 μM for $^{\Delta1-78}$ASα2 were determined by transformation of data into the linear Hanes plot and are the means of three independent determinations.

Differential Expression of Trp-Sensitive and Trp-Insensitive AS Isoenzymes in *R. graveolens*

To ensure that differential Trp sensitivity of $^{\Delta 1-61}$ASα1 and $^{\Delta 1-78}$ASα2 expressed as GST fusions in *E. coli* and observed with the purified $^{\Delta 1-61}$ASα1 and $^{\Delta 1-78}$ASα2 enzymes in vitro reflects the properties of the native plant isoenzymes, we measured AS-NH$_3$ activity in the absence of Trp and in the presence of 10 μM L-Trp in desalted extracts of elicitor-treated *R. graveolens* cell cultures. In nonelicited cells AS activity remained constant as determined in both assay systems. Elicitor treatment of *R. graveolens* cells induces increased AS-NH$_3$ activity preceded by transcriptional activation of the *ASα1* gene and followed by alkaloid accumulation (Bohlmann et al., 1995). When assayed in the absence of Trp, specific AS-NH$_3$ activity increases from 22.9 pkat/mg protein prior to elicitation to 121.5 pkat/mg protein 12 h after elicitation (Fig. 4). When 10 μM L-Trp was included in the assay, specific AS-NH$_3$ activity increases from 7.2 pkat/mg protein prior to elicitation to 94.0 pkat/mg protein 12 h after elicitation. The difference between activities measured without Trp and activities measured in the presence of Trp, 15.7 and and 27.5 pkat/mg protein prior to elicitation and 12 h after elicitation, respectively, is the activity attributable to Trp-sensitive ASα2. The 13-fold increase in Trp-insensitive AS-NH$_3$ activity verifies the presence of a Trp-insensitive, native AS isoenzyme in *R. graveolens*, the induction of which correlates well with those of the *ASα1* mRNA upon
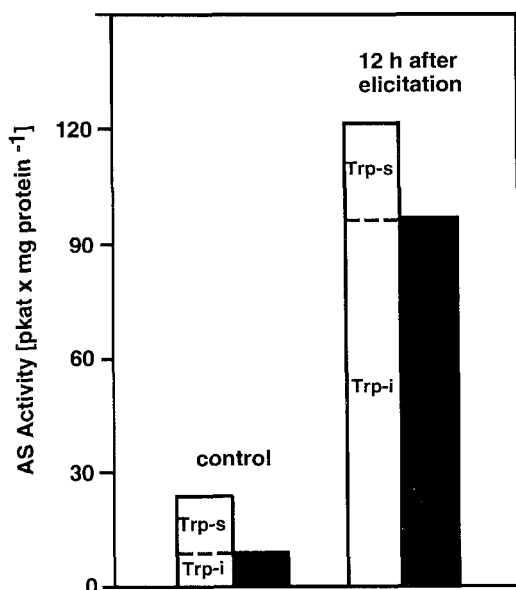

Figure 4. Elicitor induction of Trp-insensitive ASα enzyme activity in cultured cells of *R. graveolens*. AS-NH$_3$ activity was determined in the absence of Trp (white bars) and in the presence of 10 μM L-Trp (black bars) in desalted extracts of cultured cells of *R. graveolens* harvested from the same culture flask immediately after elicitation (control) and 12 h after elicitation. The difference between AS activity in the absence of Trp and Trp-insensitive (Trp-i) AS activity measured in the presence of Trp is the activity of Trp-sensitive (Trp-s) AS. Specific induction of Trp-insensitive AS-NH$_3$ activity was reproducible with independent cell culture batches.

elicitation (Bohlmann et al., 1995) and thus likely corresponds to the Trp-insensitive activity measured for $^{\Delta 1-61}$ASα1 in vitro.

DISCUSSION

Because of their low level of expression and instability (Poulsen et al., 1993; Bohlmann et al., 1995), pure isoenzymes of plant AS have never been separated and therefore relatively little is known about their regulatory properties. cDNA cloning and expression of fusion proteins in *E. coli* has proven to be a suitable tool to study the biochemistry of isoenzymes that were otherwise difficult to separate, e.g. those for Phe ammonia lyase (Appert et al., 1994). Using a similar approach we expressed the two cloned plastid ASα isoenzymes ASα1 and ASα2 from *R. graveolens* (Bohlmann et al., 1995) as GST fusion proteins in the *E. coli* mutant ΔtrpED27 (Jackson and Yanofsky, 1974). Expression of plant ASα in *E. coli* ΔtrpED provided us with in vivo selection for production of active ASα subunits and eliminated potentially contaminating bacterial ASα activity in fusion proteins. Both *R. graveolens* GST-ASα fusion proteins were active with ammonia as the amino donor in vivo as well as in vitro. The two ASα isoenzymes retained AS-NH$_3$ enzyme activity during affinity purification and cleavage of the fusion proteins. This allowed us to study enzymatic properties of pure plant ASα isoenzymes.

We tested *E. coli* strains ΔtrpED27/pGASα2-1 and ΔtrpED27/pGASα1-1 for growth inhibition by 5-MT and tested both ASα isoenzymes in vitro after purification and processing for their sensitivity toward L-Trp. The sensitivity of ΔtrpED27/pGASα2-1 to 5-MT compared to ΔtrpED27/pGASα1-1 suggests that strong Trp-dependent inhibition of ASα2 occurs in vivo. In vitro experiments revealed strong Trp-dependent inhibition of ASα2. Results from both in vivo and in vitro experiments demonstrate that different N-terminal extensions do not affect sensitivity toward Trp. Similar to AS from *Catharanthus roseus* (Poulsen et al., 1993) and to the *A. thaliana* GST-ASA1 fusion protein (Bernasconi et al., 1994), enzyme activity of ASα2 is more than 90% inhibited by 10 μM Trp. The apparent K_i value of 2.8 μM L-Trp for $^{\Delta 1-78}$ASα2 is remarkably similar to the K_i value of 3 μM reported for wild-type Arabidopsis AS (Li and Last, 1996). Since plant cells contain about 1 to 15 μM free Trp (Radwanski and Last, 1995), this result indicates that ASα2 is regulated by feedback inhibition in planta. In contrast, the same Trp concentration does not affect the activity of the inducible ASα1 isoenzyme, and even 10-fold higher Trp-levels have little or no effect on ASα1 either in vitro or in vivo.

The results described here demonstrate the existence of Trp-resistant and Trp feedback-regulated ASα isoenzymes ASα1 and ASα2 in alkaloid-producing, wild-type plants of *R. graveolens*, from which cDNAs were obtained (Bohlmann et al., 1995), as well as in elicitor-inducible cell cultures. In a previous study we showed that the two *R. graveolens* ASα genes are differentially regulated at the transcriptional level in response to elicitation (Bohlmann et al., 1995). Both *ASα* genes are constitutively expressed at a low level, but gene expression of *ASα1* is strongly and specifically in-

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duced upon elicitation. Although others have proposed a function in secondary metabolism for elicitor-inducible shikimate pathway genes in parsley (Henstrand et al., 1992), Arabidopsis (Keith et al., 1991; Niyogi and Fink, 1992), and tomato (Görlach et al., 1995), we have provided some concrete data for the role of the elicitor-inducible *ASα1* gene in the regulation of alkaloid biosynthesis. Elicitor induction of the *ASα1* gene, which encodes the Trp-insensitive isoenzyme, correlates with increased Trp-insensitive AS activity in cultured cells and with increased biosynthesis and accumulation of acridone and furoquinoline alkaloids (Eilert and Wolters, 1989; Bohlmann and Eilert, 1994; Bohlmann et al., 1995) as well as with transcriptional activation of acridone synthase (Junghanns et al., 1995). Differential enzymatic regulation of the *R. graveolens* isoenzymes ASα1 and ASα2 at the level of substrate affinity and catalytic activity strongly supports the hypothesis of different roles of ASα1 and ASα2 in primary and secondary metabolism, ASα2 being primarily involved in feedback-regulated biosynthesis of Trp and ASα1 providing precursor for elicitor-inducible alkaloid biosynthesis. We therefore propose that partitioning of chorismate toward anthranilate-derived alkaloids does not entail plastid/cytosol separation of pathway-specifc ASα isoenzymes but involves both differential transcriptional and differential enzymatic regulation (Fig. 5). This hypothesis can account for many observations, including differential Trp
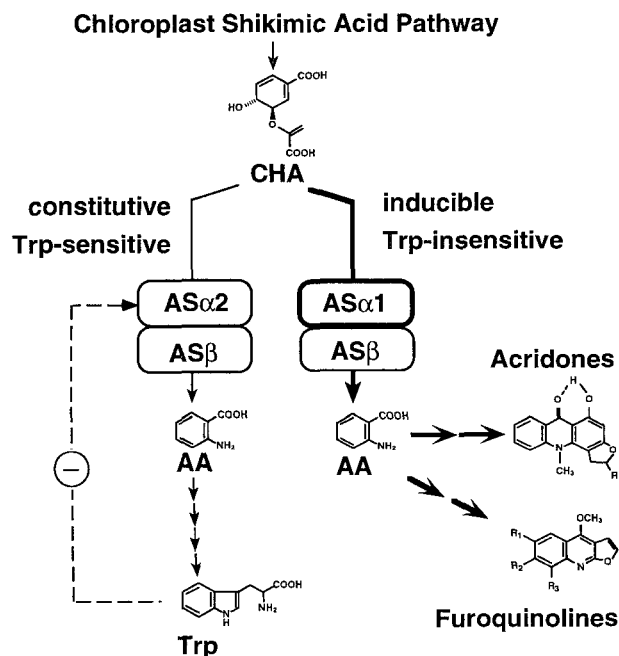
Chloroplast Shikimic Acid Pathway

Figure 5. Differentially regulated ASα isoenzymes and their proposed roles in primary and secondary metabolism of *R. graveolens*. Two plastidial ASα isoenzymes are expressed in *R. graveolens*. ASα2 is constitutively expressed and is subject to strict feedback inhibition by Trp. Constitutive gene expression of ASα1 is strongly increased upon elicitation coordinated with elicitor-induced biosynthesis and accumulation of anthranilate-derived acridone and furoquinoline alkaloids. Catalytic properties of ASα1 are not affected by physiological concentrations of Trp. AA, Anthranilate; CHA, chorismate.

sensitivity of ASα isoenzymes. Further work should address additional mechanisms that must exist to defer anthranilate from Trp accumulation subsequent to elicitation. Such mechanisms could entail the role of pathway-specific, multifunctional ASβ subunits as suggested earlier (Radwanski and Last, 1995), sequestering of ASα1 in specialized cells or plastids, substrate channeling by anthranilate utilizing enzymes of secondary metabolite pathways, or conceivably novel down-regulation of subsequent enzymes in the Trp pathway by products or intermediates of secondary metabolism.

Trp-sensitive and Trp-resistant ASα isoenzymes specific to primary and secondary metabolism, respectively, were previously cloned from the phenazine-producing prokaryote *Pseudomonas aeruginosa* (Essar et al., 1990) and therefore might as well exist in other prokaryotic and eukaryotic organisms producing anthranilate-derived secondary metabolites. The only other cloned plant ASα isoenzyme that has been investigated for sensitivity toward Trp is the inducible *A. thaliana* ASA1 isoenzyme (Niyogi and Fink, 1992; Bernasconi et al., 1994). Unfortunately, ASA1 was enzymatically inactive after expression in *E. coli* and processing of the GST-ASA1 fusion, but the fusion protein was reported to be inhibited by Trp (Bernasconi et al., 1994). Although ASα1 of *R. graveolens* and the *A. thaliana* ASA1 enzyme appear to share a common evolutionary origin (Bohlmann et al., 1995), these two enzymes have further evolved different regulatory properties that may reflect their roles in distinct secondary metabolite pathways. For example, members of the Cruciferae family produce indolic secondary metabolites, e.g. Trp-derived indole glucosinolates, whereas in *R. graveolens* alkaloid biosynthesis is directly derived from anthranilate and therefore requires Trp-independent formation of this precursor.

Plant mutants and cell culture strains of a variety of plant species have been obtained by selection for resistance toward Trp analogs (reviewed in Radwanski and Last, 1995; Li and Last, 1996). These mutants express AS enzymes with reduced sensitivity toward Trp. Trp-sensitive and Trp-insensitive isoenzymes were separated from such mutants (Carlson and Widholm, 1978; Brotherton et al., 1986), but only very recently Li and Last (1996) reported the first sequence analysis of a mutant Trp-insenstive ASα enzymes of plant origin. The mutated *A. thaliana* *ASA1* gene revealed a single amino acid substitution from Asp to Asn, which causes a 3-fold reduced sensitivity toward allosteric inhibition by Trp. It is likely that more amino acid residues will be identified to be involved in Trp inhibition of plant AS (Li and Last, 1996). In fact, the substituted Asp residue of mutant *A. thaliana* ASA1 is conserved in both wild-type ASα isoenzyme from *R. graveolens* (Bohlmann et al., 1995), and therefore other amino acids must be involved in Trp insensitivity of the *R. graveolens* isoenzyme ASα1. Sequence analysis of putative Trp-binding sites have been performed with mutant Trp-insensitive ASα enzymes from bacteria (Matsui et al., 1987; Caliguri and Bauerle, 1991) and yeast (Graf et al., 1993). Two conserved sequence motifs, $LLESX_{10}S$ and NPSPYM, involved in Trp feedback inhibition (Matsui et al., 1987; Caliguri and Bauerle, 1991; Graf et

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                        131                148
plant consensus         LFESVEPGSQ.SSVGRYS

R. graveolens  ASα1 i   LFESVEPG.RISTVGRYS
R. graveolens  ASα2 s   LFESVEPGSQASSIGRYS
A. thaliana    ASA1 s   LFESVEPGSQMSSVGRYS
A. thaliana    ASA2 n   LFESVEPGSQSSNIGRYS
microbial consensus     LLES..........S
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Figure 6. Alignment of plant ASα sequences covering a microbial regulatory element in Trp-responsive AS (Graf et al., 1993). Numbering of amino acid residues is based on a previous alignment of plant ASα sequences (Bohlmann et al., 1995). Underlined residues vary from the plant consensus motif. Ser_{139} and Gln_{140} are conserved in all Trp-sensitive ASα enzymes but not in Trp-insensitive ASα1 of *R. graveolens*. i, Trp insensitive; s, Trp sensitive; n, sensitivity toward Trp has not been investigated.

al., 1993) were proposed, with emphasis on the importance of two conserved Ser residues in the $LLESX_{10}S$ element (Graf et al., 1993). The sequence motif NPSPYM is completely conserved in all cloned plant ASα subunits (Niyogi and Fink, 1992; Bohlmann et al., 1995). However, the proposed microbial regulatory element $LLESX_{10}S$ shows some variations in plant ASα enzymes (Fig. 6). It is interesting that among all known plant ASα sequences the Trp-insensitive *R. graveolens* ASα1 isoenzyme displays the most variability within the $LLESX_{10}S$ consensus element, including deletion of Ser_{139} and substitution of Gln_{140}, conserved residues in Trp-sensitive plant ASα proteins. Although more residues involved in Trp sensitivity might be identified based on primary structure and mutagenesis of plant ASα enzymes, the ultimate structural analysis of the Trp-binding site and binding geometry of Trp-responsive ASα requires protein crystallization (Crawford, 1989).

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