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The expression of 1-deoxy-D-xylulose synthase and geraniol-10-hydroxylase or anthranilate synthase increases terpenoid indole alkaloid accumulation in *Catharanthus roseus* hairy roots

Christie A.M. Peebles [a,1], Guy W. Sander [b,2], Erik H. Hughes [c,3], Ryan Peacock [a], Jacqueline V. Shanks [b], Ka-Yiu San [a,c,*]

[a] Department of Bioengineering, Rice University, Houston, TX, United States
[b] Department of Chemical and Biological Engineering, Iowa State University, IA, United States
[c] Department of Chemical and Biomolecular Engineering, Rice University, Houston, TX, United States

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ABSTRACT

The terpenoid indole alkaloid (TIA) pathway in *Catharanthus roseus* produces two important anticancer drugs, vinblastine and vincristine, in very low yields. This study focuses on overexpressing several key genes in the upper part of the TIA pathway in order to increase flux toward downstream metabolites within hairy root cultures. Specifically, we constructed hairy root lines with inducible overexpression of 1-deoxy-D-xylulose synthase (DXS) or geraniol-10-hydroxylase (G10H). We also constructed hairy root lines with inducible expression of DXS and anthranilate synthase α subunit (ASA) or DXS and G10H. DXS overexpression resulted in a significant increase in ajmalicine by 67%, serpentine by 26% and lochnericine by 49% and a significant decrease in tabersonine by 66% and hörhammericine by 54%. Co-overexpression of DXS and G10H caused a significant increase in ajmalicine by 16%, lochnericine by 31% and tabersonine by 13%. Likewise, DXS and ASA overexpression displayed a significant increase in hörhammericine by 30%, lochnericine by 27% and tabersonine by 34%. These results point to the need for overexpressing multiple genes within the pathway to increase the flux toward vinblastine and vincristine.

1. Introduction

Since their discovery in the late 1950s (Pearce and Miller, 2005), vinblastine and vincristine have been used as powerful anticancer drugs for a variety of cancer treatments. These compounds are classified as terpenoid indole alkaloids and were discovered during a screening for novel anti-diabetic drugs in *Catharanthus roseus* (van der Heijden et al., 2004). Vinblastine and vincristine are produced in small quantities within the leaves of the plant. Approximately 500 kg of dried leaves are needed to produce 1 g of vinblastine (Noble, 1990). For commercial production, a semi-synthetic route has been used to couple vindoline and catharanthine; both compounds are present in higher concentrations within the leaves (van der Heijden et al., 2004). The importance of these anticancer drugs has lead researchers to study the biosynthesis and regulation of the terpenoid indole alkaloid pathway and to explore ways to engineer the increased production of these metabolites.

The terpenoid indole alkaloids (TIAs) are formed by the condensation of the indole moiety tryptamine and the monoterpenoid secologanin by strictosidine synthase (STR) to form strictosidine, the precursor to a wide variety of TIAs (Fig. 1). Throughout the TIA pathway there are a number of key enzymes that regulate the flux through the pathway. Anthranilate synthase (AS) catalyzes the first committed step in the synthesis of tryptophan and is subjected to feedback inhibition by tryptophan (Li and Last, 1996). AS 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 the amino subunit. The inhibition by tryptophan can be overcome by expressing a feedback-insensitive ASα. This technique has been successfully used to increase tryptophan content in rice, soybean, and *C. roseus* (Dubouzet et al., 2007; Hong et al., 2006b, 2004a; Inaba et al., 2007). In *C. roseus* hairy roots,

Abbreviations: 16-OMT, 16-hydroxytabersonine-16-O-methyltransferase; AS, anthranilate synthase; CPR, cytochrome P450 reductase; D4H, desacetoxyvindoline-4-hydroxylase; DAT, deacetylvindoline acetyltransferase; DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-d-xylulose-5-phosphate; DXS, 1-deoxy-d-xylulose-5-phosphate synthase; E4P, erthrose-4-phosphate; G10H, geraniol-10-hydroxylase; G3P, glyceraldehyde-3-phosphate; IPP, isopentenyl diphosphate; PEP, phosphoenolpyruvate; PRX, class III peroxidase; SGD, strictosidine β-d-glucosidase; SLS, secologanin synthase; STR, strictosidine synthase; T16H, tabersonine-16-hydroxylase; TDC, tryptophan decarboxylase; TIA, terpenoid indole alkaloid

* Corresponding author at: Department of Bioengineering, Rice University, Houston, TX, United States. Fax: +1 713 348 5877.
E-mail address: ksan@rice.edu (K.-Y. San).
[1] Current address: Department of Chemical and Biological Engineering, Colorado State University, Fort Collins, CO, United States.
[2] Current address:Department of Chemical Engineering, University of Minnesota Duluth, Duluth, MN, United States.
[3] Current address: Biogen Idec, Research Triangle Park, NC, United States.

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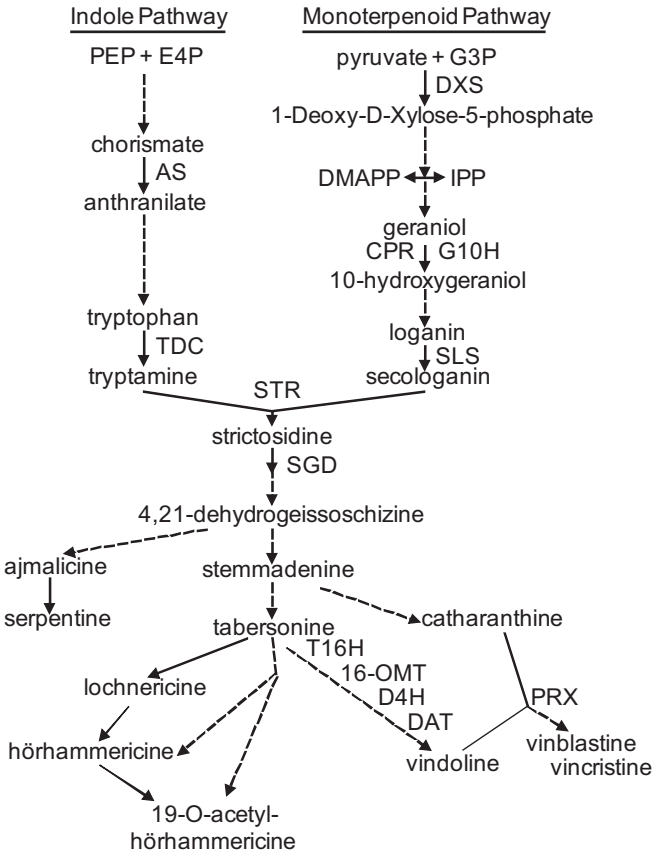

Fig. 1. Terpenoid indole alkaloid pathway. **PEP**, phosphoenolpyruvate; **E4P**, erthrose-4-phosphate; **AS**, anthranilate synthase; **TDC**, tryptophan decarboxylase; **G3P**, glyceraldehyde-3-phosphate; **DXS**, 1-deoxy-D-xylulose-5-phosphate synthase; **IPP**, isopentenyl diphosphate; **DMAPP**, dimethylallyl diphosphate; **G10H**, geraniol-10-hydroxylase; **CPR**, cytochrome P450 reductase; **SLS**, secologanin synthase; **STR**, strictosidine synthase; **SGD**, strictosidine β-D-glucosidase; **T16H**, tabersonine-16-hydroxylase; **16-OMT**, 16-hydroxytabersonine-16-O-methyltransferase; **D4H**, desacetoxyvindoline 4-hydroxylase; **DAT**, deacetylvindoline acetyltransferase; **PRX,** Peroxidase.

the overexpression of a feedback-insensitive ASα caused huge increases in tryptophan and tryptamine concentrations (> 300 fold and 10 fold, respectively), but only led to the modest increase (2 fold) of one TIA, lochnericine (Hughes et al., 2004a). The conversion of tryptophan to tryptamine is another branch point in the pathway and a potential control step. When tryptophan decarboxylase (TDC) was overexpressed in *C. roseus* hairy roots, tryptamine did not increase but serpentine showed a significant increase (Hughes et al., 2004b). The overexpression of both ASα and TDC in *C. roseus* hairy roots caused a 6-fold increase in tryptamine levels while the TIAs levels did not increase (Hughes et al., 2004b).

1-deoxy-D-xylulose 5-phosphate synthase (DXS) catalyzes the first step in the methyl-erythritol phosphate (MEP) pathway and is hypothesized to be an important control step within the MEP pathway. The following examples highlight the role of the DXS pathway in plants. In *Arabidopsis* the overexpression of DXS led to an increase in terpenoid concentrations while the repression of DXS decreased terpenoid concentrations (Estevez et al., 2001). DXS expression pattern followed the accumulation pattern of carotenoids during fruit development in tomato (Lois et al., 2000). Deoxyxylulose feeding caused an increase of 30–60% in the concentration of two indole alkaloids, lochnericine and tabersonine, in *C. roseus* hairy roots (Hong et al., 2003). In *C. roseus* cell suspension cultures, geraniol-10-hydroxylase (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). In native roots high levels of G10H activity have been reported (Meijer et al., 1993) highlighting a potential difference between root and cell culture. DXS and G10H mRNA levels are upregulated by a number of signaling molecules including jasmonic acid, ethylene, and cytokinin (Collu et al., 2001; Papon et al., 2005; van der Fits and Memelink, 2000).

The use of feeding studies can help us to determine potential limiting steps within a pathway. For the TIA pathway, there are conflicting reports as to whether the terpenoid or indole pathway is the limiting pathway in the production of TIAs. In cell suspension cultures, it has been shown that tryptophan feeding (indole pathway) has resulted in increased serpentine levels in one study, in reduced alkaloid levels in another, and in no effect in another (Kargi and Ganapathi, 1991; Knobloch and Berlin, 1980; Zenk et al., 1977). Cell cultures that overexpressed TDC exhibited an increase in tryptophan accumulation but no change in TIA accumulation (Canel et al., 1998). Loganin or secologanin feeding (terpenoid pathway) increases TIAs in cell suspension cultures (Mérillon et al., 1989; Moreno et al., 1993) and increases TIAs in cells overexpressing STR or TDC (Whitmer et al., 2002a, 2002b). The timing of precursor feeding may affect which pathway is limiting in hairy roots. In the exponential growth phase, tryptophan feeding significantly increased the production of TIAs but the feeding of geraniol, 10-hydroxygeraniol, and loganin had no significant effect. In early stationary growth phase, feeding geraniol, 10-hydroxygeraniol, or loganin increased the amount of tabersonine that accumulated, while the addition of tryptophan had no effect (Morgan and Shanks, 2000). These reports suggest that both pathways may be limiting—when the limitation of one pathway is overcome, likely the other pathway becomes limiting.

This paper explores the effect of overexpressing several important TIA pathway genes alone and in concert with other genes in hairy root cultures of *C. roseus*. Hairy roots were chosen due to the fact that they produce significantly higher concentrations of TIAs than cell suspension cultures (Montiel et al., 2007). Specifically, the single effects of overexpressing DXS, G10H, and previously published ASA (Hughes et al., 2004a) are investigated. The mixed results of single gene overexpression lead us to explore the effect of overexpressing two genes simultaneously. The effect of co-expressing DXS and G10H or DXS and ASA resulted in an increase in several TIA metabolites. These results suggest that expression of multiple key enzymes maybe necessary to overcome the native regulation and increase TIA metabolite accumulation.

2. Materials and methods

2.1. Plasmid construction

The *Arabidopsis* DXS gene, ClaI, was provided as a cDNA clone in pBluescript by Dr. Patricia Leon. G10H was amplified from cDNA prepared from RNA purified from *C. roseus* hairy roots using polyT primers and M-MLV reverse transcriptase according to manufacturer's instructions (Promega). The primers used for G10H amplification were 5′-ACTTCCATTCCATGGATTACCTTACCA-3′ and 5′-TTAAAGGGTGCTT GGTACAGCAC-3′. The sequence from the resulting gene matched the published sequence for G10H. G10H was moved from pCR2.1-TOPO (Invitrogen) into pBluescript as an EcoRV/SpeI fragment. G10H and DXS were then removed as an XhoI/SpeI fragment and ligated into the glucocorticoid-inducible plasmid pTA7002 to form p7002DXS and p7002G10H. The construction of p7002ASA was previously described (Hughes et al., 2004a). The XhoI/SpeI DXS gene fragment was also ligated into the XhoI/SpeI site of pUCGALA (Hughes et al., 2004b) to form pUCGALADXS. The Sse8387I (SdaI) restriction site was used to move DXS along with the GAL4-UAS promoter from pUCGALADXS into the Sse8387I (SdaI) restriction site of p7002ASA and p7002G10H to form p7002ASA/DXS and p7002G10H/DXS. These plasmids carry 2 genes under the control of a glucocorticoid-inducible promoter.

2.2. Generation of hairy root lines

The plasmids p7002DXS, p7002G10H and p7002ASA/DXS were electroporated into *Agrobacterium rhizogenes* 15834. Transformed colonies from YEM plates (1 g/L yeast extract, 0.2 g/L NaCl, 0.2 g/L $MgSO_4 \cdot 7H_20$, 4 mg/L $FeCl_3$, 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. The plasmid p7002G10H/DXS was electroporated into *Agrobacterium tumefaciens* strain GV3101 carrying the plasmid pPZPROL containing the rolABC genes (Hong et al., 2006a). Transformed colonies were screened on LB plates including spectinomycin (100 mg/L) and kanamycin (50 mg/L). Restriction digest was used to analyze the presence of the p7002G10H/DXS. A single colony was then used to start a 5 mL culture grown at 28 °C and 200 rpm for 36 h in liquid media. 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.

2.3. Hairy root culture conditions

Hairy roots were grown in a filter-sterilized solution of 30 g/L sucrose (Sigma), half strength Gamborg's B5 salts (Sigma), and full strength Gamborg's vitamins (Sigma) adjusted to a pH of 5.7. Cultures were initiated by placing five tips into a 250 mL Erlenmeyer flask with 50 mL of media. The cultures were grown at 26 °C at 100 rpm in the dark. Solid media plates were made from 30 g/L sucrose, half-strength Gamborg's B5 salts, full strength Gamborg's vitamins (pH 5.7), and 6 g/L agar (Sigma).

2.4. Genomic PCR analysis

Genomic DNA was extracted from hairy root cultures using the GenElute Plant Genomic DNA Miniprep Kit (Sigma) according to the manufacturer's instructions. One primer specific to the inducible promoter region outside the gene and one primer specific to a region inside the gene were used to confirm the insertion of each gene into the genome. ConfGAL1 is specific to the inducible promoter region: 5′-AGCTTGCATGCCGGTCGACTCTAGAGGATCC-3′. ConfASA is specific to ASα: 5′-TCACAAATGCAGATTCAGCCAAGTCGATGGCTCGAG-3′. ConfDXS is specific to DXS: 5′-TCAAAACAGAGCTTCCCTTGGTGCAC-3′. ConfG10H is specific to G10H: 5′-CACAGTCCTTAGTGCCAATGG-CAACC-3′. The expected PCR band size is ~2.1 kb for ASα, ~1.7 kb for G10H, and ~2.5 kb for DXS. Taq polymerase was used to perform the PCR with the following conditions: 95 °C for 1 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min with a final extension time of 10 min at 72 °C.

2.5. 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) according to 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. 12 μg of total RNA were loaded per well, and northerns were performed 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 QIAEX II kit. Probes were then made using Stratagene Prime-It II kit.

2.6. Induction study and HPLC analysis

Hairy root cultures were treated with 3 μM dexamethasone (induced cultures) or with an equal volume of ethanol (uninduced cultures) as a negative control during late exponential growth phase and harvested 72 h later (Hughes et al., 2004a). The fresh weight of the harvested hairy root cultures were measured after cultures were blotted to remove excess media. The cultures were then frozen at −80 °C. The dry weight was recorded after lyphoilization. Approximately 50 mg dry weight of the ground tissue was extracted in 25 mL of methanol by ultrasonication for 10 min (Model W-225, Heat Systems. Ultrasonics Inc., USA). The extracts were clarified by centrifugation at 1300g for 15 min at 4 °C. The supernatants were concentrated to 2 mL using a vortex evaporator, and passed through a 0.22 μm nylon filter (13 mm). 10 μL of each sample was analyzed for the following metabolites: tryptophan, tryptamine, ajmalicine, serpentine, catharanthine, hörhammericine, lochnericine, and tabersonine on the HPLC as previously described (Peebles et al., 2005).

3. Results

Hairy root cultures were generated by infecting *C. roseus* seedlings with *A. rhizogenes* strain ATCC 15834 carrying the Ri plasmid and p7002DXS, p7002G10H, or p7002ASA/DXS or *A. tumefaciens* strain GV3101 carrying the plasmid pPZPROL containing the rolABC genes (Hong et al., 2006a) and p7002G10H/DXS. The resulting hairy roots were screened on hygromycin selection plates and adapted to liquid media for several generations. pTA7002 contains a glucocorticoid-inducible promoter system which uses the constitutive CaMV35S promoter to drive the expression of a glucocorticoid-inducible transcription factor (GVG). This promoter system has previously been used to induce the expression of several genes within *C. roseus* hairy roots (Hong et al., 2006b; Hughes et al., 2004a, 2004b, 2002). Each gene introduced into *C. roseus* in this study is under control of this glucocorticoid-inducible promoter system. p7002DXS contains a fully characterized *Arabidopsis* DXS gene (Estevez et al., 2001, 2000). p7002G10H contains the *C. roseus* G10H gene (Collu et al., 2001). p7002ASA contains an *Arabidopsis* ASα gene with a point mutation to confer feedback resistance to tryptophan (Hughes et al., 2004a; Li and Last, 1996). p7002ASA/DXS and p7002G10H/DXS contain two genes that are individually controlled by the same glucocorticoid-inducible promoter.

As described earlier, the use of precursor feeding studies to determine limiting steps within the TIA pathway has lead to mixed results suggesting that both the indole and terpenoid pathways could potentially limit the flux toward TIA metabolite production. To explore in greater detail which pathway limits these fluxes and how to overcome these limitations, a series of hairy root lines expressing known or potential rate limiting genes was created. Hairy root line EHIASA-1 was previously shown to have inducible expression of a feedback-insensitive ASA (Hughes et al., 2004a) from the indole pathway. While the increase in feedback-insensitive ASA lead to increased pools of the TIA precursors, tryptophan and tryptamine (Table 1), only one TIA metabolite significantly increased ($p < 0.1$), lochnericine (Fig. 2, Table S1). In fact, two TIA metabolites, tabersonine and hörhammericine, exhibited a significant decrease ($p < 0.05$) in concentrations.

A fast growing hairy root line, EHIDXS-4-1, which showed strong induction of the terpenoid gene DXS, was chosen for further analysis. Northern blot analysis of EHIDXS-4-1 showed the inducible expression of DXS upon treatment with dexamethasone (Fig. S1). Ubiquitin 3 was used as the loading control for both the

Table 1
Tryptophan and tryptamine content of 4 different hairy root lines. Cultures were induced with 3 μM dexamethasone (I) or fed with an equivalent volume of ethanol (UI) during exponential growth phase and harvested 72 h later.

		Tryptophan (μg/g DW)	Tryptamine (μg/g DW)
EHIASA-1[a]	UI	241 ± 20	117 ± 1
	I	1895 ± 174[b]	524 ± 57[b]
EHIDXS-4-1	UI	23 ± 16	14 ± 9
	I	33 ± 15	7 ± 2
G10H/DXS-21	UI	13 ± 6	3 ± 1
	I	6 ± 1	4 ± 1
ASA/DXS-2	UI	174 ± 1	169 ± 5
	I	162 ± 7[b]	219 ± 18[b]

[a] Is taken from Peebles et al. (2006).
[b] Indicates significant results ($p < 0.05$) between the UI and I cultures. Data represent the mean of triplicate cultures ± standard deviation.

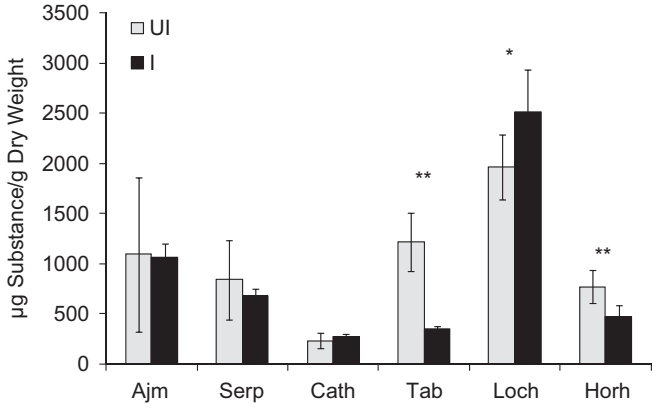

Fig. 2. The terpenoid indole alkaloid profile of EHIASA-1. Cultures were induced with 3 μM dexamethasone (I) or fed with an equivalent volume of ethanol (UI) during exponential growth phase and harvested 72 h later. Data is taken from Peebles et al. (2006). HPLC analysis was used to determine the concentration of serpentine (Serp), ajmalicine (Ajm), catharanthine (Cath), hörhammericine (Horh), lochnericine (Loch), and tabersonine (Tab). "*" indicates significant results ($p < 0.1$) between the UI and I cultures. "**" indicates significant results ($p < 0.05$) between the UI and I cultures. Data represent the mean of triplicate cultures ± standard deviation.

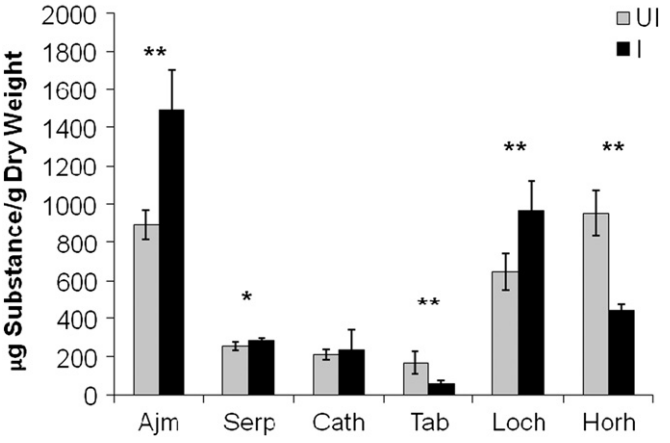

Fig. 3. The terpenoid indole alkaloid profile of EHIDXS-4-1. Cultures were induced with 3 μM dexamethasone (I) or fed with an equivalent volume of ethanol (UI) during exponential growth phase and harvested 72 h later. HPLC analysis was used to determine the concentration of serpentine (Serp), ajmalicine (Ajm), catharanthine (Cath), hörhammericine (Horh), lochnericine (Loch), and tabersonine (Tab). "*" indicates significant results ($p < 0.1$) between the UI and I cultures. "**" indicates significant results ($p < 0.05$) between the UI and I cultures. Data represent the mean of triplicate cultures ± standard deviation.

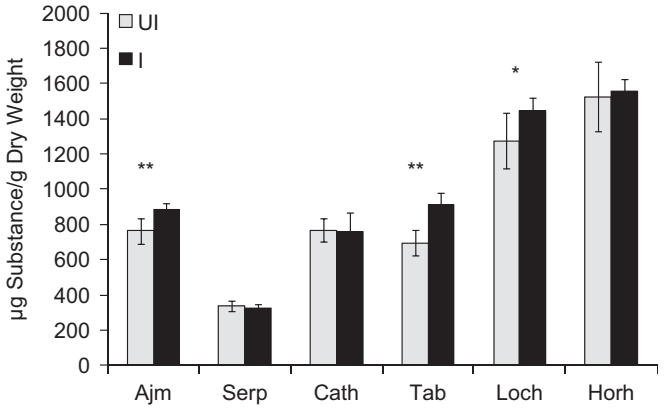

Fig. 4. The terpenoid indole alkaloid profile of G10H/DXS-21. Cultures were induced with 3 μM dexamethasone (I) or fed with an equivalent volume of ethanol (UI) during exponential growth phase and harvested 72 h later. HPLC analysis was used to determine the concentration of serpentine (Serp), ajmalicine (Ajm), catharanthine (Cath), hörhammericine (Horh), lochnericine (Loch), and tabersonine (Tab). "*" indicates significant results ($p < 0.1$) between the UI and I cultures. "**" indicates significant results ($p < 0.05$) between the UI and I cultures. Data represent the mean of triplicate cultures ± standard deviation.

uninduced and induced clones. These results were compared to the results from our previously reported negative control line EHINC-12-1 (Hughes et al., 2004a). The effect of overexpression of DXS on TIA metabolites showed mixed results with a significant increase ($p < 0.05$) in ajmalicine and lochnericine and a significant decrease ($p < 0.05$) in tabersonine and hörhammericine (Fig. 3, Table S1).

Numerous attempts at generating a hairy root line capable of overexpressing the terpenoid gene G10H under the control of a glucocorticoid-inducible promoter failed. While the integration of both GVG and G10H could be demonstrated, an increase in G10H mRNA levels could not be seen upon induction. As a control, an increase in GVG transcripts could be seen upon induction (data not shown). High levels of G10H naturally occur within the roots and young leaves of *C. roseus* plants (Burlat et al., 2004). The native level of G10H expression may be highly regulated and thus an increase in the inducible expression of G10H could be balanced by a decrease in native G10H expression. The regulation mechanism of G10H is relatively unknown although recently 3 sites for binding of unknown transcriptional enhancers were discovered within the promoter region (Suttipanta et al., 2007).

The individual overexpression of the terpenoid genes DXS and G10H resulted in mixed results in regards to the accumulation of TIA metabolite pools. Considering their location with respect to the isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) branch point (Fig. 1), the precursors for the various classes of terpenoid compounds, it is conceivable that the overexpression of one gene alone is unable to overcome the regulation around this branch point while the overexpression of two genes simultaneously may act in concert to push and pull the flux toward the TIA metabolites. The presence of an inducible G10H and DXS was confirmed by genomic PCR in the best performing hairy root line G10H/DXS-21 (Fig. S2). The inducible overexpression of G10H and DXS lead to a significant increase ($p < 0.1$) in the concentrations of ajmalicine, tabersonine, lochnericine, and total TIAs (Fig. 4, Table S1). The overexpression of both G10H and DXS alleviates the negative impact that DXS overexpression exhibited on some of the TIA metabolites.

The large increase in tryptophan and tryptamine pools that occurred upon the overexpression of ASA (Table 1) along with a previously published precursor feeding study which showed that AS overexpression coupled with terpenoid feeding resulted in an increase in TIA metabolites (Peebles et al., 2006) lead us to explore the effects of

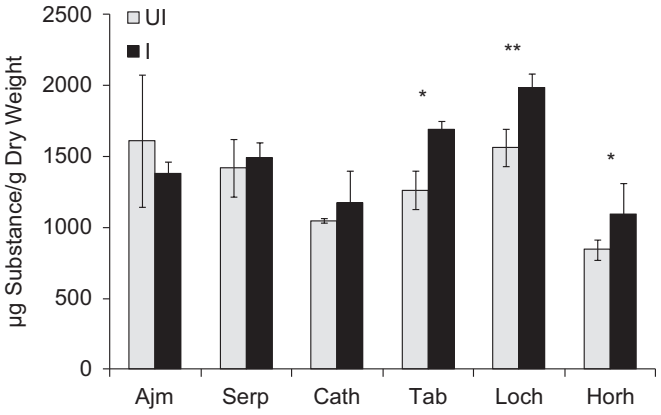

Fig. 5. The terpenoid indole alkaloid profile of ASA/DXS-2. Cultures were induced with 3 µM dexamethasone (I) or fed with an equivalent volume of ethanol (UI) during exponential growth phase and harvested 72 h later. HPLC analysis was used to determine the concentration of serpentine (Serp), ajmalicine (Ajm), catharanthine (Cath), hörhammericine (Horh), lochnericine (Loch), and tabersonine (Tab). "*" indicates significant results ($p < 0.1$) between the UI and I cultures. "**" indicates significant results ($p < 0.05$) between the UI and I cultures. Data represent the mean of triplicate cultures ± standard deviation.

overexpressing the indole gene ASA with the terpenoid gene DXS. The presence of an inducible ASA and DXS was confirmed by genomic PCR (Fig. S3). Metabolite analysis of the resulting lines showed that line ASA/DXS-2 exhibited the largest changes in TIA concentrations. This line was chosen for further study. Upon induction of ASA and DXS overexpression, a significant increase ($p < 0.1$) in tabersonine, lochnericine, and hörhammericine was observed (Fig. 5, Table S1). These results differ from the single overexpression lines which show a decrease in several TIA metabolites. The large accumulation in tryptophan and tryptamine seen when overexpressing ASA disappears when both ASA and DXS are overexpressed (Table 1).

4. Discussion

DXS overexpression in hairy roots showed mixed results in TIA profiles. Three metabolites, ajmalicine, serpentine, and lochnericine, exhibited an increase in concentration while two others, tabersonine and hörhammericine, showed a decrease in concentration upon the overexpression of DXS (Fig. 3). These differences make it hard to generalize the overall effect of DXS on TIAs. Another puzzling result is the difference seen in TIA metabolites between DXS overexpression and deoxyxylulose feeding in hairy roots. When deoxyxylulose was fed to hairy roots during late exponential growth phase, tabersonine significantly ($p < 0.05$) increased by 56% (Hong et al., 2003). This differs from the dramatic decrease seen in tabersonine when DXS is overexpressed in hairy roots (Fig. 3). One possible explanation for the difference is that DXS overexpression may have unintended consequences such that it puts significant stress on central metabolism as it pulls on the pyruvate pools. DXS may also be feedback-inhibited, reducing the flux downstream. A study looking at the role of the MEP pathway in the production of isoprene in *Eucalyptus globules* provides evidence that DXS may be feedback-inhibited by high levels of DMAPP or 1-deoxy-D-xylulose-5-phosphate (Wolfertz et al., 2004).

These results highlight some of the difficulties of engineering plants. In particular the manipulation of single enzymes to increase flux through the pathway can display mixed results. Single gene manipulations tend to target slow kinetic steps within the pathway or to target irreversible reaction steps that may act as key regulation steps within the pathway. The overexpression of single genes may lead to unintended consequences within the system. For example, phytoene synthase was constitutively expressed in tomato to increase lycopene content. Instead of increasing lycopene, lower levels were observed along with dwarf plants. Upon closer examination, it was discovered that the increase in phytoene synthase caused a decrease in the flux through the gibberellin pathway, a competing pathway and an important plant hormone (Fray et al., 1995). Thus the increase in phytoene synthase disrupted the hormone balance of the transgenic tomato plant, causing unintended consequences. In contrast, targeting the expression of phytoene synthase to canola seeds led to a significant increase in carotenoid concentrations (Shewmaker et al., 1999).

Another potential problem with overexpressing single enzymes that may act as key regulation steps is the potential for feedback inhibition. In some cases feedback inhibition can be overcome by engineering the enzyme to be insensitive to the feedback signal. For example, anthranilate synthase (AS) is feedback-inhibited by tryptophan (Li and Last, 1996). When a feedback-insensitive AS was overexpressed in rice, soybean, and *C. roseus*, an increase in the concentration of tryptophan within the plant was observed (Dubouzet et al., 2007; Hong et al., 2006b; Hughes et al., 2004a; Inaba et al., 2007).

While the overexpression of single genes may increase flux through the pathway, the overexpression of multiple genes may be necessary to achieve significant gains in product accumulation. This may especially be true in highly branched pathways in which precursors can be channeled into a variety of metabolites away from the desired product. These pathways may have complicated feedback and feed-forward regulation mechanisms. In the terpenoid pathway, IPP and DMAPP can be converted into a large array of different metabolites. The overexpression of one of the genes upstream of IPP and DMAPP such as DXS may increase flux toward all terpenoid metabolites, resulting in little or no gain in the desired product.

Overexpressing enzymes upstream and downstream of a branch point may act as a push-pull mechanism in which flux is pushed toward the branch point and then pulled toward the desired product. For example, overexpressing DXS can push the flux toward IPP and DMAPP while overexpressing G10H pulls the flux toward the monoterpenoids. *C. roseus* hairy root line G10H/DXS-21, which overexpresses both DXS and G10H, shows a positive gain in TIA metabolites (Fig. 4) compared to the mixed results of overexpressing DXS alone (Fig. 3). This point is further illustrated when we compare the change in specific productivity of each metabolite of the induced culture with the uninduced cultures (Fig. 6).

This push-pull effect may also be working with the hairy root line overexpressing ASα and DXS. This time the pull effect could come from an abundant supply of the indole precursor tryptamine which combines with the terpenoid precursor secologanin. A quicker turnover of secologanin could pull the terpenoid flux toward secologanin. This effect could be responsible for increasing the metabolite concentrations of tabersonine, lochnericine, and hörhammericine (Fig. 5). The double construct of ASα and DXS out performs the hairy root lines overexpressing either single construct where ASα alone only increases lochnericine and DXS displays mixed results (Figs. 2, 3, 5, and 6).

Alternatively the overexpression of transcription factors that positively regulate the TIA pathway could be used to overexpress multiple pathway genes at one time. This approach can be more challenging than it initially seems. ORCA3 is a jasmonate-responsive AP2-domain transcription factors that promote transcription of TIA genes. When ORCA3 was overexpressed in *C. roseus* hairy roots, a decrease in several TIA metabolites was observed. Closer examination of the mRNA transcripts showed that several but not all of the genes within the TIA pathway were indeed up-regulated when ORCA3 was overexpressed. Interestingly, the known negative TIA pathway regulators ZCT1, ZCT2, and ZCT3 mRNA transcripts were also up-regulated (Peebles et al., 2009). These results indicate that the TIA pathway is tightly regulated adding a new layer of

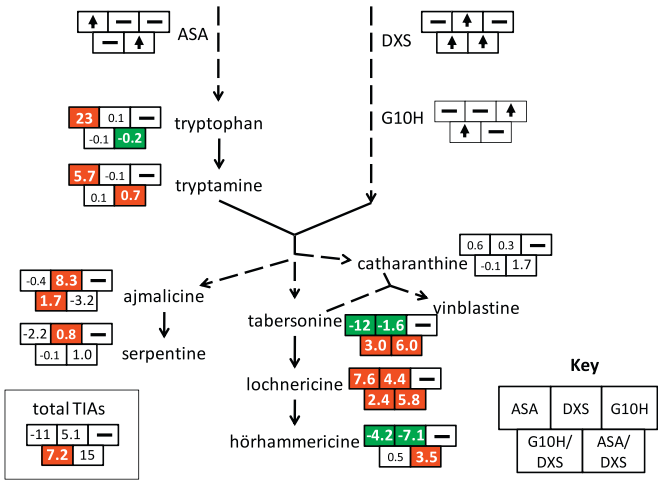

Fig. 6. Change in specific productivity of the induced culture compared to the uninduced culture. The top row of boxes represent lines that overexpress single genes while the bottom row represents lines that overexpress 2 genes. In Row 1, Box 1 is for ASA overexpression, Box 2 is for DXS overexpression, and Box 3 is for G10H overexpression. In Row 2, Box 1 is for G10H and DXS overexpression and Box 2 is for ASA and DXS overexpression. To illustrate this point, there are arrows in the corresponding boxes for each gene that is overexpressed. For the metabolites, the numbers represent the change in specific productivity between the uninduced and the induced cultures overexpressing the appropriate gene(s) [μg/g DW/h]. Red represent a significant increase ($p < 0.1$) in the specific productivity of the induced culture compared to the uninduced culture. Green represent a significant decrease ($p < 0.1$) in the specific productivity of the induced culture compared to the uninduced culture. The specific productivity box for G10H overexpression was left blank since we were unable to confirm the overexpression of G10H in our hairy root lines (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

complexity to engineer the increased production of TIA metabolites. One way to bypass this regulation is to express the pathway in a heterologous host (Leonard et al., 2006; Liu et al., 2007; Petrie et al., 2010). Unfortunately at the present time, this approach is not an option due to incomplete knowledge of all of the genes involved in the pathway. Thus a better understanding of the regulation of the TIA pathway is necessary to successfully engineer the increased production of TIAs. It is also likely that a combination of pathway genes and regulators will need to be manipulated to balance flux through the TIA pathway and increase TIA metabolite production.

5. Conclusion

This paper demonstrates some of the potential problems associated with the overexpression of single genes within an organism such as *C. roseus*. Simultaneously overexpression of several multiple enzymes within the TIA pathway are needed to see significant increases in the metabolites of interest. In addition, optimization of the expression of each enzyme in the pathway may be necessary in order to maximize metabolite production.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2010.11.005.

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