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Expression of the *Arabidopsis* feedback-insensitive anthranilate synthase holoenzyme and tryptophan decarboxylase genes in *Catharanthus roseus* hairy roots

Seung-Beom Hong [a,1], Christie A.M. Peebles [b], Jacqueline V. Shanks [c], Ka-Yiu San [b], Susan I. Gibson [d,*]

[a] *Department of Biochemistry and Cell Biology, MS-140, 6100 Main St., Rice University, Houston, TX 77005, USA*
[b] *Department of Bioengineering, MS-142, 6100 Main St., Rice University, Houston, TX 77005, USA*
[c] *Department of Chemical and Biological Engineering, Iowa State University, 3031 Sweeney Hall, Ames, IA 50011, USA*
[d] *Department of Plant Biology, University of Minnesota, 122 Cargill Building for Microbial and Plant Genomics, 1500 Gortner Ave., St. Paul, MN 55108, USA*

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Abstract

In plants, the indole pathway provides precursors for a variety of secondary metabolites. In *Catharanthus roseus*, a decarboxylated derivative of tryptophan, tryptamine, is a building block for the biosynthesis of terpenoid indole alkaloids. Previously, we manipulated the indole pathway by introducing an *Arabidopsis* feedback-insensitive anthranilate synthase (AS) α subunit (*trp5*) cDNA and *C. roseus* tryptophan decarboxylase gene (*TDC*) under the control of a glucocorticoid-inducible promoter into *C. roseus* hairy roots [Hughes, E.H., Hong, S.-B., Gibson, S.I., Shanks, J.V., San, K.-Y. 2004a. Expression of a feedback-resistant anthranilate synthase in *Catharanthus roseus* hairy roots provides evidence for tight regulation of terpenoid indole alkaloid levels. Biotechnol. Bioeng. 86, 718–727; Hughes, E.H., Hong, S.-B., Gibson, S.I., Shanks, J.V., San, K.-Y. 2004b. Metabolic engineering of the indole pathway in *Catharanthus roseus* hairy roots and increased accumulation of tryptamine and serpentine. Metabol. Eng. 6, 268–276]. Inducible expression of either or both transgenes did not lead to significant increases in overall alkaloid levels despite the considerable accumulation of tryptophan and tryptamine. In an attempt to more successfully engineer the indole pathway, a wild type *Arabidopsis* ASβ subunit (*ASB1*) cDNA was constitutively expressed along with the inducible expression of *trp5* and *TDC* in *C. roseus* hairy roots. Transgenic hairy roots expressing both *trp5* and *ASB1* show a significantly greater resistance to feedback inhibition of AS activity by tryptophan than plants expressing only *trp5*. In fact, a 4.5-fold higher concentration of tryptophan is required to achieve 50% inhibition of AS activity in plants overexpressing both genes than in plants expressing only *trp5*. In addition, upon a 3 day induction during the exponential phase, a *trp5:ASB1* hairy root line produced 1.8 times more tryptophan (specific yield ca. 3.0 mg g^{-1} dry weight) than the *trp5* hairy root line. Concurrently, tryptamine levels increase up to 9-fold in the induced *trp5:ASB1* line (specific yield ca. 1.9 mg g^{-1} dry weight) as compared with only a 4-fold

* Corresponding author. Tel.: +1 612 624 7408; fax: +1 612 624 6264.
E-mail address: gibso043@tc.umn.edu (S.I. Gibson).
[1] Present address: Department of Molecular Biotechnology, Konkuk University, 1 Whayang-dong, Kwangjin-gu, Seoul, Korea.

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tryptamine increase in the induced *trp5* line (specific yield ca. 0.3 mg g^{-1} dry weight). However, endogenous TDC activities of both *trp5:ASB1* and *trp5* lines remain unchanged irrespective of induction. When *TDC* is ectopically expressed together with *trp5* and *ASB1*, the induced *trp5:ASB1:TDC* hairy root line accumulates tryptamine up to 14-fold higher than the uninduced line. In parallel with the remarkable accumulation of tryptamine upon induction, alkaloid accumulation levels were significantly changed depending on the duration and dosage of induction.

Keywords: *Catharanthus roseus*; Hairy roots; Anthranilate synthase holoenzyme; Tryptophan decarboxylase

1. Introduction

The periwinkle, *Catharanthus roseus* (Apocynaceae), is widely used as an ornamental plant as well as a medicinal plant. As part of its secondary metabolism, this plant produces pharmaceutically valuable terpenoid indole alkaloids (TIA) such as the anticancer drugs vincristine and vinblastine. A very low yield of these compounds is a major motivation of the research interest in this plant. Although hairy root cultures do not produce these two bisindole alkaloids that consist of catharanthine and vindoline, they have been shown to produce catharanthine and tabersonine, a precursor of vindoline. Based on the chemical structure of TIA, *C. roseus* root alkaloids have been divided into three families: iboga, corynanthe and aspidosperma (Fig. 1).

Biosynthesis of TIAs begins with condensation of an indole moiety of tryptamine and a monoterpenoid component of secologanin. This condensation produces strictosidine, which is a universal precursor of all TIA compounds. Although tryptamine is derived directly from tryptophan by tryptophan decarboxylase (TDC), synthesis of tryptophan requires a series of six consecutive enzymes among which anthranilate synthase (AS) catalyzes the first committed step by the amination of chorismate to anthranilate. This is known to be a rate-limiting step of tryptophan synthesis with tryptophan inhibiting AS activity via at least two mechanisms. First, tryptophan feedback inhibits AS activity directly. Secondly, tryptophan activates chorismate mutase, which catalyzes the conversion of chorismate into prehenate, a precursor of the amino acids phenylalanine and tyrosine (Siehl, 1999). Thus, feedback regulation of these two competing enzymes plays an important role in controlling the flux of intermediates in the indole pathway.

In plants, the AS holoenzyme is believed to be a heterotetramer that consists of two α subunits and two β subunits (Poulsen et al., 1993). The α subunit has a catalytic activity and the β subunit has an aminotransferase activity that transfers an amino group from glutamine to the α subunit. Although this glutamine-dependent AS reaction requires both the α and β subunits, the α subunit alone is able to synthesise anthranilate from chorismate when ammonium is present at high concentrations. Feedback inhibition of AS activity by tryptophan results from the binding of tryptophan to the α subunit. In this regard, there have been several reports on isolation and expression of feedback-insensitive forms of ASα genes in an attempt to increase free tryptophan and secondary metabolite levels in several plants (Li and Last, 1996; Tozawa et al., 2001; Cho et al., 2000; Hughes et al., 2004a). Although the ASβ subunit is not subject to feedback inhibition, the *trp4* mutation in the gene encoding the *Arabidopsis* subunit (*ASB1*) suppresses accumulation of anthranilate in the *trp1-100* mutant by reducing the rate of conversion of chorismate to anthranilate. In addition, the *trp4* and *trp1-100* single mutants are able to grow normally without tryptophan, whereas the *trp4:trp1-100* double mutant is a tryptophan auxotroph (Niyogi et al., 1993). These results suggest an important role for ASβ in tryptophan synthesis in vivo. Thus far there has been no report that shows the impact of over-expression of both feedback-resistant ASα and ASβ on the level of tryptophan and its derivative metabolites.

The studies presented here are focused on the flux of the engineered indole pathway in relation to TIA production in *C. roseus* hairy roots. This work builds on two recent studies that focused on analysis of hairy root lines transgenic for a feedback-resistant *Arabidopsis* ASα subunit (Trp5) and/or TDC (Hughes et al., 2004a,b). In these previous studies, inducible

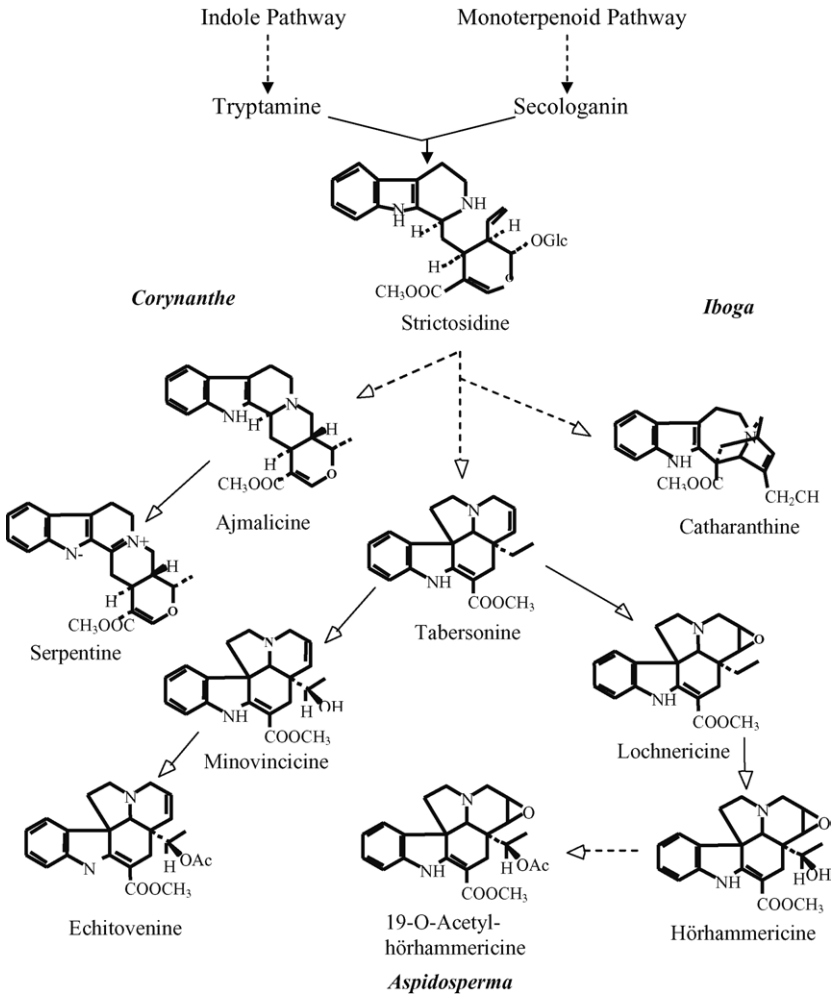

Fig. 1. Terpenoid indole alkaloid families found in *C. roseus* roots. Aspidosperma type alkaloids include epoxidation, hydroxylation, and acetylation derivatives of tabersonine (Rodriguez et al., 2003; Laflamme et al., 2001). Ajmalicine and serpentine are corynanthe type alkaloids. Catharanthine is an iboga type alkaloid.

expression of Trp5 alone during the exponential phase transiently increased accumulation of only one TIA, lochnericine, which was accompanied by increases in tryptophan and tryptamine (Hughes et al., 2004a). In contrast, inducible expression of both Trp5 and TDC had no effects on alkaloid levels despite the considerable accumulation of tryptamine (Hughes et al., 2004b). As neither of these previous studies examined the role of the ASβ subunit on TIA accumulation, such a study is presented here. In this paper, we report that ectopic expression of wild-type *Arabidopsis* ASβ subunit (ASB1) in conjunction with Trp5 causes Trp5 to have a 4.5-fold higher resistance to tryptophan-feedback inhibition, leading to a higher yield of tryptophan, than the expression of Trp5 alone. In addition, tryptamine accumulates at 6-fold higher levels in *C. roseus* hairy roots transgenic for ASB1 and Trp5 (specific yield 1.9 mg g^{-1} DW) as compared with those transgenic for Trp5 despite the fact that endogenous TDC activities of both transgenic lines remain unchanged. Ectopic expression of TDC together with the mutant AS holoenzyme further increased accumulation of tryptamine. Although the effect of these changes on alkaloid levels depends on the lines and

the inducing conditions, TDC activity correlates with alkaloid accumulation in a transient manner.

2. Materials and methods

2.1. Construction of plant expression cassettes

An *Xho*1/*Eco*R1 fragment of plasmid pCGN1547 (McBride and Summerfelt, 1990) that contains the promoter of the mannopine synthase (*mas*) gene was cloned into the pBluescript II-KS vector to yield pMAS5. Another *Xho*1/*Eco*R1 fragment of plasmid pCGN1547 that contains the *mas* terminator was cloned into pBluescript II-KS to yield pMAS3. A full-length wild-type *Arabidopsis thaliana ASB1* cDNA in plasmid pKN51 (Niyogi et al., 1993) was amplified by PCR and fused to the *mas* terminator in pMAS3. DNA sequencing confirmed a complete match of the amplified *ASB1* cDNA sequence to the published sequence (GenBank accession. No. L22585). A 1.6 kb *Xho*I fusion fragment carrying the *ASB1* cDNA and *mas* terminator was then fused to the *mas* promoter in pMAS5 in such a way that the *mas* promoter is placed behind the *mas* terminator. Finally, a 2.3 kb gene fusion cassette containing the three consecutive *ASB1* cDNA, *mas* terminator and promoter fragments was blunt-ended with T4 DNA polymerase and inserted into a single *Pme*1 site located between the CaMV 35S promoter and the GVG gene of p7002ASA and p7002ASATDC (Hughes et al., 2004a,b) to yield constructs named pASαβ and pASαβT, respectively. In both constructs, the *ASB1* cDNA is flanked by the CaMV 35S promoter and the *mas* terminator and the GVG gene is present behind the *mas* promoter.

2.2. Transformation and culture conditions

The constructs pASαβ and pASαβT were transformed into the agropine-type *Agrobacterium rhizogenes* strain 15834 by electroporation (Wen-Jan and Forde, 1989). The transformants that grew on kanamycin (50 μg ml^{-1}) selection YEM plates (1 g yeast extract, 0.2 g NaCl, 0.2 g $MgSO_4 7H_2O$, 4 mg $FeCl_3$, 0.66 g $K_2HPO_4 3H_2O$, 10 g mannitol, 15 g agar per liter) were analyzed for the presence of the plasmids by restriction enzymes. Wound infection of *C. roseus* seedlings, selection of transgenic hairy root lines and adaptation to liquid medium were followed as previously described (Bhadra et al., 1993). Liquid adapted hairy roots were sub-cultured when they reached late exponential phase at around 20, 40 and 90 days for ASαβ1, ASαβT2 and ASαβ3, respectively. At the beginning of one of these sub-culture cycles, hairy root tips were inoculated into 50 ml filter-sterilized medium (3% sucrose, half-strength Gamborg's B5 salts, and full strength Gamborg's vitamins, pH 5.7) in a 250 ml Erlenmeyer flask. The cultures were shaken at 100 rpm and kept at 26 °C in the dark. For induction experiments, dexamethasone dissolved in ethanol was added to the culture media to the indicated final concentrations at 15, 30 and 80 days for ASαβ1, ASαβT2 and ASαβ3, respectively. The fresh and dry weights of the cultures were determined after blotting and lyophilization, respectively. The generation of the tryptophan feedback-insensitive mutant ASα and empty vector control lines used in this study was previously described (Hughes et al., 2004a).

2.3. Assay of AS and TDC activities

Protein extracts for AS and TDC enzyme assays were prepared as described previously (Hughes et al., 2004a,b) by adding 2 ml extraction buffer to 1 g of pulverized liquid nitrogen frozen tissues and were desalted using Sephadex G25. The extraction buffer contained a proteinase inhibitor cocktail (Roche). Protein concentration was determined by a dye-binding assay method (Bradford, 1976) using bovine serum albumin for reference. The enzyme assays for the glutamine-dependent AS and TDC were performed using fresh proteins per reaction (20 μg ml^{-1}) as previously described (Li and Last, 1996; Sangwan et al., 1998).

2.4. Determination of tryptophan, tryptamine and alkaloid levels

Cultures in the mid-exponential phase (dry weight between 0.1 and 0.2 g) were harvested and processed as described before (Morgan and Shanks, 2000). Lyophilized roots were pulverized and 50 mg aliquots of tissue were extracted twice each with 10 ml methanol in a sonicating bath at 16 °C for 1 h. The extracts were clarified by centrifugation, combined, evaporated at 50 °C under vacuum to a 2 ml volume and then filtered through 0.2 μm nylon membranes. Ten microliter

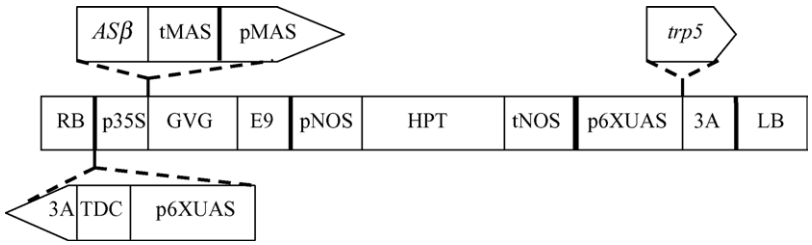

Fig. 2. ASαβ double and ASαβTDC triple gene expression cassettes under the dexamethasone (DEX)-inducible promoter. The constructs pASαβ and pASαβT were made in an empty T-DNA plasmid vector pTA7002 (Aoyama and Chua, 1997): p35S, cauliflower mosaic virus 35S promoter; GVG, hybrid transcription factor; E9, pea rbcs-E9 gene terminator; pNOS, nopaline synthase gene promoter; *HPT*, hygromycin resistance gene; tNOS, nopaline synthase gene terminator; p6XUAS, dexamethasone-inducible promoter; 3A, pea rbcs-3A gene terminator; RB, T-DNA right border; LB, T-DNA left border. tMAS and pMAS indicate terminator and promoter region of the mannopine synthase gene, respectively. *trp5* denotes the cDNA of the *Arabidopsis* feedback-insensitive anthranilate synthase α subunit gene.

aliquots of the filtered extracts were injected onto an HPLC system. HPLC runs and alkaloid quantification were performed as previously reported (Hughes et al., 2004a,b).

3. Results

3.1. Generation of transgenic C. roseus hairy root lines

C. roseus seedlings were infected with *Agrobacterium rhizogenes* 15834 harboring the chimeric binary plasmids pASαβ or pASαβT (Fig. 2). Both constructs allow inducible expression of a feedback resistant *Arabidopsis* ASα cDNA under the control of the glucocorticoid-inducible promoter and constitutive expression of an *Arabidopsis* ASβ cDNA and a hybrid transcription factor (GVG) gene under the control of the CaMV 35S and manopine synthase (*mas*) promoters, respectively. They also contain the hygromycin resistance gene as a plant selection marker. In addition, the plasmid pASαβT permits expression of the *C. roseus TDC* under the control of the glucocorticoid-inducible promoter. Of the 21 hairy root lines that grew on hygromycin selection plates, two ASαβ and three ASαβT lines were adapted to liquid media and maintained for more than eight generations before further analysis. The presence of ASα, ASβ, TDC and GVG transgenes in all of the ASαβ and ASαβT lines was confirmed by PCR analysis of genomic DNA using transgene-specific primers.

3.2. AS and TDC enzyme activities

Glutamine-dependent AS activities and the kinetics of tryptophan feedback inhibition were measured and compared among hairy root extracts. Upon induction with 0.2 μM dexamethasone (DEX), the ASα, ASαβ1 and ASαβT2 lines showed 1.8, 2.2 and 2.7-fold increases in AS activity, respectively (Table 1). Although the relative fold increases were somewhat similar, specific activities were approximately 2-fold higher in the induced ASαβ and ASαβT lines than in

Table 1
AS and TDC activities (nmole min^{-1} mg^{-1}) in hairy root lines[a]

Activity	Lines[b]					
	U-ASα	I-ASα	U-ASαβ1	I-ASαβ1	U-ASαβT2	I-ASαβT2
AS	0.47	0.84	0.77	1.70	0.62	1.71
TDC	0.56	0.51	0.43	0.49	0.48	1.60

[a] Induced extracts (I) were prepared from lines treated with 0.2 μM DEX for 72 h. Uninduced extracts (U) were prepared from lines treated with the same amount of ethanol for 72 h.

[b] ASα, ASαβ and ASαβT represent hairy root lines transgenic for *trp5*, *trp5:ASB1* and *trp5:ASB1:TDC*, respectively.

the induced ASα line. To determine whether expression of the ASβ subunit alters the insensitivity of Trp5 to tryptophan feedback inhibition, AS enzyme activities of the induced ASα, ASαβ and ASαβT lines were measured in the presence of increasing concentrations of tryptophan. As shown in Fig. 3, the induced ASαβ and ASαβT lines are much more resistant to inhibition: the tryptophan concentrations causing 50% inhibition of AS activities (apparent K_i values) are 10 μM for the ASα line and 45 μM for the ASαβ and ASαβT lines. In contrast, uninduced ASα, ASαβ and ASαβT lines displayed indistinguishable kinetics with an apparent K_i of 5 μM tryptophan.

In order to quantify changes in TDC enzyme activities of the ASα, ASαβ and ASαβT lines upon induction, protein extracts from both uninduced and induced tissues were prepared and assayed using the direct fluorometry method (Sangwan et al., 1998). No significant changes in endogenous TDC activities were noted among the uninduced and induced ASα and ASαβ lines with an average specific activity of 0.49 nmol min^{-1} mg^{-1} (Table 1). However, the induced ASαβT2 line had an activity level of 1.6 nmol min^{-1} mg^{-1}, which represents a 3-fold increase in activity relative to the uninduced line. The apparent K_m of TDC for L-tryptophan was determined to be approximately 100 μM for both the uninduced and induced ASαβT lines (Fig. 4).

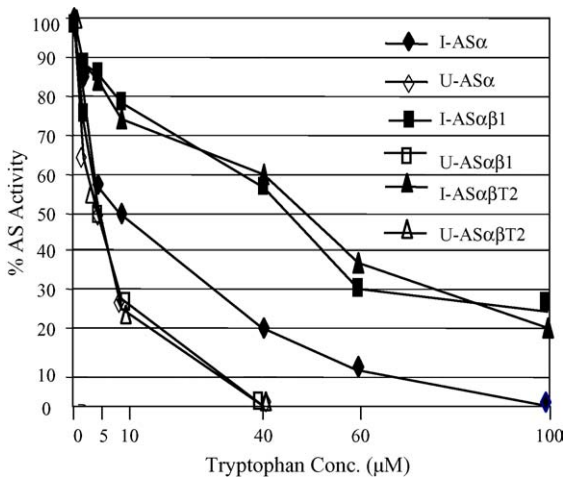

Fig. 3. Effect of tryptophan concentration on AS activity in the absence (U) and presence (I) of 0.2 μM DEX for 72 h.

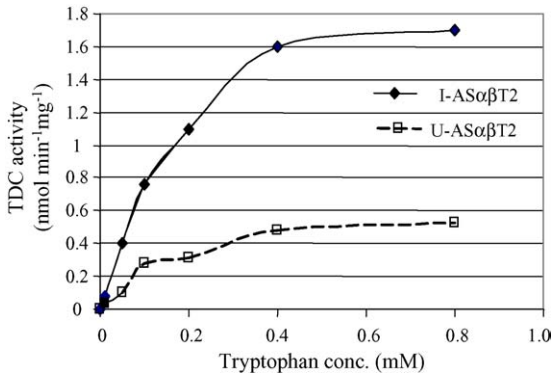

Fig. 4. TDC activity kinetics in the ASαβT2 line. U and I indicate uninduced and induced with 0.2 μM DEX for 72 h, respectively.

3.3. Tryptophan and tryptamine levels

The observed change in K_i value of the ASαβ1 and ASαβT2 lines suggested that the levels of tryptophan and its direct derivative tryptamine may be elevated as a result of the increases in K_i value of the AS holoenzyme and the substrate concentration, respectively. The lines in the exponential growth phase were analyzed in parallel with the mutant ASα and empty vector control lines for the levels of tryptophan and tryptamine (Fig. 5). Accumulation of tryptophan and tryptamine is dramatic in all of the lines when induced, whereas it remains unchanged in the negative control line transgenic for the empty vector regardless of induction. Tryptophan and tryptamine in the negative control line accumulated to approximately 60 and 108 μg g^{-1} dry weight tissue, respectively. When the ASαβ and ASαβT lines were induced, they produced 1.8 times more tryptophan, with specific yield of 3.0 mg g^{-1} dry

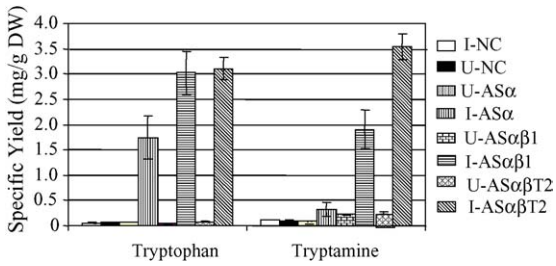

Fig. 5. A comparison of tryptophan and tryptamine yields in the absence (U) and presence (I) of DEX. Mid-exponential phase cultures were induced with 3 μM DEX for 72 h. NC indicates an empty vector control line.

Table 2
Tryptophan and tryptamine levels ($\mu g\, g^{-1}$ DW) in uninduced hairy root lines[a]

Yields	Lines[b]				
	NC	ASα	ASαβ1	ASαβ3	ASαβT2
Tryptophan	56 ± 7.8	–	35 ± 3.2	44 ± 1.1	76 ± 7.0
Tryptamine	106 ± 1.6	81 ± 5.0	223 ± 3.8	128 ± 1.6	258 ± 2.4

[a] Data represent means of triplicate cultures with standard deviations.
[b] NC indicates an empty vector control line.

weight, than the induced ASα line. Accumulated levels of tryptamine are much greater in the induced ASαβ and ASαβT lines as compared to the induced ASα line, with 4, 9 and 14-fold increases in tryptamine observed in the ASα, ASαβ1 and ASαβT2 lines, respectively. It is notable that the level of tryptamine accumulation in the induced ASαβT2 line is extreme, with up to 3.5 mg g^{-1} dry weight of specific yield despite the fact that the tryptophan specific yield for this line is very similar to that of the ASαβ1 line. Even under the uninduced condition, ASαβ1 and ASαβT2 lines accumulated more than 2-fold higher levels of tryptamine than the ASα and empty vector control lines, although none of the lines exhibit significant differences in tryptophan content (Table 2).

3.4. Alkaloid levels

We examined whether or not the substantial accumulation of tryptamine leads to increased synthesis of terpenoid indole alkaloids. When exponential phase ASαβ1 and ASαβT2 lines were induced with 3 μM DEX for 72 h, levels of the aspidosperma family alkaloids, tabersonine, lochnericine and hörhammericine, were greatly diminished, whereas ajmalicine levels were significantly elevated (data not shown). Under this condition, both hairy root lines exhibited a strong brown color. Thus, the reduced levels of some alkaloids may not accurately reflect the effects of elevated levels of tryptophan and tryptamine. To eliminate the browning effect, a lower DEX dosage and shorter exposure time were applied to the lines before harvesting the cultures for alkaloid analyses. Neither the browning effect nor significant effects on biomass growth were observed when the lines were induced with 0.2 μM DEX. Under this dosage, different durations of induction led to significant differences in alkaloid accumulation levels. As shown in Fig. 6, 8 h induction of the ASαβ1 line caused a 1.8-fold increase in tryptamine but did not affect overall alkaloid levels. In contrast, 24 h induction led to a significant increase in ajmalicine with a concomitant decrease in tabersonine, which was accompanied by a 5-fold increase in tryptamine. Further induction times caused even greater decreases in accumulation of the aspidosperma family alkaloids. A similar result of the transient alkaloid profiles was also

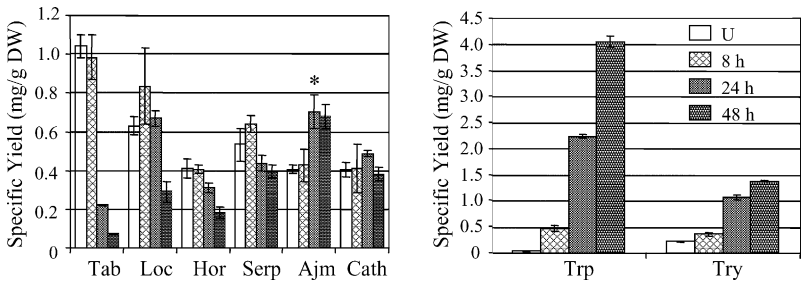

Fig. 6. Levels of alkaloids (Tab, tabersonine; Loc, lochnericine; Hor, hörhammericine; Serp, serpentine; Ajm, ajmalicine; Cath, catharanthine), tryptophan (Trp) and tryptamine (Try) in mid-exponential phase cultures of the ASαβ1 line. Cultures were induced with 0.2 μM DEX for 8, 24 and 48 h. Ethanol was added to the uninduced control (U). Uninduced cultures were harvested 48 h after induction was initiated. Data represent means of triplicate cultures with standard deviations indicated. An asterisk above the bar indicates a statistically significant increase between the induced and uninduced samples using a Student's *t*-test ($P < 0.05$).

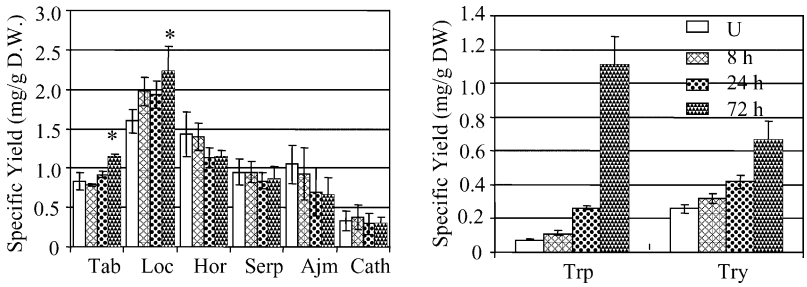

Fig. 7. Levels of alkaloids (abbreviations are the same as those in Fig. 6) tryptophan and tryptamine in mid-exponential phase cultures of the ASαβT2 line. Cultures were induced with 0.2 μM DEX for 8, 24 and 72 h. Uninduced cultures were harvested 72 h after induction was initiated. Asterisks above the bars indicate statistically significant increases between the induced and uninduced samples using a Student's *t*-test ($P < 0.05$).

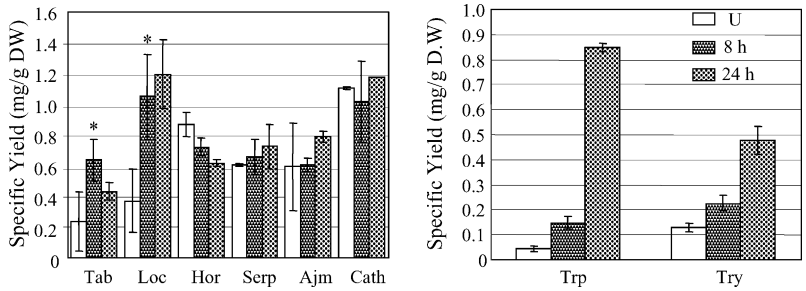

Fig. 8. Levels of alkaloids (abbreviations are the same as those in Fig. 6) tryptophan and tryptamine in mid-exponential phase cultures of the ASαβ3 line. Cultures were induced with 0.2 μM DEX for 8 and 24 h. Uninduced cultures were harvested 24 h after induction was initiated. Asterisks above the bars indicate statistically significant increases between the induced and uninduced samples using a Student's *t*-test ($P < 0.05$).

noted when a late exponential phase culture was used (data not shown).

Although the ASαβT2 line exhibited a 2.6-fold increase in tryptamine, unlike the ASαβ1 line an 8–72 h induction of the ASαβT2 line did not give rise to a reduction of aspidosperma family alkaloids (Fig. 7). During a 72 h induction period, both tabersonine and lochnericine continued to accumulate, exhibiting a 1.4-fold increase relative to the uninduced control. On the other hand, even 8 h induction of the ASαβ3 line caused significant increases in tabersonine and lochnericine with a 1.8-fold increase in tryptamine (Fig. 8). These two alkaloids did not accumulate further after a 24 h induction despite a 3.7-fold increase in tryptamine. These results indicate that the effects of increased tryptamine accumulation on measured alkaloid yields are highly dependent on the lines and the duration and dosage of induction. In addition, all three uninduced lines exhibited significant variations in accumulation of alkaloids. For example, tabersonine specific yields of uninduced ASαβ1, ASαβ3 and ASαβT2 lines are 1040, 240 and 830 $\mu g\, g^{-1}$ dry weight biomass, respectively. Catharanthine specific yields of uninduced ASαβ1, ASαβ3 and ASαβT2 lines are 404, 1095 and 330 $\mu g\, g^{-1}$ dry weight, respectively. Despite wide variations in alkaloid yields among the lines, the observed elevated levels of alkaloids are consistently accompanied by increased levels of tryptamine.

4. Discussion

In plants, the indole pathway provides the essential amino acid tryptophan as well as precursors for many secondary metabolites. In addition to the positive effects of our engineered indole pathway on TIA accumulation, the dramatic increases in tryptophan and tryptamine levels observed in the present study may have significant biotechnological applications. Tryptophan is the most limiting essential amino acid in cereal crops, such as rice, maize, wheat, oats and forage legume plants (Tozawa et al., 2001; Cho et al.,

2000). Although further analyses will be required to determine the exact mechanism(s) by which expression of the ASβ subunit along with Trp5 results in very high accumulation of tryptamine, this phenomenon can potentially be exploited as a useful biocontrol agent of insect pests. Accumulation of tryptamine is known to greatly inhibit reproduction of whiteflies in plants (Thomas et al., 1995). Besides, tryptamine is an important precursor of mammalian hallucinogenic and psychedelic drugs (Berlin et al., 1993; Zhang et al., 2004). Tryptamine is also known to inhibit the activity of the CYP83B1 enzyme that catalyzes the first committed step in the biosynthesis of indole glucosinolates in Cruciferous plants (Bak et al., 2001). These toxic metabolites reduce meal palatability in oilseed crops (Chavadej et al., 1994). TDC was proposed as a novel biochemical selection marker because a toxic compound, 4-methyl-L-tryptophan, is efficiently metabolized by TDC (Goddijin et al., 1993). Thus, the expression gene cassette we constructed can potentially be used not only to increase tryptamine but also to directly select transformants without use of antibiotic selection.

As compared with the ASα hairy root line, the ASαβ line has a 4.5-fold higher K_i value for tryptophan. In addition, the ASαβ line exhibits a 2-fold higher specific activity than the ASα line and accumulates approximately 1.8- and 6-fold higher levels of tryptophan and tryptamine, respectively, when both lines are treated with 0.2 μM DEX for 72 h. These findings demonstrate that ASβ plays an important role in tryptophan synthesis, as previously suggested by the results of double mutant analysis (Niyogi et al., 1993). Cho et al. (2000) reported that the free tryptophan levels in *Astragalus sinicus* hairy roots transgenic for a tobacco tryptophan feedback-resistant ASα increased with increasing K_i values, up to K_i values of approximately 10 μM. Increasing K_i values above 10 μM did not result in higher levels of tryptophan accumulation. The differences between this work and the results reported here could be attributed to different ASα mutations and/or AS holoenzyme expression. In addition, the effects of ectopic expression of ASβ could be further augmented in other plant tissues that have a limited access to ammonium ions. Interestingly, the ASαβ line greatly enhances production of tryptamine in addition to tryptophan, and this is not due to the increased endogenous TDC activity. A transgenic TDC hairy root line was reported to show no significant increase in tryptamine content upon induction (Hughes et al., 2004b). However, the ASαβT2 line accumulated a 1.8-fold higher level of tryptamine than the ASαβ line. These results together indicate that cellular concentrations of substrate tryptophan and TDC enzyme are rate limiting in tryptamine synthesis in *C. roseus* hairy roots. Alternatively, expression of the AS holoenzyme may somehow affect metabolism of tryptamine, leading to increased tryptamine accumulation.

Previous reports (Geerlings et al., 1999; Canel et al., 1998) showed that ectopic expression of TDC and strictosidine synthase led to increased pools of tryptamine and alkaloids. In contrast, overexpression of TDC alone in actively growing *C. roseus* cell cultures did not elevate TIA accumulation despite a high level of tryptamine (Whitmer et al., 2002). However, our results with three independent lines show that enhanced production of tryptamine during the exponential phase results in increased production of alkaloids. A possible explanation for this difference is that, unlike *C. roseus* cell suspension cultures, strictosidine synthase activity is high in hairy roots (Meijer et al., 1993). Tabersonine and lochnericine are the most dynamically changed alkaloids and are sensitive to the induction that brings about an increase in tryptamine. Tabersonine and lochnericine tend to rapidly and transiently accumulate at early stages of induction, whereas ajmalicine accumulates at later stages of induction, though their precise timing of accumulation differs depending on the transgenic line being analyzed. Moreover, tabersonine yields of the uninduced lines appear to correlate with growth rate, as the faster the hairy root lines grow, the higher levels of tabersonine they accumulate. Consistent with this, accumulation of tabersonine was previously found to exhibit a growth-associated trend (Bhadra and Shanks, 1997). Tryptamine accumulation is also strongly associated with growth as the mid-exponential, late-exponential and stationary phase cultures of an empty vector control line have specific yields of about 100, 50 and 15 $\mu g\, g^{-1}$ DW, respectively. Yields of tryptophan and tryptamine in the ASα hairy root line are also more sensitive to the duration and dosage of induction during the exponential stage as compared to the stationary stage (Hughes et al., 2004a). However, persistently high levels of tryptophan and tryptamine from prolonged induction caused a sharp decline in tabersonine, lochnericine and hörhammericine accumulation (Fig. 6). Excessive

levels of tryptophan and tryptamine might accelerate either breakdown of alkaloids or over-production of auxin by both endogenous and T-DNA associated genes encoding enzymes involved in auxin (IAA) biosynthesis (Bartel, 1997; Cardarelli et al., 1985). Auxin is known to be a strong negative regulator of TIA gene expression (Meijer et al., 1993; Pasquali et al., 1992). Alternatively, metabolic flux through the pathways downstream of tabersonine could be pushed further into unknown pathways or other metabolites, such as minovincine, echitovenine and acetyl-hörhammericine (Fig. 1). *C. roseus* hairy roots were estimated to contain a total of at least 24 TIAs with differing molecular weights as analyzed by LC/MS and 2D TLC (Parr et al., 1988). This reflects the incredibly complex downstream pathways of TIA biosynthesis. Accordingly, determination of the exact metabolic flux changes will await the availability of all these compounds together with extensive monitoring of transient metabolite profiles over time.

Increased production of lochnericine in ASαβ3 and ASαβT2 lines upon induction with a low dosage of DEX suggests a high activity of tabersonine 6,7-epoxidase that converts tabersonine to lochnericine (Rodriguez et al., 2003). Unlike tabersonine and lochnericine, hörhammericine did not significantly accumulate under the same induction condition. This may happen if the conversion rate of hörhammericine to 19-O-acetyl-hörhammericine is as fast as that of lochnercinine to hörhammericine (Fig. 1). In any case, our results clearly show that TDC activity correlates with TIA accumulation in a transient manner during the exponential phase and that newly synthesised tabersonine is rapidly diverted to terminal end products despite the significant disparities of hairy root lines in biosynthetic capacities for TIA. In contrast, induction has little effect on the pools of catharanthine and serpentine. This is consistent with the previous report that serpentine accumulation is associated with a non-growth trend (Bhadra and Shanks, 1997). Apparently either expression of branch-specific genes or activation of the gene products involved in the biosynthesis of this compound is delayed until the later stage of development. Although a low dosage of jasmonic acid positively affected all of the above alkaloid levels, their transient profiles are different (Rijhwani and Shanks, 1998). Chitin treatment of *C. roseus* hairy roots selectively enhanced ajmalicine levels by 50% with no significant effects on other alkaloids (Shanks et al., 1998). All these results suggest that the pathways leading to the three families of alkaloids are finely tuned and may be controlled by distinct elicitor perception and signaling mechanisms. In contrast to the upstream indole and monoterpenoid pathways, little information on the regulation of the downstream branch pathways is currently available and many of the enzymatic activities and genes involved in these pathways remain to be identified and characterized. Elucidation of the downstream pathways and molecular cloning of the corresponding genes would pave the way for successful metabolic engineering of *C. roseus* alkaloids.

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