Assessing the Limitations to Terpenoid Indole Alkaloid Biosynthesis in *Catharanthus roseus* Hairy Root Cultures Through Gene Expression Profiling and Precursor Feeding

Sheba Goklany
Dept. of Chemical Engineering, Northeastern University, 360 Huntington Avenue, Boston, MA 02115

Ralph H. Loring
Dept. of Pharmaceutical Sciences, Northeastern University, 360 Huntington Avenue, Boston, MA 02115

James Glick
Dept. of Chemistry and Chemical Biology, Northeastern University, 360 Huntington Avenue, Boston, MA 02115

Carolyn W. T. Lee-Parsons
Dept. of Chemical Engineering, Northeastern University, 360 Huntington Avenue, Boston, MA 02115

DOI 10.1002/btpr.204
Published online August 31, 2009 in Wiley InterScience (www.interscience.wiley.com).

The production of pharmaceutically important terpenoid indole alkaloids (TIAs) from Catharanthus roseus *is partly regulated at the transcriptional level. In this study, limitations in TIA biosynthesis from* C. roseus *hairy root cultures were assessed through gene expression profiling and precursor feeding. The transcript levels of key TIA pathway genes (G10h, Tdc, Str, and Sgd) and metabolite levels associated with the TIA pathway (tryptamine, loganin, secologanin, strictosidine, ajmalicine, serpentine, and tabersonine) were monitored using quantitative RT-PCR and HPLC, respectively. In cultures elicited with methyl jasmonate (250 μM MeJA on day 21), G10h, Tdc, Str, and Sgd expression increased by 9.1, 3.1, 6.7, and 8.3-fold, respectively, after 24 h. Up-regulation of gene expression was followed by a 160, 440, and 420% increase in strictosidine, ajmalicine, and tabersonine levels, respectively, after 5 days. Precursors loganin, tryptamine, or their combination were fed to noninduced and MeJA-induced cultures to complement the above studies. TIA production was not significantly enhanced in either noninduced or MeJA-induced cultures with precursor feeding. In noninduced cells, steps downstream of loganin and tryptamine were limiting (SLS, STR, or SGD) because either loganin or tryptamine accumulated in the cells with precursor feeding. These bottlenecks were partly overcome in MeJA-induced cultures as the expression of* Str *and* Sgd *genes and TIA production increased. However, secologanin accumulated in MeJA-induced cultures with precursor feeding, suggesting that STR was likely limiting under MeJA-induced conditions.* © 2009 American Institute of Chemical Engineers *Biotechnol. Prog.,* 25: 1289–1296, 2009
Keywords: Catharanthus roseus; *hairy root cultures, gene expression, methyl jasmonate, quantitative RT-PCR, alkaloid, precursor feeding*

Introduction

The *Catharanthus roseus* plant produces over 130 different terpenoid indole alkaloids (TIAs).[1] Important TIAs from this plant include vincristine and vinblastine (anti-cancer), ajmalicine (anti-hypertensive), and serpentine (sedative). Critical early steps in the biosynthesis of TIAs include the reactions catalyzed by tryptophan decarboxylase (TDC), geraniol 10-hydroxylase (G10H), and strictosidine synthase (STR). TDC and G10H catalyze the first committed steps towards TIA biosynthesis in the indole and terpenoid precursor branches, respectively (Figure 1). STR then condenses tryptamine and secologanin to yield strictosidine, the common backbone structure of the TIAs. Because yields from whole plants are low and subject to weather conditions, cell and organ cultures of *C. roseus* are being pursued as a means of producing valuable TIAs. In this article, we are investigating TIA production from *C. roseus* hairy root cultures, a differentiated and genetically stable culture system.[6,7]

One strategy for enhancing alkaloid production from plant cell cultures is by using elicitors; these are compounds or mixtures that induce plant stress responses leading to enhanced gene expression and alkaloid biosynthesis.[2,8,9] Common elicitors include methyl jasmonate (MeJA, a plant stress hormone) and fungal homogenates.[2,10–12] In *C. roseus*, elicitors appear to regulate alkaloid production at the

Additional Supporting Information may be found in the online version of this article.

Correspondence concerning this article should be addressed to C. W. T. Lee-Parsons at clee@coe.neu.edu.

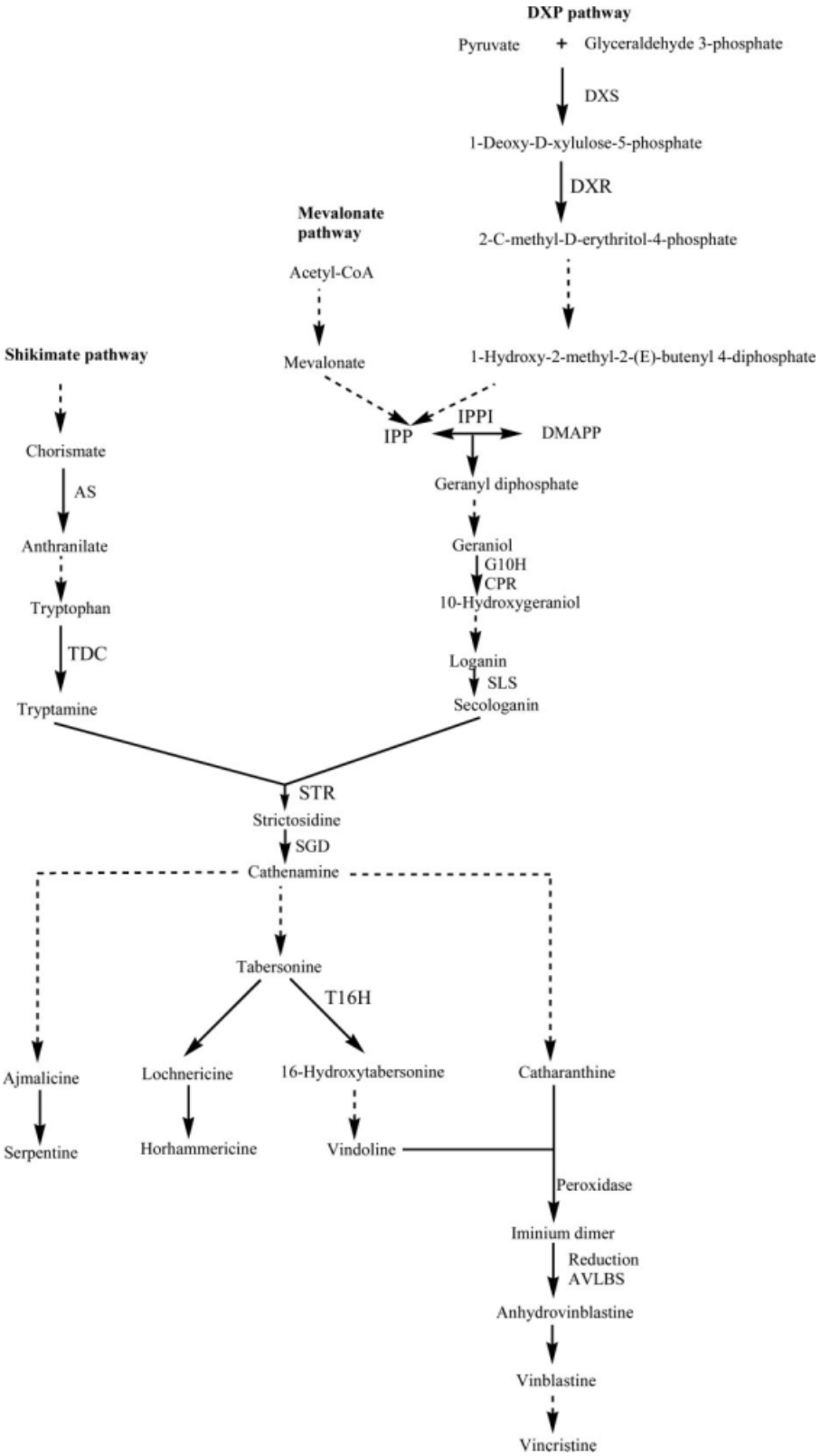

Figure 1. The biosynthesis of terpenoid indole alkaloids from the primary metabolic pathways (shikimate, mevalonate, and DXP pathways).[2–5]

The precursors tryptophan and geraniol are derived from the shikimate, mevalonate, and MEP pathways. Abbreviations of the enzymes are: DXS, 1-Deoxy-D-xylulose 5-phosphate synthase; DXR, 1-Deoxy-D-xylulose 5-phosphate reductoisomerase; IPPI, Isopentenyl diphosphate isomerase; G10H, Geraniol 10-hydroxylase; CPR, Cytochrome P450 reductase; SLS, Secologanin synthase; AS, Anthranilate synthase; TDC, Tryptophan decarboxylase; STR, Strictosidine synthase; SGD, Strictosidine β-D-glucosidase; T16H, Tabersonine 16-hydroxylase. Single arrows represent single steps and dotted arrows represent multiple or unidentified steps.

transcriptional level, that is, through inducing the expression of genes that code for enzymes involved in TIA biosynthesis.[2,10,13] Van der Fits and Memelink (2000) showed that jasmonates activate the transcriptional regulator protein ORCA (octadecanoid-responsive *Catharanthus* AP2-domain protein) in *C. roseus* cell cultures. ORCA then induces the

expression of multiple genes by binding to their promoter regions, which ultimately increases TIA production.[2,9] For instance, the addition of MeJA to *C. roseus* cell suspension cultures up-regulated upstream (*G10h*, *Cpr*, *10Hgo*, *Tdc*, *Str*, and *Sgd*) and downstream TIA pathway genes (*T16h*, *Dat*, and *D4h*), as observed using Northern Blots.[2,13,14] Differences in the gene expression response to MeJA appear to depend on cell line and conditions of elicitation.

In this article, limitations in TIA biosynthesis in *C. roseus* hairy root cultures were investigated through profiling gene expression and alkaloid production and through precursor feeding. In the literature, precursor feeding is applied to assess enzymatic bottlenecks to secondary metabolite production.[15–19] Transcript levels of TIA pathway genes, including the critical early genes (i.e., *G10h*, *Tdc*, *Str*, and *Sgd*), were monitored through quantitative RT-PCR. Metabolite levels of early TIA precursors (tryptamine, loganin, and secologanin) and TIAs (strictosidine, ajmalicine, serpentine, and tabersonine) were also monitored. Integrating the results from gene expression, alkaloid profiling, and precursor feeding implicated potential limitations in the TIA biosynthetic pathway, which were partly overcome following MeJA addition.

Materials and Methods

Maintenance of C. roseus *hairy root cultures*

C. roseus hairy root cultures were obtained from Dr. Jacqueline V. Shanks (Iowa State University, Ames, IA) and Dr. Ka-Yiu San (Rice University, Houston, TX). These hairy roots were subcultured every 4 weeks in 250 mL Erlenmeyer flasks as described by Morgan and Shanks (2000)[16]; 50 mL of media was inoculated with five 3–4 cm long root tips (~0.04 g). These cultures were placed in a dark incubator-shaker (Forma Scientific, Marietta, OH) maintained at 26°C and agitated at 100 rpm. Growth media consisted of sucrose (30 g/L, Sigma-Aldrich, St. Louis, MO), half-strength Gamborg's B5 Basal Salt Mixture (1.55 g/L, Sigma-Aldrich), and full-strength Gamborg's Vitamin Solution (1 mL/L, Sigma-Aldrich). Sterile water was added to the cultures every week to compensate for evaporation loss.

Elicitation with methyl jasmonate (MeJA)

C. roseus hairy root cultures were elicited with 250 μM MeJA (assay $\geq$95+%, SAFC, St. Louis, MO) on day 21 (late-exponential growth phase). Previously, maximum alkaloid production was obtained under these elicitation conditions.[20] Stock solutions of MeJA (460 mM) were prepared in ethanol (200 proof, ACS/USP grade, Pharmco-AAPER, Brookfield, CT), and 27.2 μL of the stock solution was added to 50 mL of the media to achieve a final concentration of 250 μM.

Cultures were harvested 0 and 24 h following elicitation for RNA extraction because transcript levels reached maximum and remained constant after 8 h or increased gradually between 8 and 24 h.[20] Cultures were harvested 5 days following elicitation for alkaloid analysis, that is when maximum alkaloid levels were observed.[20] The effect of MeJA on gene expression and alkaloid production was reproduced at least thrice. All statistical significance tests on gene expression and alkaloid production were performed using the Student's *t*-test in Excel.

Precursor feeding studies in MeJA-elicited and non-induced cultures

Precursor feeding studies have been used to determine the limiting branch in TIA biosynthesis in *C. roseus* cultures.[15–19] Tryptamine, loganin, or a combination of both precursors was added to MeJA-elicited and noninduced cultures to assess the limiting branch of the alkaloid biosynthetic pathway in *C. roseus* hairy root cultures. For the precursor feeding experiment, 125 mL Erlenmeyer flasks were inoculated with three 3–4 cm long root tips in 25 mL of media. These cultures were elicited with 250 μM MeJA on day 21 (late-exponential growth phase) and the precursors loganin, tryptamine, or their combination were added to MeJA-elicited and noninduced cultures on day 22.

Stock solution of tryptamine (250 mM, Acros Organics, Morris Plains, NJ) was prepared in ethanol and 20 μL of the stock solution was added to 25 mL of the media to achieve a final concentration of 0.2 mM.[18] Stock solution of loganin (100 mM, Extrasynthese, France) was prepared in water and 100 μL was added to 25 mL media to obtain a final concentration of 0.4 mM.[18] Similar tryptamine and loganin concentrations were used in precursor feeding studies performed with other *C. roseus* cultures.[15,16,19]

Monitoring gene expression using qRT-PCR

Hairy root cultures were flash-frozen in liquid nitrogen upon harvesting, ground in liquid nitrogen using a mortar and pestle, and extracted for RNA using the methods described by Chang et al.[21] RNA was quantified using a NanoDrop spectrophotometer (ThermoScientific, Wilmington, DE) and its integrity was verified by running a formaldehyde gel and visualized using a UV transilluminator (FOTO/UV®15, Fotodyne Incorporated, Hartland, WI). Extracted RNA (200 ng) was treated with DNase to remove genomic DNA using a kit available from Invitrogen (Deoxyribonuclease I Amplification Grade, Carlsbad, CA). cDNA was synthesized from the DNase-treated RNA (8 μL) using a kit available from Invitrogen (SuperScript First-Strand Synthesis System for RT-PCR).

The genes monitored in this study were *G10h*, *Tdc*, *Str*, and *Sgd*. Primer sequences for *G10h*, *Tdc*, and *Sgd* were designed using the database available from the National Center for Biotechnology Information (www.pubmed.com) and Integrated DNA Technologies (www.idtdna.com). Primer sequences for *Str* were obtained from Dutta et al (2005).[22] The forward and reverse primers for *G10h*, *Tdc*, *Str*, *Sgd*, and *Rps9* (the housekeeping gene[2,14]) are listed in Table 1. Preparation for quantitative RT-PCR was performed using a kit available from Stratagene (Brilliant SYBR Green QPCR Master Mix, La Jolla, CA). The upstream and downstream primer concentrations were optimized for each gene monitored (Table 2, Supporting Information).

qRT-PCR was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The initial thermocycler protocol set-up consisted of a heating cycle at 50°C for 2 min and then 95°C for 10 min. The thermocycler was then set for 40 cycles; each cycle consisted of (1) 30 s at 95°C, (2) 45 s at 60°C, and (3) 60 s at 72°C. After 40 cycles, the extension step at 72°C was repeated for another 3 min followed by a final cooling step at 4°C for 2 min. The size of the RT-PCR products was verified by running the products on an agarose gel (Table 1).

Table 1. Sequences for Forward and Reverse Primers of the TIA Biosynthetic Pathway Genes Monitored (*G10h*, *Tdc*, *Str*, and *Sgd*)

Gene	Primer Sequence
G10h	
Forward primer	TAGCAGGGACGGACACAACATCAA
Reverse primer	TCACGTCCAATTGCCCAAGCATTC
Product size (bp)	304*
Tdc	
Forward primer	ACCTACGACCGTCGAAACGGATTT
Reverse primer	AAACTCGGGACATATACAGGCGCT
Product size (bp)	232*
Str	
Forward primer†	CCTTCCTATGCTCCGAATGC
Reverse primer†	CCATCGTGCTCTTGAATCTG
Product size (bp)	836†
Sgd	
Forward primer	ATGAGAGCTCTTGTAGGAAGCCGT
Reverse primer	GCGCACTTCCTTCCCATCAACTTT
Product size (bp)	210*
Rps9	
Forward primer	TCCACCATGCCAGAGTGCTCATTA
Reverse primer	TCCATCACCACCAGATGCCTTCTT
Product size (bp)	203*

Rps9 was used as the housekeeping gene. Products were analyzed by running the RT-PCR products against a DNA ladder on a 1% agarose gel and verified by sequencing the recovered DNA.

*Product sizes were obtained from Integrated DNA Technologies (www.idtdna.com) and confirmed using agarose gel electrophoresis and DNA sequencing. †Primer sequences for *Str* were obtained from Dutta et al (2005).[22] The product size was verified using agarose gel electrophoresis and DNA sequencing.

The RT-PCR product was extracted from the gel using a QIAquick Gel Extraction kit (QIAgen, Valencia, CA) and was sequenced to confirm the formation of the correct product at the Molecular Genetics Core facility (Children's Hospital, Boston, MA).

The amplification efficiency for *G10h*, *Tdc*, *Str*, *Sgd*, and *Rps9* was calculated using Ct values for different cDNA dilutions and was ~100% for each gene monitored.[20] *Rps9* was validated as an appropriate housekeeping gene for the MeJA-induced *C. roseus* hairy root cultures by verifying that the Ct profile for *Rps9* remained nearly constant over time in cultures elicited with MeJA.

Extraction and analysis of alkaloids from hairy root cultures using HPLC and MS

Frozen hairy root cultures were freeze-dried using a Flexi-Dry MP Freeze-Dryer (Kinetics Thermal Systems, Stone Ridge, NY) and then ground to a very fine powder using a mortar and pestle. Dried crushed root powder (0.05 g) was extracted using 5 mL of methanol twice[3,23]; the pooled extract was evaporated to dryness, redissolved in 1 mL of methanol, and passed through 0.45 μm filters (Millipore Millex Nonsterile Syringe Filters, Millipore, MA) before HPLC analysis.

Alkaloids were analyzed using a HPLC system consisting of Waters 2695 Separations Module, a Waters 996 Photodiode Array Detector, and Millennium 32 Software (Waters Corp., Milford, MA). Separation of TIAs (strictosidine, ajmalicine, serpentine, and tabersonine) was performed using a reversed phase C18 column (Luna, 150 × 4.60 mm ID column, particle size of 5 μm, Phenomenex, Torrance, CA); separation of terpenes (secologanin) was performed using a LiChrospher® 100 RP-18 column (250 × 4.00 mm ID column, particle size of 5 μm, Agilent Technologies, Santa Clara, CA). The sample injection volume was 10 μL.

The HPLC mobile phase for TIA separation consisted of water with 0.1% formic acid as the aqueous phase and acetonitrile with 0.1% formic acid as the organic phase. This HPLC protocol consisted of the following steps: (1) 10% organic as the initial condition, (2) gradient to 27% organic over 40 min, (3) gradient to 100% organic over 20 min, (4) 100% organic for 20 min to wash the column, (5) gradient to 10% organic over 10 min, (6) maintain at 10% organic for 19 min to equilibrate the column for the next injection (adapted from Lee-Parsons and Ertürk, 2005[24]). All the flow rates were maintained at 1.0 mL/min.

The HPLC mobile phase for secologanin separation consisted of an aqueous mixture containing 1% formic acid (89.88%), acetonitrile (8.98%), and a 20% w/v trichloroacetic acid solution (1.14%) and acetonitrile with 0.1% formic acid as the organic phase. This HPLC protocol consisted of the following steps: (1) 100% aqueous mixture as the initial condition, (2) isocratic at 100% aqueous over 40 min at 1.2 mL/min, (3) gradient to 100% organic over 20 min at 1.0 mL/min, (4) maintain at 100% organic for 20 min at 1.0 mL/min to wash the column, (5) gradient to 100% aqueous over 10 min at 1.2 mL/min, (6) maintain at 100% aqueous for 19 min at 1.2 mL/min to equilibrate the column for the next injection (adapted from Dagnino et al, 1996[23]).

Strictosidine (a gift from Dr. Sarah O' Connor, Massachusetts Institute of Technology, Cambridge, MA) was monitored at 274 nm; secologanin (Sigma-Aldrich), ajmalicine (TCI America, Portland, OR), and serpentine (The Sigma-Aldrich Library of Rare Chemicals, Milwaukee, WI) were monitored at 254 nm; yohimbine (Tocris Bioscience, Ellisville, MO), catharanthine, and tabersonine (both were gifts from Dr. Martin Kuehne, University of Vermont, Burlington, VT) were monitored at 329 nm. The retention times for yohimbine, strictosidine, ajmalicine, catharanthine, serpentine, tabersonine, and secologanin were ~15.3, 19.5, 22.8, 24.2, 25.5, 26.7, and 34.0 min, respectively. Peaks were collected at the above respective wavelengths (i.e., 254, 274, and 329 nm) and identified by comparing retention time, UV absorbance spectra, and MS analysis with authentic standards.

MS analysis was performed using a Thermo Finnigan LCQ Classic ion trap (San Jose, CA). MSn analysis was performed on all standards in positive ESI mode using a custom built nanospray interface as previously described.[25] Stock solutions of strictosidine, ajmalicine, serpentine, and tabersonine (1 mg/mL in methanol) were diluted to 10 μg/mL in 70:30:0.1% methanol:water:formic acid and infused at 10 μL/min. The flow was split using the nanospray interface to a final flow rate of 300 nL/min and the emitter tip was a metal distal coated tip (360 μm OD × 75 μm ID and 10 μm tip, New Objective, Woburn, MA). The MS parameters were as follows: ESI voltage = +2.0 kV, capillary temperature = 190°C, capillary voltage = 37.8 V, and tube lens offset = 0.0 V. For all MSn fragmentation experiments, the mass isolation window was ±1.0 Da and the relative collision energy was set to achieve ~75% fragmentation of the selected ion. The relative collision energy values typically ranged from 25–40%.

The presence of strictosidine, ajmalicine, serpentine, and tabersonine in the *C. roseus* extracts was confirmed by comparing the MS^3 spectra of the collected peaks with those of the standards. The presence of secologanin in the *C. roseus* extracts was confirmed by comparing the MS/MS spectra of the collected peak with that of the standard. No yohimbine and catharanthine was produced by these hairy roots. The

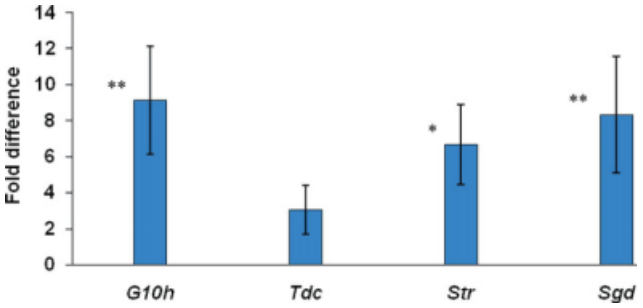

Figure 2. Increased transcript levels of *G10h*, *Tdc*, *Str*, and *Sgd* in *C. roseus* hairy root cultures 24 h following MeJA elicitation.

Cultures were elicited with 250 μM MeJA on day 21 and harvested 24 h after treatment. Transcript levels of the target genes were normalized relative to *Rps9* as the housekeeping gene. Fold differences are based on averages of two biological replicates and error bars denote standard deviations. Significance for the treated cultures was calculated relative to the fold difference for the untreated cultures. All significance tests were performed using the Student's *t*-test in Excel (*denotes $0.05 < P < 0.1$.; **denotes $P < 0.05$).

MS/MS data for confirmation of the metabolites is available in the Supporting Information section.

Results and Discussion

Monitoring TIA gene expression and TIA production in MeJA-elicited C. roseus *hairy root cultures*

TIA gene expression and TIA production were monitored in *C. roseus* hairy root cultures treated with MeJA at the optimum conditions of 250 μM on day 21 (late-exponential growth phase).[20] Rijhwani and Shanks (1998) also observed maximum specific yields of ajmalicine, serpentine, lochnericine, and horhammericine with 237 μM jasmonic acid added on day 21 (also late-exponential growth phase).[26] However, so far, the effect of jasmonates on the expression of TIA biosynthetic pathway genes in *C. roseus* hairy root cultures has not been investigated.

Transcript levels of *G10h*, *Tdc*, *Str*, and *Sgd* were monitored 24 h after elicitation and are shown in Figure 2. When compared with noninduced cultures at 0 h, *G10h*, *Tdc*, *Str*, and *Sgd* were up-regulated by 9.1, 3.1, 6.7, and 8.3-fold, respectively. Similarly, MeJA enhanced the expression of these TIA pathway genes *G10h*, *Tdc*, *Cpr*, *Str*, and *Sgd* in *C. roseus* cell suspension cultures.[2,14] Hence, MeJA increased TIA gene expression in hairy root cultures similarly to that observed in cell suspensions.

The increased expression of TIA genes with MeJA was followed by enhanced TIA production as shown in Figure 3. For instance, 5 days after elicitation, strictosidine, ajmalicine, and tabersonine levels in MeJA-elicited cultures were 16.2 $\pm$ 2.2 mg/L, 3.4 $\pm$ 0.59 mg/L, and 29.3 $\pm$ 0.10 mg/L, respectively, representing a 160, 440, and 420% increase when compared with controls. No increase in serpentine levels was observed in the MeJA-induced cultures, possibly because the enzyme catalyzing the reaction between ajmalicine and serpentine is activated by light[27] and these hairy root cultures were grown in the dark. In Loyola-Vargas et al (1992), serpentine accumulation increased by ~1,000% when *C. roseus* callus cultures were transferred to white light from the dark.[28]

Increased alkaloid production following jasmonate elicitation has been linked to enhanced transcript levels, suggesting

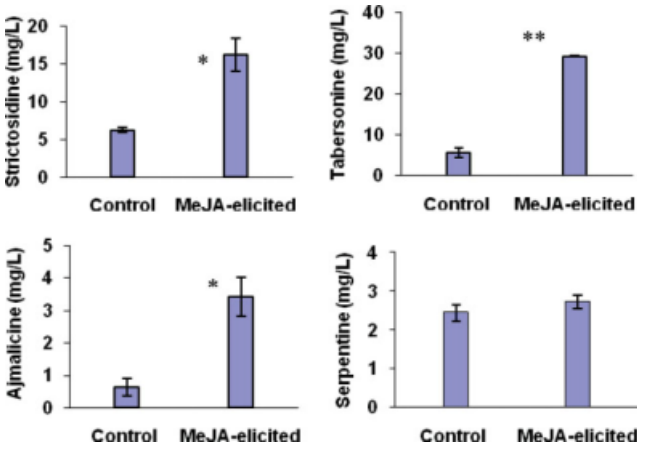

Figure 3. Strictosidine, ajmalicine, serpentine, and tabersonine production in *C. roseus* hairy root cultures treated with MeJA.

Cultures were elicited with 250 μM MeJA on day 21 and harvested on day 26. Alkaloid levels are based on averages of two biological replicates and the error bars represent standard deviations. Strictosidine, ajmalicine, and tabersonine levels were significantly higher in the MeJA-elicited cultures when compared with noninduced cultures. All significance tests were performed using the Student's *t*-test in Excel (*denotes $0.05 < P < 0.1$.; **denotes $P < 0.05$).

that TIA production is partly regulated at the gene expression level.[2,10,13] For example, Rischer et al (2006) observed an enhanced expression of TIA pathway genes (*G10h*, *10Hgo*, *Tdc*, *Sgd*, and *T16h* but not *Cpr*, *Sls*, *Mat*, *D4h*, and *Dat*) with an increased production of TIAs (ajmalicine, tabersonine, and catharanthine) after MeJA addition.[13] Similarly, van der Fits and Memelink (2000) observed an enhanced expression of the TIA pathway genes (*G10h*, *Cpr*, *Tdc*, *Str*, *Sgd*, *D4h*, and *Dat*) with a ~3.2-fold increase in TIA levels (strictosidine, ajmalicine, and a lochnericine-like TIA) with MeJA elicitation when compared with untreated cultures.[2] In addition, Memelink's group identified transcriptional regulators known as ORCAs whose expression is induced or whose existing proteins are activated in the presence of jasmonates.[2,9] The overexpression of ORCA3 coordinately induced genes associated with the indole (*Asα* and *Tdc*), DXP (*Dxs*), and TIA biosynthetic pathways (*Cpr*, *Str*, *Sgd*, and *D4h*).[2]

Determining precursor limitations in C. roseus *hairy root cultures*

In the previous section, addition of MeJA to the hairy root cultures altered the expression of TIA pathway genes and TIA production. In the following study, precursors loganin, tryptamine, or their combinations were fed to noninduced and MeJA-induced cultures to identify enzymatic limitations in TIA biosynthesis. As in the previous section, *C. roseus* hairy root cultures were elicited with 250 μM MeJA on day 21; precursors loganin (0.4 mM) and tryptamine (0.2 mM) were added to noninduced and MeJA-induced hairy root cultures on day 22 with cultures harvested on day 26.

Addition of loganin, tryptamine, or their combination did not significantly increase strictosidine, ajmalicine, serpentine, and tabersonine production in noninduced cultures (Figure 4), indicating that the supply of indole and terpenoid precursors was not likely limiting in these cultures. In fact, loganin or tryptamine accumulated in these cultures with individual

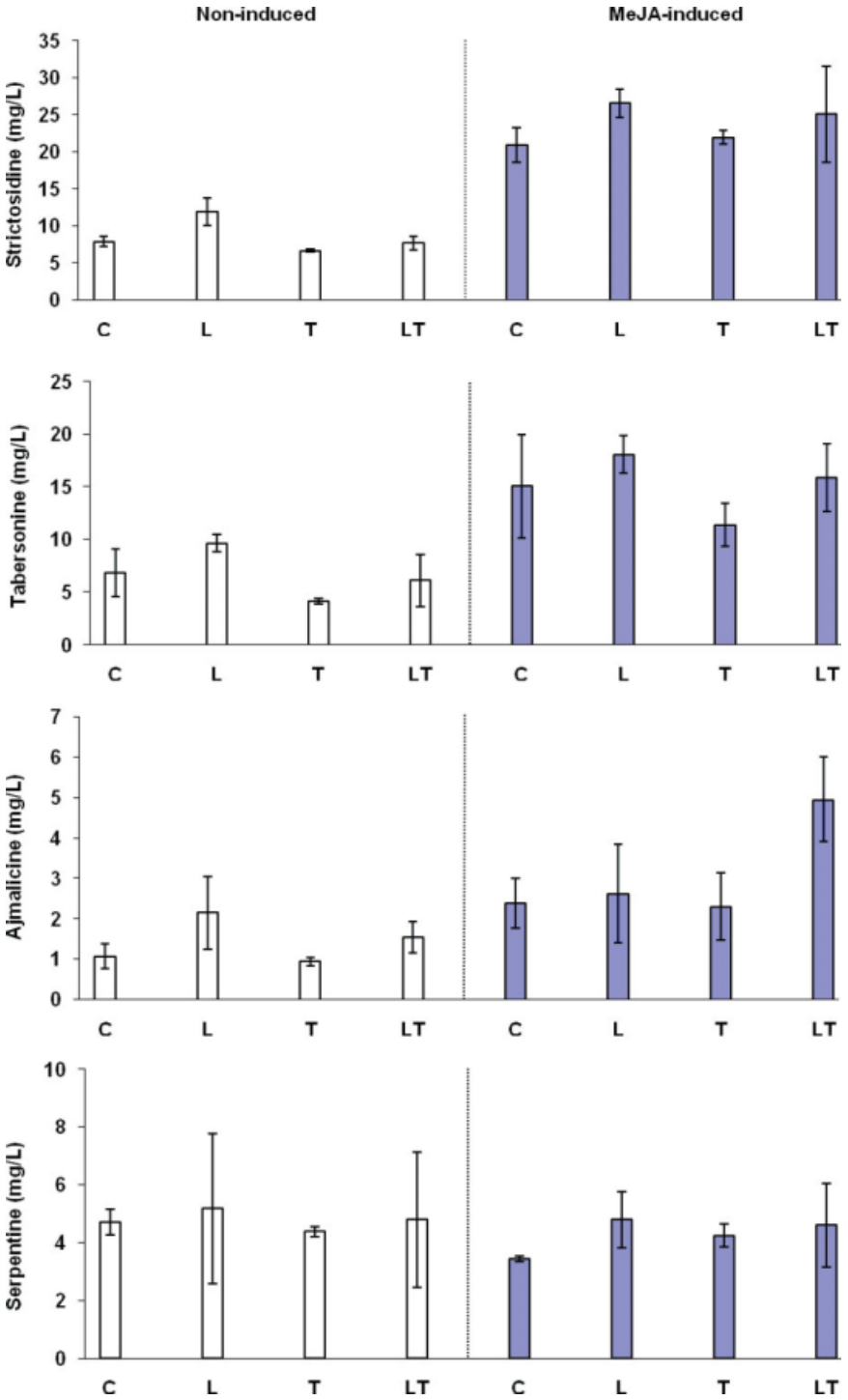

Figure 4. Strictosidine, ajmalicine, serpentine, and tabersonine production in *C. roseus* hairy root cultures fed with loganin (L, 0.4 mM), tryptamine (T, 0.2 mM), or their combinations (LT).

Hairy root cultures were elicited with 250 μM MeJA on day 21, fed with precursors the following day, and harvested on day 26. Alkaloid levels are based on averages of two biological replicates and error bars denote standard deviations. Statistical significance for the MeJA-elicited or noninduced precursor-fed cultures was calculated relative to the nonfed cultures using the Student's *t*-test in Excel. The *C. roseus* hairy root cultures in this experiment was a sub-cell line derived from the original cultures investigated in Figure 3 and was pursued for these studies due to the unexpected loss of the original cell line during the course of the study.

feeding, respectively (as observed from the HPLC chromatograms), suggesting that enzymatic steps downstream of these precursors were limiting (i.e., SLS, STR, or SGD), resulting in their accumulation.

In MeJA-induced cultures, loganin or tryptamine feeding also did not significantly enhance strictosidine, ajmalicine, serpentine, and tabersonine production (Figure 4). Only the addition of both loganin and tryptamine enhanced ajmalicine levels by 110% when compared with MeJA-induced nonfed cultures. Unlike noninduced cultures, tryptamine did not accumulate in the MeJA-induced cultures, even with the tryptamine feeding. Loganin also did not accumulate in the MeJA-induced cultures, except for one replicate fed with loganin; hence, SLS was not likely limiting.

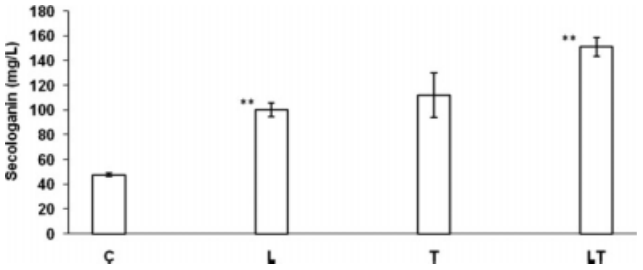

Figure 5. Secologanin accumulation in MeJA-elicited *C. roseus* hairy root cultures fed with loganin (L, 0.4 mM), tryptamine (T, 0.2 mM), or their combinations (LT).

Hairy root cultures were elicited with 250 μM MeJA on day 21, fed with precursors the following day, and harvested on day 26. If the fed loganin were completed converted by SLS, the secologanin levels would be expected to reach 155 mg/L. Secologanin levels are based on averages of two biological replicates and error bars denote standard deviations. Significance for the precursor-fed cultures was calculated relative to the nonfed cultures using the Student's *t*-test in Excel (*denotes $0.05 < P < 0.1$.; **denotes $P < 0.05$).

Secologanin levels were then measured in MeJA-elicited cultures to assess if the addition of loganin or loganin combined with tryptamine resulted in secologanin accumulation (Figure 5). If the fed loganin (0.4 mM) were completed converted by SLS, the secologanin level would have reached 155 mg/L. Secologanin levels in MeJA-elicited cultures fed with loganin, tryptamine, or their combination were 100.1, 112.0, and 151.1 mg/L, representing a 110, 135, and 220% increase in levels when compared with nonfed MeJA-elicited cultures. The accumulation of secologanin in MeJA-elicited cultures with precursor feeding suggests that STR may be limiting in the MeJA-elicited cultures. However, the accumulation of secologanin (from the terpenoid precursor branch) with tryptamine addition (from the indole precursor branch) was unexpected.

In noninduced cells, steps downstream of loganin and tryptamine were limiting (i.e., SLS, STR, or SGD) because both loganin and tryptamine accumulated in cells with the individual precursor feeding. But these bottlenecks were partly overcome in MeJA-induced cultures as the expression of *Str* and *Sgd* genes increased, TIA production increased, and loganin or tryptamine did not accumulate. The enhanced accumulation of secologanin in MeJA-elicited cultures with precursor feeding when compared with nonfed cultures suggests that STR was limiting in the MeJA-elicited cultures.

In Lee-Parsons and Royce (2005), the indole branch was also not limiting in noninduced and MeJA-induced *C. roseus* suspension cultures. However, the addition of loganin or a combined feeding enhanced alkaloid levels (i.e., ajmalicine), indicating steps downstream of loganin and tryptamine were not likely limiting.[15] In Morgan and Shanks (2000), the terpenoid branch was limiting in *C. roseus* hairy root cultures during the late-exponential growth phase (day 21).[16] However, in jasmonic acid-induced cultures, the addition of precursors loganin and tryptamine did not increase alkaloid levels above jasmonic acid-induced cultures alone,[16] similar to this present study.

In conclusion, the addition of MeJA to these hairy root cultures enhanced *G10h*, *Tdc*, *Str*, and *Sgd* levels which was followed by an increase in alkaloid production in these cultures. The combined gene expression, alkaloid profile, and precursor feeding studies suggest that TIA biosynthesis was likely limited (1) by the expression of SLS, STR, or SGD in noninduced cultures which were partly overcome following MeJA addition and (2) by STR in MeJA-elicited cultures.

Acknowledgments

This work was supported by the National Science Foundation (NSF-CAREER, Grant No. BES-0134511). We would like to thank Dr. Jacqueline V. Shanks (Iowa State University, Ames, IA), Dr. Ka-Yiu San (Rice University, Houston, TX), and Christie Peebles for providing the *C. roseus* hairy root cultures and for help on culturing methods. We are grateful to Dr. Martin Kuehne (University of Vermont, Burlington, VT) for providing the tabersonine and catharanthine standards and to Dr. Sarah O'Connor (Massachusetts Institute of Technology, Cambridge, MA) for providing the strictosidine standard and help with the MS analysis.

Literature Cited

1. van der Heijden R, Jacobs DI, Snoeijer W, Hallard D, Verpoorte R. The *Catharanthus* alkaloids: pharmacognosy and biotechnology. *Curr Med Chem*. 2004;11:607–628.
2. van der Fits L, Memelink J. ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. *Science*. 2000;289:295–297.
3. Hong SB, Hughes EH, Shanks JV, San KY, Gibson SI. Role of the non-mevalonate pathway in indole alkaloid production by *Catharanthus roseus* hairy roots. *Biotechnol Prog*. 2003;19: 1105–1108.
4. El-Sayed M, Verpoorte R. *Catharanthus* terpenoid indole alkaloids: biosynthesis and regulation. *Phytochem Rev*. 2007;6:277–305.
5. Loyola-Vargas VM, Galaz-Avalos RM, Ku-Cauich R. *Catharanthus* biosynthetic enzymes: the road ahead. *Phytochem Rev*. 2007;6:307–339.
6. Baíza A, Quiroz-Moreno A, Ruíz J, Loyola-Vargas V. Genetic stability of hairy root cultures of *Datura stramonium*. *Plant Cell Tissue Organ Cult*. 1999;59:9–17.
7. Shanks JV, Morgan J. Plant 'hairy root' culture. *Curr Opin Biotechnol*. 1999;10:151–155.
8. Radman R, Saez T, Bucke C, Keshavarz T. Elicitation of plants and microbial cell systems. *Biotechnol Appl Biochem*. 2003;37: 91–102.
9. Memelink J, Verpoorte R, Kijne JW. ORCAnization of jasmonate-responsive gene expression in alkaloid metabolism. *Trends Plant Sci*. 2001;6:212–219.
10. Vazquez-Flota F, De Luca V, Carrillo-Pech M, Canto-Flick A, de Lourdes Miranda-Ham M. Vindoline biosynthesis is transcriptionally blocked in *Catharanthus roseus* cell suspension cultures. *Mol Biotechnol*. 2002;22:1–8.
11. Menke FL, Parchmann S, Mueller MJ, Kijne JW, Memelink J. Involvement of the octadecanoid pathway and protein phosphorylation in fungal elicitor-induced expression of terpenoid indole alkaloid biosynthetic genes in *Catharanthus roseus*. *Plant Physiol*. 1999;119:1289–1296.
12. Gundlach H, Muller MJ, Kutchan TM, Zenk MH. Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. *Proc Natl Acad Sci USA*. 1992;89:2389–2393.
13. Rischer H, Oresic M, Seppanen-Laakso T, Katajamaa M, Lammertyn F, Ardiles-Diaz W, Van Montagu MC, Inze D, Oksman-Caldentey KM, Goossens A. Gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cells. *Proc Natl Acad Sci USA*. 2006;103:5614–5619.
14. Collu G, Unver N, Peltenburg-Looman AM, van der Heijden R, Verpoorte R, Memelink J. Geraniol 10-hydroxylase, a cytochrome P450 enzyme involved in terpenoid indole alkaloid biosynthesis. *FEBS Lett*. 2001;508:215–220.
15. Lee-Parsons CWT, Royce AJ. Precursor limitations in methyl jasmonate-induced *Catharanthus roseus* cell cultures. *Plant Cell Rep*. 2006;25:607–612.

16. Morgan JA, Shanks JV. Determination of metabolic rate-limitations by precursor feeding in *Catharanthus roseus* hairy root cultures. *J Biotechnol*. 2000;79:137–145.
17. Whitmer S, Canel C, Hallard D, Goncalves C, Verpoorte R. Influence of precursor availability on alkaloid accumulation by transgenic cell line of *Catharanthus roseus*. *Plant Physiol*. 1998;116:853–857.
18. Whitmer S, van der Heijden R, Verpoorte R. Effect of precursor feeding on alkaloid accumulation by a tryptophan decarboxylase over-expressing transgenic cell line T22 of *Catharanthus roseus*. *J Biotechnol*. 2002;96:193–203.
19. Whitmer S, van der Heijden R, Verpoorte R. Effect of precursor feeding on alkaloid accumulation by a strictosidine synthase over-expressing transgenic cell line S1 of *Catharanthus roseus*. *Plant Cell Tissue Organ Cult*. 2002;69:85–93.
20. Goklany S. Monitoring the expression of TIA pathway genes in *Catharanthus roseus* hairy root cultures under optimum elicitation conditions. Master's thesis, Northeastern University, Boston, 2007.
21. Chang S, Puryear J, Cairney J. A simple and efficient method for isolating RNA from pine trees. *Plant Mol Biol Rep*. 1993; 11:113–116.
22. Dutta A, Batra J, Pandey-Rai S, Singh D, Kumar S, Sen J. Expression of terpenoid indole alkaloid biosynthetic pathway genes corresponds to accumulation of related alkaloids in *Catharanthus roseus* (L.) G. Don. *Planta*. 2005;220:376–383.
23. Dagnino D, Schripsema J, Verpoorte R. Analysis of several iridoid and indole precursors of terpenoid indole alkaloids with a single HPLC run. *Planta Med*. 1996;62:278–280.
24. Lee-Parsons CWT, Ertürk S. Ajmalicine production in methyl jasmonate-induced *Catharanthus roseus* cell cultures depends on Ca^{2+} level. *Plant Cell Rep*. 2005;24:677–682.
25. Gangl ET, Annan M, Spooner N, Vouros P. Reduction of signal suppression effects in ESI-MS using a nanosplitting device. *Anal Chem*. 2001;73:5635–5644.
26. Rijhwani SK, Shanks JV. Effect of elicitor dosage and exposure time on biosynthesis of indole alkaloids by *Catharanthus roseus* hairy root cultures. *Biotechnol Prog*. 1998;14:442–449.
27. Zhao J, Zhu W-H, Hu Q. Effects of light and plant growth regulators on the biosynthesis of vindoline and other indole alkaloids in *Catharanthus roseus* callus cultures. *Plant Growth Regul*. 2001;33:43–49.
28. Loyola-Vargas VM, Mendez-Zeel M, Monforte-Gonzalez M, Miranda-Ham MDL. Serpentine accumulation during greening in normal and tumor tissues of *Catharanthus roseus*. *J Plant Physiol*. 1992;140:213–217.

Manuscript received July 9, 2008, and revision received Nov. 26, 2008.