Effects of Terpenoid Precursor Feeding on *Catharanthus roseus* Hairy Roots Over-Expressing the Alpha or the Alpha and Beta Subunits of Anthranilate Synthase

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Abstract: Among the pharmacologically important terpenoid indole alkaloids produced by *Catharanthus roseus* are the anti-cancer drugs vinblastine and vincristine. These two drugs are produced in small yields within the plant, which makes them expensive to produce commercially. Metabolic engineering has focused on increasing flux through this pathway by various means such as elicitation, precursor feeding, and introduction of genes encoding specific metabolic enzymes into the plant. Recently in our lab, a feedback-resistant anthranilate synthase α subunit was over-expressed in *C. roseus* hairy roots under the control of a glucocorticoid inducible promoter system. Upon induction we observed a large increase in the indole precursors, tryptophan, and tryptamine. The current work explores the effects of over-expressing the anthranilate synthase α or α and β subunits in combination with feeding with the terpenoid precursors 1-deoxy-D-xylulose, loganin, and secologanin. In feeding 1-deoxy-D-xylulose to the hairy root line expressing the anthranilate synthase α subunit, we observed an increase of 125% in hörhammericine levels in the induced samples, while loganin feeding increased catharanthine by 45% in the induced samples. Loganin feeding to the hairy root line expressing anthranilate synthase α and β subunits increases catharanthine by 26%, ajmalicine by 84%, lochnericine by 119%, and tabersonine by 225% in the induced samples. These results suggest that the terpenoid precursors to the terpenoid indole alkaloids are important factors in terpenoid indole alkaloid production.

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INTRODUCTION

Catharanthus roseus produces vinblastine and vincristine, two terpenoid indole alkaloids, which are valuable chemotherapy drugs used in combination with other drugs to treat lymphoma and leukemia (Gidding et al., 1999; van der Heijden et al., 2004). Since these drugs are produced in small amounts within the plant (Tyler, 1988), research has focused on increasing the production of terpenoid indole alkaloids within cell suspension or hairy root cultures. These research efforts include media optimization (Bhadra et al., 1993), elicitation (Moreno et al., 1993; Rijhwani and Shanks, 1998), precursor feeding (Moreno et al., 1993; Morgan and Shanks, 2000; Whitmer et al., 1998), and the over-expression of enzymes within the pathway (Canel et al., 1998; Whitmer et al., 2002a,b). Results from these studies have begun to form a picture of the complex network that leads to the formation of the terpenoid indole alkaloids. Even though there are still many unknown compounds and uncharacterized enzymes within this pathway (Hughes and Shanks, 2002; van der Heijden et al., 2004), researchers can focus on metabolic engineering of the known enzymes and compounds in order to further characterize this complex network. Precursor feeding studies in particular can help identify limiting enzymes in the pathway in order to focus future research efforts on these limiting enzymes.

In *C. roseus*, tryptamine from the indole pathway is coupled with secologanin from the terpenoid pathway to form strictosidine, which then is converted to a broad range of terpenoid indole alkaloids (Fig. 1). The first committed step in tryptophan synthesis is the conversion of chorismate to

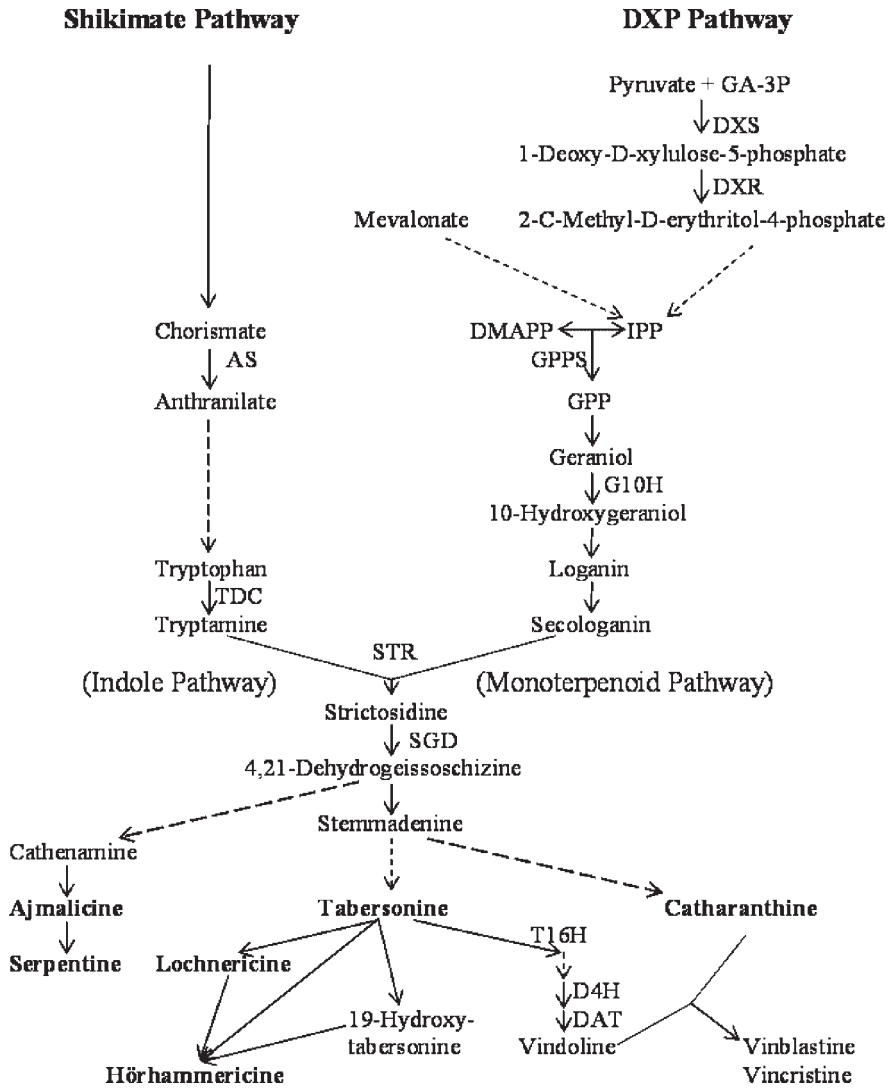

Figure 1. Biosynthesis of terpenoid indole alkaloids in *Catharanthus roseus.* DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; GPPS, geranyl pyrophosphate synthase; G10H, geraniol 10-hydroxylase; AS, anthranilate synthase; TDC, tryptophan decarboxylase; STR, strictosidine synthase; SGD, strictosidine β-D-glucosidase; T16H, tabersonine 16-hydroxylase; D4H, desacetoxyvindoline 4-hydroxylase; DAT, deacetylvindoline acetyltransferase.

anthranilate by anthranilate synthase (AS). The AS enzyme is a tetramer composed of two α subunits (ASα) and two β subunits (ASβ). The α subunit catalyzes the aromatization of chorismate while the β subunit donates an amino group from glutamine (Hughes et al., 2004). The native anthranilate synthase enzyme is inhibited by increased tryptophan levels (Verpoorte et al., 1999). This inhibition has been overcome via the constitutive expression of a feedback resistant ASα (Cho et al., 2000; Tozawa et al., 2001). We have been successful in introducing and expressing a mutant *Arabidopsis ASα* gene resistant to tryptophan analogues (Li and Last, 1996) in *C. roseus* hairy roots (Hong et al., 2005; Hughes et al., 2004).

It has been previously reported that the expression of a feedback-resistant anthranilate synthase alpha subunit (ASα) from *Arabidopsis* in *Catharanthus roseus* hairy roots under the control of the glucocorticoid-inducible promoter system caused significant increases in both tryptophan and tryptamine levels within hairy roots over a 3-day induction period in late-exponential growth phase with 3 μM dexamethasone (Hughes et al., 2004). However, the increase in tryptamine and tryptophan was not accompanied by an increase in terpenoid indole alkaloids except for lochnericine. These results suggest that there is tight regulation of the terpenoid indole alkaloid levels (Hughes et al., 2004). It was also previously reported that when the α subunit of AS was overexpressed it may combine with the naturally available β unit of AS to form a functional enzyme and cause an increase in the tryptophan and tryptamine pools (Hong et al., 2005; Hughes et al., 2004). In cell suspension cultures of *C. roseus*, precursor feeding studies have suggested that the terpenoid pathway is limiting as feeding cultures with loganin or secologanin led to a significant increase in terpenoid indole alkaloids (Whitmer et al., 1998, 2002a,b).

Along with the ASα hairy root line, we have also constructed an AS α/β hairy root line that expresses a feedback-resistant α subunit from *Arabidopsis* under the control of a glucocorticoid inducible promoter system and the β subunit from *Arabidopsis* under the control of the constitutive CaMV 35S promoter. This AS α/β line, designated ASAB-1, showed a greater increase in tryptophan and tryptamine at a lower induction level of 0.2 μM dexamethasone than the AS α line. This lower dexamethasone level was used because significant browning occurred in the samples at 3 μM dexamethasone (Hong et al., 2005).

In this paper, we examined the effects of feeding various precursors on the terpenoid indole alkaloid levels in the ASα (EHIASA-1) and ASαβ (ASAB-1) *C. roseus* hairy root lines.

MATERIALS AND METHODS

Chemicals

1-deoxy-D-xylulose (Omicron, South Bend, IN) was added directly to the culture media as a liquid. Loganin and secologanin (Fluka, Switzerland) were first dissolved in double deionized water and then filter-sterilized through a 0.22 μm nylon filter (13 mm) before adding to the culture media. Dexamethasone (Sigma, St. Louis, MO) was dissolved in ethanol and added to the culture media. Serpentine (Sigma), ajmalicine (Sigma), catharanthine (Qventas, Bradford, CT), and tabersonine (a gift from Dr. Hamada, Okayama University, Japan) were dissolved in methanol and used as HPLC standards.

Culture Conditions and Hairy Root Lines

The culture media for the hairy roots consisted of a filter-sterilized (0.22 μm filter) solution of 30 g/L sucrose (Sigma), half-strength Gamborg's B5 salts (Sigma), and full-strength Gamborg's vitamins (Sigma) with the pH adjusted to 5.7. Hairy root cultures were initiated by placing five root tips in 50 ml of the culture media in a 250 ml Erlenmeyer flask. These cultures were grown in the dark at 26°C at 100 rpm. The generation of EHIASA-1 and ASAB-1 were previously described (Hong et al., 2005; Hughes et al., 2004). EHIASA-1 expresses a feedback-resistant AS α subunit from *Arabidopsis* under the control of a glucocorticoid inducible promoter. ASAB-1 carries this same construct and, in addition, expresses an AS β subunit from *Arabidopsis* under the control of the constitutive CaMV 35S promoter.

Precursor Feeding

Earlier work suggested that the biggest effects on TIA production occur in late exponential phase (Morgan and Shanks, 2000). EHIASA-1 transitioned from late exponential phase to stationary phase around day 45 (data not shown) and ASAB-1 transitioned to stationary phase around day 23 (data not shown). In this study, precursors were fed on day 40 for EHIASA-1 and day 18 for ASAB-1 and harvested 72 h later. For EHIASA-1, cultures were either induced with 3 μM dexamethasone or uninduced with an equal amount of ethanol as a control and were fed simultaneously with 208 μM 1-deoxy-D-xylulose, 104 μM loganin, or left unfed. For ASAB-1, cultures were either induced with 0.2 μM dexamethasone or uninduced with an equal amount of ethanol as a control and were fed simultaneously with 104 μM loganin, 104 μM secologanin, or left unfed. Each combination was repeated in triplicate.

Alkaloid Extraction

The fresh weights of the harvested hairy root cultures were measured after the cultures were blotted to remove excess media. The cultures were then frozen at −80°C. The dry weights were measured after lyophilization. Approximately 50 mg dry weight of the ground tissues were extracted with 10 ml of MeOH in a sonicating bath for 1 h at 15°C. The extracts were then clarified by centrifugation at 1,300*g* for 15 min at 15°C. The supernatant was removed and the cellular debris was re-extracted in the same manner. The supernatants from both extracts were combined, concentrated to 2 ml using a vortex evaporator, and passed through a 0.22 μm nylon filter (13 mm).

HPLC Analysis

Ten microliters of the alkaloid extract was injected onto a Phenomenex (Torrance, CA) Luna 5A C18(2) HPLC column (250 × 4.6 mm) under two different solvent systems. The HPLC system used was a Thermo Separations Products (San Jose, CA) Spectrasystem HPLC consisting of a P4000 pump, an AS3000 autosampler, and an UV2000 detector. To detect tryptophan and tryptamine, a protocol was adapted from the literature with UV detection at 218 nm (Hughes et al., 2004; Tikhomiroff and Jolicoeur, 2002). For the first 12 min, a 15:85 mixture of MeCN: 100 mM phosphoric acid (pH 2) was maintained at a flow rate of 1 ml/min. The column was then washed with an 85:15 mixture and re-equilibrated. Another solvent system was used to detect the indole alkaloids ajmalicine, serpentine, catharanthine, hörhammericine, lochnericine, and tabersonine (Hughes et al., 2004). Ajmalicine, serpentine, and catharanthine were detected at 254 nm and were quantified by comparison with authentic standard curves. Tabersonine, hörhammericine, and lochnericine were detected at 329 nm by retention time of standards and were quantified based on a tabersonine standard curve as previously reported (Morgan et al., 2000). For the first 5 min, the mobile phase consisted of a 30:70 mixture of 50% MeOH/50% MeCN: 5 mM $(NH_4)_2HPO_4$ (pH 7.3) at a flow rate of 1 ml/min. Over the next 10 min, the mobile phase was linearly ramped to a 64:36 mixture, where it was maintained for 15 min at a flow rate of 1 ml/min. Over the next 5 min, the flow rate was linearly ramped to 1.4 ml/min. During the next 5 min, the ratio was increased to 80:20 where it was maintained for 15 min at a flow rate of 1.4 ml/min. The ratio was then brought back to 30:70 at 1 ml/min and re-equilibrated.

Statistical Analysis

Data was analyzed using the Student's *t*-test.

RESULTS AND DISCUSSION

Precursor Feeding of ASα

Induction of the *C. roseus* hairy root line EHIASA-1, which expresses ASα under the control of a glucocorticoid inducible promoter system, with 3 μM dexamethasone for 3 days in late exponential growth phase followed the same trend as previously described (Hughes et al., 2004). It has been previously reported that unphosphorylated 1-deoxy-D-xylulose can be efficiently utilized for the biosynthesis of carotenoids and phytols in *C. roseus* cell cultures and used to rescue the albino phenotype of an *Arabidopsis* mutant (Arigoni et al., 1997; Estevez et al., 2000). As expected, feeding a terpenoid precursor decreased the tryptophan and tryptamine pools ($P \leq 0.05$) when ASα was over-expressed (Fig. 2a). It is interesting to note that feeding 1-deoxy-D-xylulose decreased these pools more than feeding with loganin, a compound located further downstream in the terpenoid pathway.

It is difficult to analyze the effects of feeding a terpenoid precursor on the terpenoid indole alkaloids because of limited characterization of these alkaloids and their metabolic pathways. Nevertheless, we evaluated the metabolic effects of ASα overexpression and feeding of a terpenoid precursor on the production of terpenoid indole alkaloids by the hairy roots. The alkaloids analyzed include ajmalicine and serpentine from the coryanthe family, catharanthine from the iboga family, and tabersonine, hörhammericine, and lochnericine from the aspidosperma family. Vinblastine and vincristine have not been observed in *C. roseus* hairy roots or cell suspension cultures because of an absence of vindoline (Shanks et al., 1998). Feeding the precursors 1-deoxy-D-xylulose or loganin has little effect on overall alkaloid levels, regardless of whether AS α was or was not induced (Figs. 2b, and 3). However, some significant differences were observed in the levels of individual alkaloids. Feeding 1-deoxy-D-xylulose significantly ($P \leq 0.05$) increases hörhammericine in the fed cultures compared to their unfed counterparts. Similarly, feeding loganin results in a significant ($P \leq 0.05$) increase in catharanthine.

Precursor Feeding of ASαβ

Induction of the *C. roseus* ASAB-1 hairy root line expressing ASα under the control of a glucocorticoid inducible promoter system and ASβ constitutively (ASαβ) was performed using a lower concentration of dexamethasone, 0.2 μM, as compared to inducing ASα with 3 μM dexamethasone. The higher concentration of dexamethasone was found to cause significant browning of tissue from the ASαβ line but not the ASα line; thus a lower concentration was used to induce the ASαβ line to reduce any non-specific metabolic effects due to

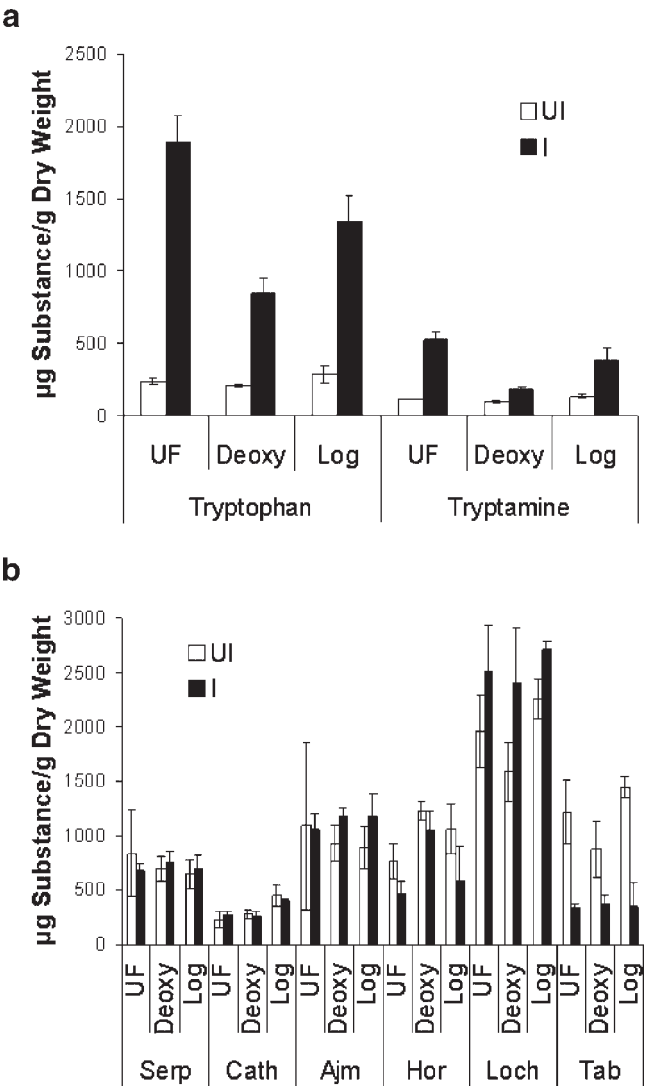

Figure 2. **a**: Tryptophan, tryptamine, (**b**) serpentine (Serp), catharanthine (Cath), ajmalicine (Ajm), hörhammericine (Hor), lochnericine (Loch), and tabersonine (Tab) levels for ASα (EHIASA-1). Cultures were induced (I) by feeding to a final concentration of 3 μM dexamethasone or un-induced (UI) by feeding a comparable amount of ethanol to each culture. At the time of induction, cultures were either unfed (UF) or fed with 208 μM 1-deoxy-D-xylulose (Deoxy) or 104 μM loganin (Log). These cultures were fed in late-exponential growth phase and harvested after 3 days. Compounds were determined by HPLC analysis from crude extracts. Data represent mean of triplicate cultures ± standard deviation.

browning (Hong et al., 2005). The lower, 0.2 μM, concentration of dexamethasone may decrease the ASα expression level by around 20% compared to 3 μM dexamethasone (Hughes et al., 2002). There were no significant effects observed on biomass growth or appearance of the induced lines for ASα or ASαβ.

Induction of ASαβ with 0.2 μM dexamethasone for 3 days in late exponential growth phase showed the same trend that was previously reported (Hong et al., 2005). Feeding with the terpenoid precursors loganin or secologanin has no effect on the pools of tryptophan and tryptamine in the uninduced samples (Fig. 4a). Feeding with loganin increases the pools of both tryptophan ($P \leq 0.05$) and tryptamine ($P \leq 0.11$) when

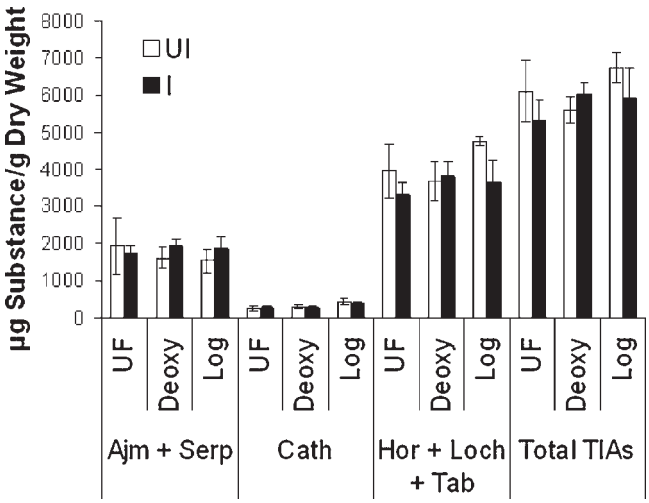

Figure 3. Pools of terpenoid indole alkaloids for ASα (EHIASA-1). Total TIAs represent the total amount of serpentine, catharanthine, ajmalicine, hörhammericine, lochnericine, and tabersonine within the cultures. Cultures were induced (I) by feeding to a final concentration of 3 µM dexamethasone or un-induced (UI) by feeding a comparable amount of ethanol to each culture. At the time of induction, cultures were either unfed (UF), or fed with 208 µM 1-deoxy-D-xylulose (Deoxy) or 104 µM loganin (Log). These cultures were fed in late-exponential growth phase and harvested after 3 days. Compounds were determined by HPLC analysis from crude extracts. Data represent mean of triplicate cultures ± standard deviation.

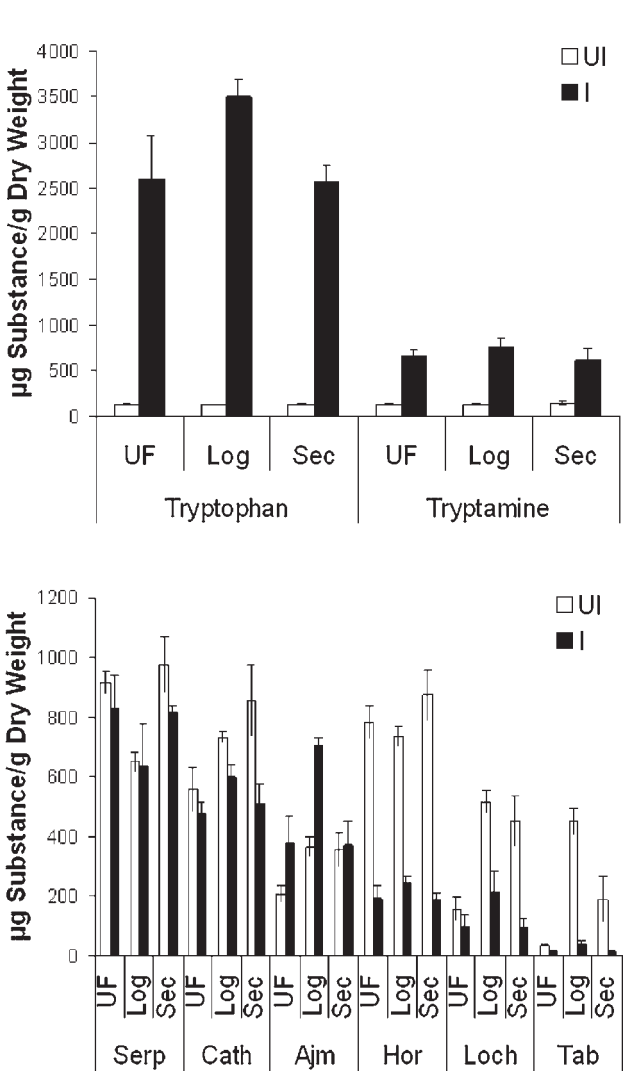

Figure 4. **a**: Tryptophan, tryptamine, (**b**) serpentine (Serp), catharanthine (Cath), ajmalicine (Ajm), hörhammericine (Hor), lochnericine (Loch), and tabersonine (Tab) levels for ASαβ (ASAB-1). Cultures were induced (I) by feeding to a final concentration of 0.2 µM dexamethasone or un-induced (UI) by feeding a comparable amount of ethanol to each culture. At the time of induction, cultures were either unfed (UF) or fed with 104 µM loganin (Log) or 104 µM secologanin (Sec). These cultures were fed in late-exponential growth phase and harvested after 3 days. Compounds were determined by HPLC analysis from crude extracts. Data represent mean of triplicate cultures ± standard deviation.

the induced samples are compared. This is counterintuitive because one would expect to see a decrease in tryptophan and tryptamine levels when the cultures are fed with loganin rather than an increase in these levels when compared to the unfed cultures since there should be a greater availability of the terpenoid precursor secologanin to combine with tryptamine.

Precursor feeding with loganin or secologanin led to many significant ($P \leq 0.05$) effects on the terpenoid indole alkaloids when compared to the unfed cultures (Fig. 4b). When comparing loganin fed samples, a significant ($P \leq 0.05$) increase in catharanthine, ajmalicine, lochnericine, and tabersonine was observed compared to their unfed counterpart. Loganin feeding also significantly ($P \leq 0.05$) decreases serpentine in the uninduced samples. Secologanin feeding significantly ($P \leq 0.05$) increases catharanthine, ajmalicine, lochnericine, and tabersonine in the uninduced samples but has no significant effects when ASαβ is over-expressed. The combined effects of feeding loganin and induction of ASαβ resulted in significant ($P \leq 0.05$) increases in ajmalicine and decreases in serpentine and hörhammericine when compared to unfed and un-induced samples of ASαβ. The combined effects of feeding secologanin and induction of ASαβ resulted in significant ($P \leq 0.05$) increases in ajmalicine and decreases in tabersonine and lochnericine when compared to unfed and uninduced samples of ASαβ. These effects are made even more evident by examining the combined levels of the alkaloids in each family (Fig. 5). Feeding either loganin or secologanin alone leads to significant increases in terpenoid indole alkaloids whereas the over-expression of ASαβ in general decreases the terpenoid indole alkaloid pools. When ASαβ is over-expressed, loganin significantly ($P \leq 0.05$) increases the iboga and aspidosperma family of terpenoid indole alkaloids. The lessening effects of secologanin may be caused by poor uptake of this compound to the vacuole within the cell (van der Heijden et al., 2004). Proper compartmentalization is essential since the reactions leading to the terpenoid indole alkaloids happen within many different compartments within the cell (Meijer et al., 1993).

Comparison of the Two Hairy Root Lines

When comparing the results from the loganin feeding of both the ASα and ASαβ hairy root lines after a 3-day induction in

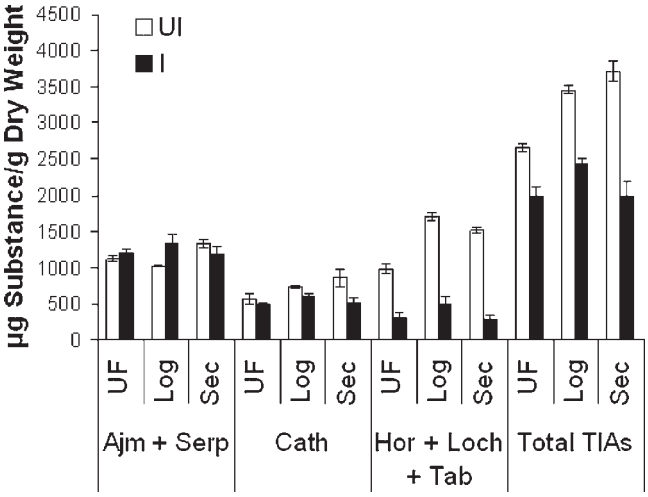

Figure 5. Pools of terpenoid indole alkaloids for ASαβ (ASAB-1). Total TIAs represent the total amount of serpentine, catharanthine, ajmalicine, hörhammericine, lochnericine, and tabersonine within the cultures. Cultures were induced (I) by feeding to a final concentration of 0.2 µM dexamethasone or un-induced (UI) by feeding a comparable amount of ethanol to each culture. At the time of induction, cultures were either unfed (UF) or fed with 104 µM loganin (Log) or 104 µM secologanin (Sec). These cultures were fed in late-exponential growth phase and harvested after 3 days. Compounds were determined by HPLC analysis from crude extracts. Data represent mean of triplicate cultures ± standard deviation.

late exponential growth phase one notices a number of trends. First, the basal levels of indole and terpenoid indole alkaloid accumulation between the lines vary significantly for the uninduced and unfed samples (Figs. 2 and 5). Second, the tryptophan and tryptamine pools are larger in ASαβ over-expressing hairy roots even though the dexamethasone induction level is lower than that of the ASα over-expressing line. Third, loganin feeding decreases tryptophan and tryptamine pools for ASα but increases these pools for ASαβ. Finally, the ASαβ over-expressing hairy roots exhibit more significant effects after loganin feeding than the ASα over-expressing hairy root line.

The differences in these two lines could be caused by a number of factors. It was previously reported that when the α subunit of AS was over-expressed it may combine with the naturally available β subunit of AS to form a functional enzyme and cause an increase in the tryptophan and tryptamine pools (Hughes et al., 2004). Thus, the availability of the β subunit of AS may be a limiting factor. If this is the case, then constitutively expressing the β subunit of AS could cause a further increase in tryptophan and tryptamine pools and may lead to the observed increase in terpenoid indole alkaloid levels upon loganin feeding. Clonal variation between these two lines may also explain the observed differences between the two lines. Since the *C. roseus* genome has not been sequenced, it is difficult to determine where the genes have inserted into the genome and thus difficult to determine if any genes were disrupted in the process of inserting the transgenes.

Terpenoid Pathway Limits Flux to the TIAs

The precursor feeding results presented in this study suggest that the terpenoid pathway limits the flux to terpenoid indole alkaloids in genetically transformed *C. roseus* hairy roots. These results are consistent with previous precursor feeding studies in genetically transformed *C. roseus* cell suspension cultures (Whitmer et al., 1998, 2002a,b). This study also suggests that enzymes upstream of 1-deoxy-D-xylulose-5-phosphate or loganin may be potential bottlenecks. Two possible bottlenecks enzymes are 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and geraniol-10-hydroxylase (G10H). DXS catalyzes the condensation of pyruvate and glyceraldehyde-3-phospate to produce 1-deoxy-D-xylulose-5-phosphate (Fig. 1). This is the first step in the non-mevalonate pathway that leads to the formation of IPP, which feeds into the monoterpenoid pathway. DXS overexpression in *Arabidopsis* and *E. coli* was shown to increase various terpenoids (Estevez et al., 2001; Matthews and Wurtzel, 2000). Feeding *C. roseus* hairy roots with deoxyxylulose increased the accumulation of two indole alkaloids, lochnericine, and tabersonine (Hong et al., 2003). G10H converts geraniol to 10-hydroxygeraniol (Fig. 1). In *C. roseus* cell suspension cultures, G10H is considered to be an important limiting enzyme in the production of TIAs (Collu et al., 2002, 2001; Dagnino et al., 1995; Schiel et al., 1987). Several factors, such as the rate of uptake of the compound and the ability of the compound to reach the correct compartment within the cell, may obscure the interpretation of precursor feeding results. Precise genetic modifications can be used to provide additional evidence of potential bottlenecks within the terpenoid pathway. This study also suggests that metabolic engineering efforts should focus on a combined effort to increase the flux through both the terpenoid and indole pathways.

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