Expression of a Feedback-Resistant Anthranilate Synthase in *Catharanthus roseus* Hairy Roots Provides Evidence for Tight Regulation of Terpenoid Indole Alkaloid Levels

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Abstract: Different plant species produce a variety of terpenoid indole alkaloids, which are of interest as plant defensive secondary metabolites and as valuable pharmaceuticals. Although significant progress has been made, the mechanisms regulating the levels of this important class of compounds require continued elucidation. Previous precursor feeding studies have indicated that alkaloid accumulation can be improved during the exponential growth phase of hairy root cultures through enhanced tryptophan availability. To test this relationship, transgenic hairy root cultures of *Catharanthus roseus* were established with a glucocorticoid-inducible promoter controlling the expression of an Arabidopsis feedback-resistant anthranilate synthase alpha subunit. Enzyme assays demonstrated that the Arabidopsis alpha subunit is compatible with the native beta subunit and that anthranilate synthase activity is more resistant to tryptophan inhibition in induced than in uninduced extracts. The metabolic effects of expressing the feedback-resistant anthranilate synthase alpha subunit were also dramatic. Over a 6-day induction period during the late exponential growth phase, tryptophan and tryptamine specific yields increased from almost undetectable levels to 2.5 mg/g dry weight and from 25 μg/g to 267 μg/g dry weight, respectively. The greater than 300-fold increase in tryptophan levels observed in these studies under certain induction conditions compares favorably with the fold increases obtained in previous constitutive expression studies. Despite the large increases in tryptophan and tryptamine, the levels of most terpenoid indole alkaloids were not significantly altered, with the exception of lochnericine, which increased 81% after a 3-day induction period. These results suggest that terpenoid indole alkaloid levels are tightly controlled.

Keywords: terpenoid indole alkaloids; anthranilate synthase; *Catharanthus roseus*; plant metabolic engineering

INTRODUCTION

Catharanthus roseus has been extensively studied due to its production of two valuable alkaloids, vincristine and vinblastine, which are used in chemotherapy. Biologically, indole alkaloids produced by plants are believed to play a role as antimicrobial and antifeeding compounds (Chockalingam et al., 1989; Luijendijk et al., 1996). Due to the economic value of certain alkaloids, studies have included efforts at increasing alkaloid flux through elicitation (Moreno et al., 1993; Rijhwani and Shanks, 1998), precursor feeding (Moreno et al., 1993; Morgan and Shanks, 2000), and attempts at medium optimization (Bhadra et al., 1993). Although these studies have elucidated some characteristics of the complex regulation governing indole alkaloid production, it still remains a complex network with a number of uncharacterized and uncloned enzymes (Hughes and Shanks, 2002). The genes that have been cloned and characterized, however, present researchers with an opportunity to metabolically engineer the system, characterize the outcome, and therefore further elucidate the network.

The broad class of terpenoid indole alkaloids in *C. roseus* is formed by the coupling of secologanin from the terpenoid pathway and tryptamine from the indole pathway. Precursor

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feeding studies have occasionally found that feeding the indole precursors, tryptamine and tryptophan, leads to increased accumulation of indole alkaloids (Kreuger and Carew, 1978; Zenk et al., 1977). Specifically, studies on *C. roseus* hairy roots show that tryptophan feeding during the late exponential growth phase results in significant increases in the accumulation of two alkaloids, tabersonine and serpentine (Morgan and Shanks, 2000). In *C. roseus* cell lines engineered to overexpress strictosidine synthase, which catalyzes the coupling of the indole and terpenoid moieties, tryptophan feeding combined with terpenoid feeding was necessary to further increase alkaloid synthesis after an initial terpenoid deficiency was overcome (Whitmer et al., 2002a). Although a systems approach rather than single gene studies will likely be necessary for substantial increases in alkaloid flux, only the indole pathway and its regulation have been extensively characterized. Studies have been reported in a number of plant systems, including rice (Tozawa et al., 2001), Arabidopsis (Li and Last, 1996), and *C. roseus* (Poulsen et al., 1993). In the first committed step of tryptophan synthesis, anthranilate synthase (AS) catalyzes the conversion of chorismate to anthranilate and is feedback-inhibited by tryptophan (Fig. 1) (Li and Last, 1996). The AS enzyme is a tetramer composed of two α subunits (ASα) and two β (ASβ) subunits. It is the α subunit that catalyzes the aromatization of chorismate and binds tryptophan as a feedback inhibitor, while the β subunit donates an amino group from glutamine.

In addition to precursor feeding indications, the increased transcription of indole pathway genes in plants has previously been associated with the increased synthesis of defensive secondary metabolites (Li and Last, 1996; van der Fits and Memelink, 2000). In *C. roseus* cell cultures, transcription of *AS*α is coordinately regulated with other alkaloid biosynthetic genes by ORCA3, a jasmonate-responsive transcriptional regulator (van der Fits and Memelink, 2000). Upon transfer to medium used for the induction of alkaloid production, AS specific activity is also known to increase (Poulsen et al., 1993). The coordinate regulation of the indole and alkaloid pathways points to the potential importance of increased indole precursor pools for improved alkaloid synthesis. With the positive indications from regulatory and feeding studies, we sought to engineer the indole pathway within our experimental system, *C. roseus* hairy roots. Recent reports of an inducible promoter useful for *C. roseus* (Hughes et al., 2002) and the generation of transgenic *C. roseus* hairy roots (Ayora-Talavera et al., 2002; Hughes et al., 2002) provide new options for pathway manipulation. However, the indole pathway presents some challenges due to its tight regulation. Increased tryptophan levels inhibit native AS activity and induce chorismate mutase thereby channeling flux into a competitive pathway (Verpoorte et al., 1999). Fortunately, the regulatory network has been overcome in a few studies through constitutive expression of a feedback-resistant ASα subunit (Cho et al., 2000; Tozawa et al., 2001). An Arabidopsis *AS*α gene from a mutant resistant to tryptophan analogues has been cloned (Li and Last, 1996) and should allow researchers to effect changes in free tryptophan levels and, potentially, indole-derived metabolites.

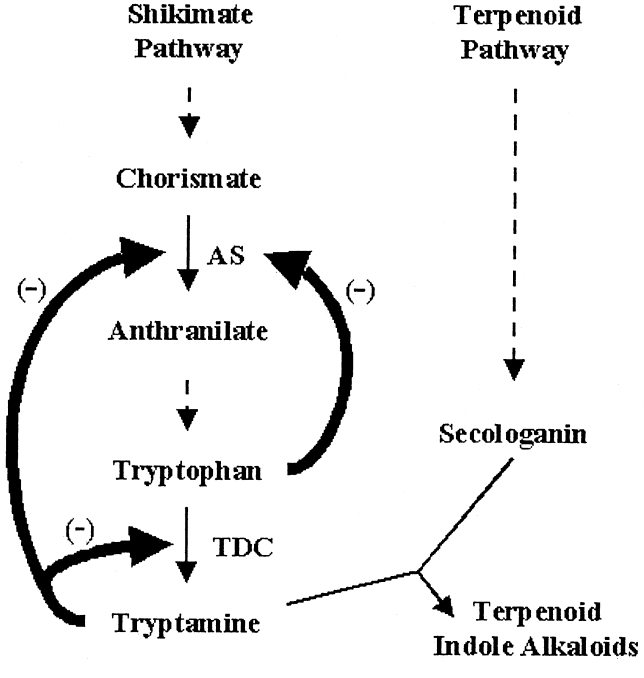

Figure 1. Regulation of the indole pathway in *C. roseus*. Arrows with minus signs indicate previously identified steps sensitive to feedback regulation. AS, anthranilate synthase. TDC, tryptophan decarboxylase.

In this article we report the generation of *C. roseus* hairy roots transgenic for a feedback-resistant Arabidopsis ASα subunit. The gene is cloned behind a glucocorticoid-inducible promoter (Aoyama and Chua, 1997) that has previously been used successfully within our system (Hughes et al., 2002) and should provide an improved negative control against clonal variation. As precursor effects have been shown to be developmentally dependent, we study the effects of induction during both the late exponential and early stationary growth phases. At each point, we report the effects of ASα induction on tryptophan, tryptamine, and specific alkaloid levels.

MATERIALS AND METHODS

Plasmid Construction

A full-length wildtype *Arabidopsis thaliana ASA1* cDNA was kindly provided by Dr. Gerald R. Fink (Whitehead Institute for Biomedical Research). Based on the previous report of G to A substitution in the sixth exon of the *ASA1* gene from tryptophan feedback-resistant *A. thaliana* (Li and Last, 1996), a site-directed mutagenesis mediated by PCR was performed using overlapping mutagenic primers (5′-ttgcaAacccttgaagtttat-3′ and 5′-aagggtTtgcaaatgttcg-ccg-3′). The final PCR product was cloned into a pBluescript KS vector and sequenced to confirm the dominant

point mutation. The mutated *ASA1* cDNA was then inserted into the XhoI/SpeI site downstream of the GAL4-UAS promoter in the binary pTA7002 vector (Aoyama and Chua, 1997). This construct was named p7002ASA. The plasmid also contains hygromycin phosphotransferase for plant antibiotic selection and the glucocorticoid-dependent transcription factor driven by the NOS and CaMV 35S promoters, respectively.

Clone Generation

The plasmids described above were electroporated into *A. rhizogenes* 15834. Transformed colonies from YEM plates (1 g/L yeast extract, 0.2 g/L NaCl, 0.2 g/L $MgS0_4 \cdot 7H_20$, 4 mg/L $FeC1_3$, 0.66 g/L $K_2HPO_4 \cdot 3H_20$, 10 g/L mannitol, 15 g/L agar) including kanamycin (50 mg/L) were analyzed for the presence of the respective plasmid by restriction digest. A single colony was then used to start a 5 mL culture grown at 28°C and 200 rpm for 36 h in liquid YEM + kanamycin (50 mg/L) medium. Scissors were dipped in the culture and used to infect aseptically grown plants as previously described (Bhadra et al., 1993). After 6 weeks, hairy roots were excised from the plants and selected on solid and liquid media as described, except that 30 mg/L hygromycin was added to the first solid subculture to select for clones carrying the transgenes of interest.

Culture Conditions

Hairy roots were grown in a filter-sterilized solution of 30 g/L Suc, half strength Gamborg's B5 salts, and full strength vitamins (pH 5.7). Cultures were initiated by placing five tips into a 250 mL Erlenmeyer flask with 50 mL of medium. The cultures were grown at 26°C at 100 rpm in the dark. Growth curves and residual medium volumes were determined for cultures grown on a fixed subculture cycle by measuring the culture fresh weight and medium volume every 3 days over a culture cycle. Line EHIASA-1 was maintained on a 54-day subculture cycle with the stationary phase beginning at 53 days. For EHINC-12-1 on a 35-day subculture cycle, 34 days marked the beginning of stationary phase. At 6 days before the beginning of stationary phase and again at the onset of stationary phase, induction was performed by adding the appropriate volume of a 5-mM dexamethasone stock in ethanol. An equal amount of ethanol was added to uninduced controls.

RNA Isolation and Northern Analysis

Hairy root flask cultures that were approximately 4 weeks old were used for transcriptional induction studies. A 5-mM stock solution of dexamethasone was used to induce each cell line, with ethanol added to the uninduced controls. Isolation of total RNA from hairy roots was performed using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was quantified by spectrophotometer according to absorbance at 260 nm and an ethidium bromide-stained test gel used to verify the quality of RNA. Twelve μg of total RNA were loaded per well and Northerns performed essentially according to a reference protocol (Brown and Mackey, 1997). All genes to be tested had been cloned into plasmids and the relevant section was removed by restriction digest and gel-purified with a Qiagen (Valencia, CA) QIAEX II kit. Probes were then made using the Stratagene (La Jolla, CA) Prime-It II kit.

Enzyme Assays

Anthranilate synthase enzyme assays were performed essentially as previously reported (Last and Fink, 1988). After a 72-h induction with 3 μM dexamethasone, approximately 1 g of fresh-weight 4-week-old biomass was harvested and ground in a mortar and pestle with grinding buffer (200 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 8 mM $MgCl_2$, 0.2 mM DTT, 60% glycerol), 200 mg glass beads, and 200 mg polyvinylpolypyrrolidone. Uninduced controls were exposed to equal amounts of ethanol. All steps were performed at 4°C unless otherwise noted. Supernatant was cleared by centrifugation and desalted on a Bio-Rad (Hercules, CA) Econo-Pac l0DG column preequilibrated in column buffer (50 mM Tris-HCl pH 7.5, 0.05 mM EDTA, 2 mM $MgCl_2$, 0.05 mM DTT, 5% glycerol). AS activity was measured in a 2.0 mL reaction with 1 mL column buffer plus 0.05 mM chorismic acid, 10 mM glutamine, 1 mM MgC12, and 12.5 mM Tris-HCl (pH 8), and 20 μg crude protein as determined by the Bradford Assay (Sigma, St. Louis, MO). Tryptophan from a 10-mM stock was added at the desired concentration to the assay volume to test the inhibition of the anthranilate synthase activity. The reaction was terminated after 30 min at 30°C by adding 0.2 mL of 1.0 M HCl. The anthranilate produced was extracted into 3.5 mL ethyl acetate and quantified on a fluorescence sprectrophotometer (excitation 340 nm, emission 400 nm). Standard curves were generated by adding known amounts of anthranilic acid to the reaction assay volume without protein and following the same extraction procedure. Uninhibited AS assays were performed in triplicate and average values are reported.

HPLC Analysis

Harvested cultures were immediately frozen at −80°C. After lyophilization, a crude MeOH extract was prepared by extracting 50 mg of freeze-dried biomass with 10 mL of MeOH for 1 h in a sonicating bath. Extracts were clarified at 1,300*g* for 15 min at 15°C. The supernatant was removed and the biomass reextracted in the same manner. Extracts were combined and concentrated under vacuum to 2 mL and then passed through a 0.22 μm nylon filter (13 mm). Ten μL of this extract was injected onto a Phenomenex (Torrance, CA) Luna 5μ C18(2) HPLC column (250 x 4.6 mm) under two different solvent systems. The Thermo Separations Products (San Jose, CA) Spectrasystem HPLC system used included a P4000 pump unit, an AS3000

autosampler, and a UV2000 detector. For the detection of tryptophan and tryptamine, a protocol was adapted from the literature with UV detection at 218 nm (Tikhomiroff and Jolicoeur, 2002). For 12 min, a flow rate of 1 mL/min was maintained of a 15:85 mixture of MeCN:100 mM phosphoric acid. The column was then washed with an 85:15 mixture and reequilibrated. Another solvent system was used for the detection of the indole alkaloids ajmalicine, serpentine, hörhammericine, lochnericine, and tabersonine. Ajmalicine and serpentine were detected at 254 nm and quantified by comparison with authentic standard curves. Tabersonine, lochnericine, and hörhammericine were detected at 329 nm by retention time of standards and quantified based on a tabersonine standard curve as previously described (Morgan et al., 2000). For the first 5 min, the mobile phase consisted of a 20:80 mixture of 50% MeOH/5O% MeCN : 5 mM $(NH_4)_2HP0_4$ pH 6 pumped at 1 mL/min. Over 10 min, it was linearly ramped to a 64:36 mixture, where it was maintained for 15 min. Over the next 5 min, the flow rate was linearly ramped to 1.4 mL/min. The ratio was then increased to 80:20 over 5 min, where it was maintained for 15 min.

RESULTS

Clone Generation and Tightly Controlled Transcriptional Inducibility

Agrobacterium rhizogenes strain ATCC 15834 carrying the Ri plasmid and p7002ASA was used to infect approximately 350 5-week-old *C. roseus* seedlings. The p7002ASA plasmid contains an Arabidopsis *AS*α gene with a point mutation to confer feedback resistance (Li and Last, 1996) expressed behind a glucocorticoid-inducible promoter. In addition, the plasmid also contains a hygromycin selection marker and a glucocorticoid (dexamethasone) inducible transcription factor (GVG) under the control of the constitutive CaMV 35S promoter. Of the 161 hairy roots excised, 39 showed strong growth on solid selection medium containing 30 mg/L hygromycin. Of these, only six adapted to long-term maintenance of 10 generations on liquid medium. Negative control (NC) lines were similarly generated with ATCC 15834 carrying the Ri plasmid and p7002, which lacks the inducible *AS*α gene but contains all other elements of the inducible system. Of 379 seedlings infected, 159 yielded hairy roots, of which 43 grew on solid medium and four adapted to long-term liquid culture maintenance.

After a minimum of four liquid generations, four NC and four ASα lines were subjected to Northern blot analysis to check for ASα transcriptional inducibility. Each line was induced with 3 μM dexamethasone for 72 h. Total RNA isolated from these eight lines from both induced (3 μM dexamethasone for 72 h) and uninduced flasks was hybridized with labeled *AS*α cDNA, tryptophan decarboxylase (TDC), and GVG probes. Of those lines tested, two NC lines and two ASα lines exhibited readily detectable GVG mRNA levels in both induced and uninduced states, confirming constitutive GVG expression. The two ASα lines also showed readily detectable *AS*α mRNA levels in the induced state, with no background expression visible in the uninduced state. The NC line with higher overall GVG transcript levels and the ASα line with the highest transcriptional induction were chosen for further analysis. The Northerns for these two lines, EHIASA-1 (*AS*α line) and EHINC-12-1 (NC line), are shown in Figure 2. As an added test, both lines were also subjected to genomic PCR to confirm the presence of the transgenes. The insertion of GVG was confirmed in both, and insertion of ASα was confirmed in EHIASA-1 by using a primer specific to the promoter's *GAL4* upstream activating sequences and one at the 3' end of the *AS*α gene.

Expression of Mutated ASα Leads to Reduced Feedback Sensitivity

Initially, the overall glutamine-dependent activities of the lines were measured. Desalted protein extracts were prepared as detailed and the specific activities of the uninduced and induced states compared. Glutamine-dependent activity is dependent on the compatibility of the transgenic Arabidopsis ASα with the native ASβ and the availability of ASβ subunits. Activity measured in vitro by measurement of anthranilate produced over 30 min by 20 μg of crude desalted protein. On induction, there was a 10% increase in AS activity from 4.18 to 4.61 pmol/mg/s in EHINC-12-1,

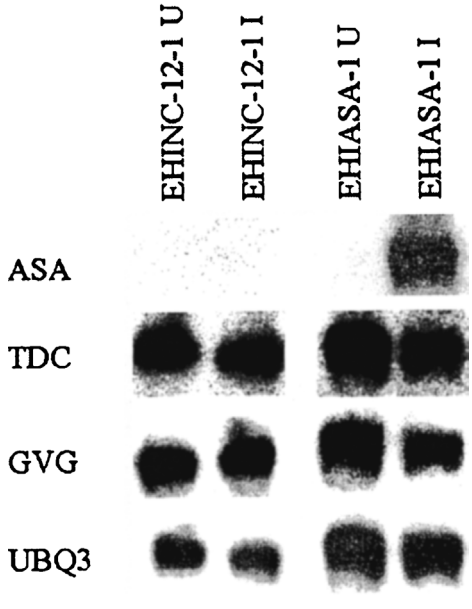

Figure 2. Northern analysis of lines EHINC-12-1 and EHIASA-1. Lines were induced (I) with 3 μM dexamethasone for 72 h and RNA prepared. Ethanol was added to uninduced (U) controls. Twelve μg RNA was loaded and blotting performed. Probes were made for Arabidopsis anthranilate synthase α (ASA), tryptophan decarboxylase (TDC), the glucocorticoid transcription factor (GVG), and ubiquitin 3 for loading control (UBQ3).

the negative control line. The AS line, EHIASA-1, showed a 36% increase from 2.75 to 3.75 pmol/mg/s. Although a substantial increase was noted, it was not as dramatic as one might initially expect from the high level of transcriptional induction. Glutamine-dependent activity requires both α and β subunits, though, and therefore activity is not simply a function of ASα levels. In addition, the in vivo flux through the indole pathway is likely to be strongly dependent on the feedback inhibition of AS activity rather than by absolute enzyme levels.

In order to test further the induction of the feedback-resistant ASα, tryptophan inhibition curves for the induced vs. uninduced protein extracts were generated. In Figure 3, the activity levels from uninduced and induced extracts of the NC and ASα lines are plotted as a percentage of their initial in vitro activity vs. tryptophan concentration. The induced AS extract clearly shows an increase in feedback resistance. Furthermore, the results are promising in that the uninduced AS extract closely mirrors the results from the NC line, indicating that expression in the absence of the inducing agent is likely limited using this inducible system, as previously reported (Hughes et al., 2002; Ouwerkerk et al., 2001).

Inducible ASα Expression Yields Large Increases in Tryptophan and Tryptamine

The use of a feedback-resistant ASα should enable continued enzymatic activity in the presence of increased tryptophan levels within the system. After confirming the transcriptional inducibility and altered enzymatic activity on dexamethasone treatment, the effect of induction on tryptophan levels was investigated. As differential effects have been noted in precursor feeding studies based on growth stage, points were taken after a 3- and 6- days 3 μM dexamethasone induction during both the late exponential and early stationary growth phases. Growth curves had previously been generated based on fresh weight measurements of actively growing cultures on a fixed subculture cycle.

The HPLC results show an extraordinary increase in tryptophan levels upon induction. From initial levels that are barely detectable, the induced AS line accumulates 2.5 mg/g dry weight (DW) after a 6-day induction in the exponential phase. As seen in Figure 4, the longer induction times lead to higher accumulations of tryptophan, and inductions during the exponential phase are more effective than those during the stationary phase. Induction has no substantial impact on tryptophan levels in the NC line, indicating that the increase is a clear consequence of ASα induction. Although the noted increase is the intended direct effect of the genetic manipulation, the underlying motivation in studying *C. roseus* using an inducible promoter was to examine the effects of tryptophan accumulation on downstream metabolites in a well-controlled system. The significant and sudden metabolic change, along with the absence of any effects on the NC line, indicates that such an analysis is possible.

Within our system, tryptophan is converted to tryptamine by TDC. Northern analysis (Fig. 2) and activity assays within our laboratory are consistent with literature reports that indicate high native levels of TDC transcription (Goddijn et al., 1995) and activity in *C. roseus* hairy roots (data not shown). In vitro assays have shown TDC activity levels in hairy roots to be at least an order of magnitude higher than those found in cell culture (Moreno-Valenzuela et al.,1998). Even in cell culture, though, native TDC activity levels were found to be sufficient for the utilization of tryptophan for increased alkaloid production (Whitmer et al., 1998). Assays of tryptamine levels in our system closely

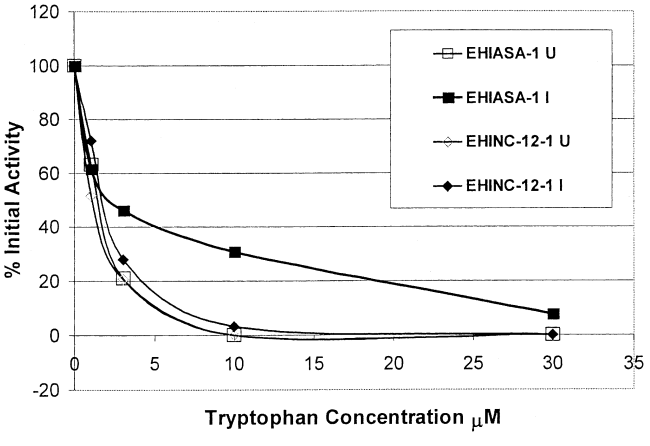

Figure 3. Tryptophan inhibition curves of protein extracts for ASα (EHIASA-1) and NC (EHINC-12-1) lines. Lines were induced (I) with 3 μM dexamethasone for 72 h and protein extracts prepared. Ethanol was added to uninduced (U) controls. Data plotted are the percent of initial in vitro activity at a range of tryptophan concentrations.

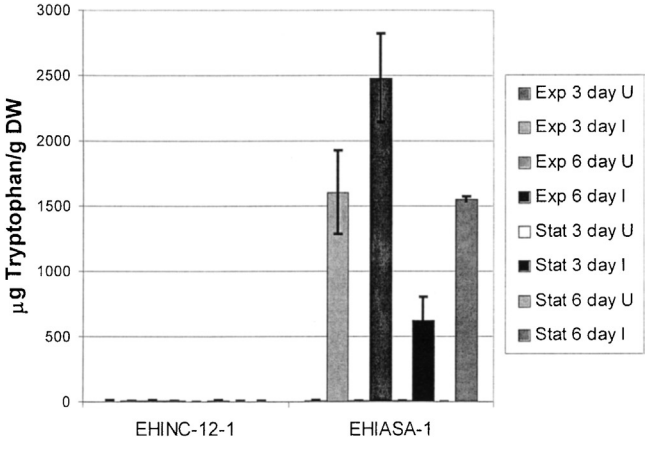

Figure 4. Tryptophan levels at various induction lengths and growth stages for ASα (EHIASA-1) and NC (EHINC-12-1) lines. Late exponential (Exp) and early stationary (Stat) growth phase cultures were induced with 3 μM dexamethasone and harvested after 3 or 6 days. Tryptophan was determined by HPLC analysis from crude extracts. Data represent mean of triplicate cultures ± standard deviation.

mirrored the trends in tryptophan levels, while showing a much smaller percentage increase. Effects of induction on the NC line were again insignificant. Data for both the NC and AS lines are displayed in Figure 5. Six-day inductions of the AS line resulted in higher tryptamine levels than those measured over 3-day inductions. Increases during the exoponential phase were larger than those measured during the stationary phase, indicating again that the metabolic activity of the line might have decreased with growth and nutrient levels. At all timepoints, the increase was greater than 3-fold, with a maximum increase of 10-fold up to 0.27 mg/g DW. Elevated tryptophan synthesis on induction clearly impacted tryptamine levels, as might be expected from the high native TDC activity levels already shown in hairy roots.

Transient Increase in One Alkaloid

Having successfully increased tryptophan and tryptamine levels, the effect of increased indole flux on the biosynthesis of terpenoid indole alkaloids was studied. Although the analysis of pathways remains difficult due to the existence of secondary effects, a limited number of fixed timepoints, and the limited characterization of alkaloids and their pathways, five alkaloids were measured at each timepoint to evaluate the metabolic effects. These included tabersonine, hörhammericine, and lochnericine from the Aspidosperma family of alkaloids and ajmalicine and serpentine from the Coryanthe family. Tabersonine data has been omitted due to very low production in one of the lines, making comparative analysis impossible. As shown in Figure 6, induction of the AS line caused no significant change in the levels of hörhammericine, ajmalicine, or serpentine. In contrast, a significant difference ($p < 0.05$) between the induced and uninduced AS line at 3 days was noted in lochnericine-specific yield, with no change noted in the NC line (Fig. 7). After a 3-day 3 μM dexamethasone induction, lochnericine levels in induced cultures were 2.03 mg/g DW vs. 1.12 mg/g DW in the uninduced state. NC lines showed no significant effects on alkaloids during the exponential growth stage (Fig. 8), but a slight browning in the NC lines induced during the stationary phase might indicate nonspecific elicitation from dexamethasone treatment, as previously noted in other reports (Kang et al., 1999; Ouwerkerk et al., 2001). Accompanying this browning was a statistically significant effect on ajmalicine levels during the stationary phase (Fig. 8). The browning noted in the NC line was not visible in the AS line and the related

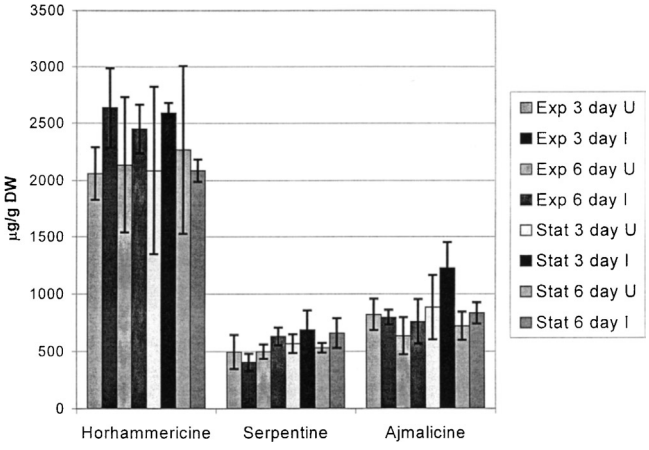

Figure 6. Levels of alkaloids in ASα line. Late exponential (Exp) and early stationary (Stat) growth phase cultures were induced with 3 μM dexamethasone and harvested after 3 or 6 days. Alkaloids were determined by HPLC analysis from crude extracts. Data represent mean of triplicate cultures ± standard deviation.

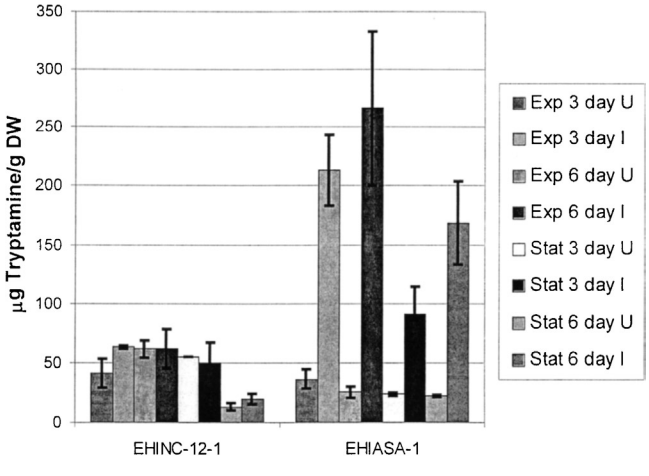

Figure 5. Tryptamine levels at various induction lengths and growth stages for ASα (EHIASA-1) and NC (EHINC-12-1) lines. Late exponential (Exp) and early stationary (Stat) growth phase cultures were induced with 3 μM dexamethasone and harvested after 3 or 6 days. Tryptamine was determined by HPLC analysis from crude extracts. Data represent mean of triplicate cultures ± standard deviation.

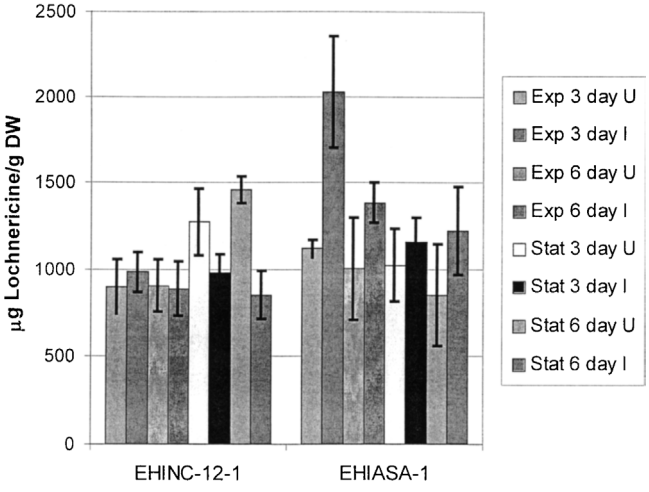

Figure 7. Lochnericine levels in ASα (EHIASA-1) and NC (EHINC-12-1) lines. Late exponential (Exp) and early stationary (Stat) growth phase cultures were induced with 3 μM dexamethasone and harvested after 3 or 6 days. Lochnericine was determined by HPLC analysis from crude extracts. Data represent mean of triplicate cultures ± standard deviation.

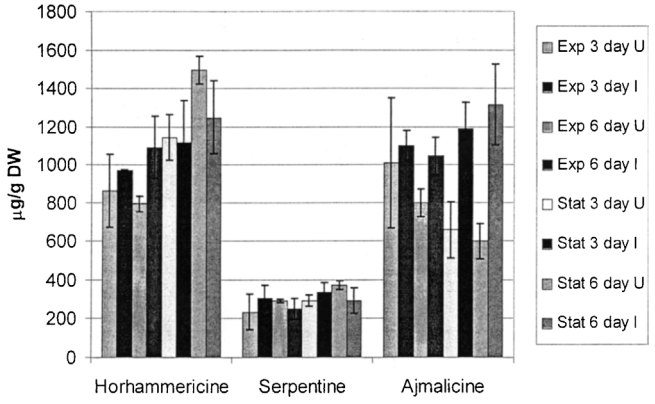

Figure 8. Levels of alkaloids in NC line. Late exponential (Exp) and early stationary (Stat) growth phase cultures were induced with 3 µM dexamethasone and harvested after 3 or 6 days. Alkaloids were determined by HPLC analysis from crude extracts. Data represent mean of triplicate cultures ± standard deviation.

metabolic effects seem limited but should not be ignored in interpreting our results.

DISCUSSION

Inducible Promoter as an Advantageous Tool for Pathway Analysis

As no regeneration protocol has been reported for *C. roseus,* cell and tissue cultures represent the only available options for metabolic engineering of the plant. Most work to date has focused on cell culture, but hairy roots have certain advantages. Differentiation is known to play a role in alkaloid accumulation, hairy roots contain higher levels of alkaloids than cell cultures (Moreno-Valenzuela et al., 1998), and hairy roots maintain genetic and biochemical stability, whereas cell cultures often do not (Baiza et al., 1999; Deus-Neumann and Zenk, 1984). Furthermore, the inducible promoter used in this study provides an improved negative control to evaluate the effects of any metabolic alteration. Negative controls in most studies are often different cell lines transformed independently. Here, the negative control is the same genetic line without the inducing agent. Given that there is wide variability in alkaloid production in our system (Bhadra et al., 1993), such a negative control allows for improved data analysis within the system. Furthermore, induction of the *ASα* gene within this study has shown that the inducible promoter allows the researcher to time a substantial metabolic change. In this study, a large metabolic change was reported at 3 days and nonspecific expression appeared insignificant. There also seemed to be minimal effects on alkaloid biosynthesis by the inducing agent. Although some changes were noted in the stationary phase accompanying weak browning, they are likely due to the specific NC line chosen for this study. Previous work had shown the activity of the promoter within our system (Hughes et al., 2002). This study demonstrates the suitability of that system for studying the alkaloid pathways in *C. roseus.*

Successful Improvement of Tryptophan Levels

Although the substantial increase in ASα transcription led to relatively small improvements in enzyme activity, the advantageous properties of the feedback-resistant enzyme were apparent. In vitro enzyme assay results from the tryptophan inhibition studies indicate that the Arabidopsis ASα subunit is compatible with the native ASβ subunit. It is the corresponding improvement in feedback resistance rather than the basal AS activity level that proves most important to the metabolic effects. Within this work, overexpression of the feedback-resistant ASα subunit led to substantial increases in both tryptophan and tryptamine levels. Although trytophan levels were almost undetectable in uninduced control cultures, tryptophan increased to 2.5 mg/g DW after a 6-day induction with 3 µM dexamethasone in the late exponential phase. Similar dramatic increases in tryptophan levels without large changes in overall activity levels have been noted in other reports (Tozawa et al., 2001) and are consistent with the hypothesis that feedback inhibition rather than absolute enzyme levels control indole flux. Furthermore, the increases in tryptophan synthesis reported here compare favorably with previous reports. The increase seen within this work exceeds 300-fold, while the original Arabidopsis mutant and an *Astragalus sinicus* hairy root line engineered for the constitutive overexpression of feedback-resistant ASα produced 3-fold and 5.5-fold increases, respectively (Cho et al., 2000; Li and Last, 1996). At 2.5 mg/g DW or 0.22 mg/g fresh weight (FW), the overall levels also exceed the tryptophan accumulation measured in the original Arabidopsis mutant (0.06 mg/g FW) (Li and Last, 1996), a 5-methyltryptophan resistant *C. roseus* cell line (0.7 mg/g DW) (Scott et al., 1979), and the engineered *Astragalus sinicus* hairy root line (0.06 mg/g FW) (Cho et al., 2000). Along with the noted increase in tryptophan, tryptamine simultaneously rose 10-fold. TDC activity within *C. roseus* has previously been reported to be extremely high in hairy root cultures (Goddijn et al., 1995; Moreno-Valenzuela et al., 1998), and it appears that basal levels are sufficient to increase tryptamine levels when supplied with an increased tryptophan pool. As often occurs with downstream products, the increases were diminished. Such a result is consistent with reports that tryptamine feedback inhibits TDC activity (Noe et al., 1984). Accumulated tryptamine may also be utilized for increased flux to the alkaloids or other metabolites. Two substantially increased peaks were noted in the chromatogram at 329 nm, and they have yet to be identified. Combined with results from other systems, the expression of a feedback-resistant ASα appears sufficient to increase tryptophan pools for potential conversion into secondary metabolites. The engineering approach presented here could also prove useful for manipulating

the accumulation of other valuable secondary metabolites in cases where there are indications that tryptophan supply may be a limiting factor (Silvestrini et al., 2002).

Our engineering of the indole pathway is subject to a notable secondary effect from auxins that has been hypothesized to play a role in alkaloid synthesis. A possible effect of increased tryptophan pools could be the synthesis of increased levels of auxins, which are known to be strong plant regulators with effects on particular alkaloid genes. The auxin IAA is known to be synthesized from tryptophan in even untransformed roots (Muller et al., 1998). Additionally, hairy roots show increased sensitivity to auxins (Shen et al., 1990) and agropine *A. rhizogenous* strains as used in this study insert genes for the synthesis of IAA (Cardarelli et al., 1985). In previous work, though, the effects of auxins on alkaloids have been contradictory, depending on the exact *C. roseus* system studied and auxin dosage (Aerts et al., 1992; Goddijn et al., 1992; Morgan and Shanks, 2000). In our system, tryptophan or auxin feeding to *C. roseus* hairy roots increased thickening and branching (Morgan and Shanks, 2000). At low levels of feeding, however, the effects on both alkaloids and morphology were limited. In this study, no such growth effects were noted, indicating that the secondary effect is likely minimal. There have also been literature reports in *Lemna gibba* indicating that increased pools of L-tryptophan do not lead to significant increases in IAA levels (Baldi et al., 1991). The presence of such secondary effects, however, is worthy of note given the number of potential metabolic fates of tryptophan.

Transient Alkaloid Increase Suggests Tight Regulation of Alkaloid Levels

Previous results have often indicated that the supply of terpenoid precursors is initially limiting to alkaloid accumulation in *C. roseus* (Merillon et al., 1986; Moreno et al., 1993; Morgan and Shanks, 2000). However, some feeding studies have shown increases in alkaloids in response to tryptophan or tryptamine feeding (Kreuger and Carew, 1978; Zenk et al., 1977). In *C. roseus* hairy roots, feeding tryptophan during the late exponential phase resulted in significant increases in serpentine and tabersonine after 3 days (Morgan and Shanks, 2000). In the early stationary phase, no significant effects were seen 3 days later, effectively demonstrating a transient effect. Within the work presented here, lochnericine-specific yield increased 81% after a 3-day induction in the late exponential phase. No such effects were seen at the 6-day exponential phase timepoint or either stationary phase timepoint. Although more detailed studies would be necessary to confirm the transient nature of the peak, these results are consistent with previous results. A feeding study on *C. roseus* cell culture reported a similar transient in that tryptamine increased the rate of alkaloid accumulation when combined with loganin feeding, but not the final accumulation value (Whitmer et al., 2002a). The lochnericine spike seen in this study is also consistent with the report of substantial increases in only certain alkaloids. It appears, therefore, that the effects of even dramatically increased indole precursor availability include a limited impact on alkaloid accumulation. There could be an increase in indole alkaloid flux demonstrated by the transiently improved specific yields that is counterbalanced over longer time periods by strict regulation of alkaloid accumulation through catabolic pathways. More detailed studies to explore the existence of these transient effects could be pursued by utilizing the flexibility of the inducible promoter.

Engineering of Complementary Pathway

As strictosidine synthase activities have been reported to be high in hairy roots (Meijer et al., 1993), a likely explanation of the limited alkaloid changes is that the availability of both indole and terpenoid precursors controls the long-term accumulation of alkaloids. Recent feeding studies using transgenic *C. roseus* cell lines have indicated such coordination is necessary (Whitmer et al., 2002a,b). In both strictosidine synthase and tryptophan decarboxylase overexpressers, indole feeding combined with terpenoid feeding increased alkaloid yields after an initial terpenoid deficiency was overcome. Such studies point to the importance of further characterization and engineering efforts focused on the nonmevalonate terpenoid pathway. As this pathway was only recently elucidated, little is known about its regulation and few of the genes encoding pathway components have been cloned (Lichtenthaler, 1999). A few attempts at engineering the pathway in other plant systems have been made, but progress is limited to date (Estevez et al., 2001; Mahmoud and Croteau, 2001). Potential limitations downstream of strictosidine synthase may also hinder gains in the particular alkaloids measured, as previously noted in cell culture (Whitmer et al., 2002a). Continued characterization of these pathways should, however, lead to an improved understanding of alkaloid regulation and metabolic engineering efforts.

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