Jasmonate-Dependent Alkaloid Biosynthesis in *Catharanthus roseus* Hairy Root Cultures Is Correlated with the Relative Expression of *Orca* and *Zct* Transcription Factors

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The effects of methyl jasmonate (MJ) dosage on terpenoid indole alkaloid (TIA) biosynthesis in Catharanthus roseus *are correlated with the relative levels of specific MJ-responsive transcription factors. In this study, the expression of transcription factors (*Orca*,* Zct*,* Gbf*,* Myc2*,* At-hook*, and* Wrky1*), TIA pathway genes (*G10h*,* Tdc*,* Str*, and* Sgd*), and TIA metabolites (secologanin, strictosidine, and tabersonine) were investigated in* C. roseus *hairy root cultures elicited with a range of MJ dosages (0–1,000 μM) during mid-exponential growth. The highest production of TIA metabolites occurs at 250 μM MJ, increasing by 150–370% compared with untreated controls. At this MJ dosage, the expression of the transcriptional activators (*Orca*) is dramatically increased (29–40 fold) while the levels of the transcriptional repressors (*Zct*) remain low (2–7 fold). Simultaneously, the expression of genes coding for key enzymes involved in TIA biosynthesis increases by 8–15 fold. In contrast, high MJ dosages (1,000 μM) inhibit the production of TIA metabolites. This dosage is correlated with elevated expression levels of* Zct *(up to 40-fold) relative to* Orca *(13–19-fold) and minimal induction of the TIA biosynthetic genes (0–6 fold). The significant changes in the expression of* Orca *and* Zct *with MJ dosage do not correspond to changes in the expression of the early-response transcription factors (*AT-hook, Myc2*, and* Wrky1*) believed to regulate* Orca *and* Zct*. In summary, these observations suggest that the dependence of alkaloid production on MJ dosage in* C. roseus *may be partly mediated through the relative levels of* Orca *and* Zct *family transcription factors.* © 2013 American Institute of Chemical Engineers *Biotechnol. Prog.*, 29:1367–1376, 2013
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Introduction

The *Catharanthus roseus* plant is the source of 130 different terpenoid indole alkaloids (TIAs),[1] including vincristine and vinblastine (anti-cancer drugs), ajmalicine (anti-hypertensive), and serpentine (sedative). The backbone of these and other TIAs is strictosidine, a condensation product of the monoterpenoid and indole pathway precursors, secologanin and tryptamine (Figure 1). The concentration of TIAs in plant tissue is low. For instance, 15 tons of dried *C. roseus* leaves produce 28 g of vinblastine.[2] Semi-synthesis of these compounds is possible but requires advanced precursors such as vindoline and catharanthine which are themselves obtained from the plant.[3,4] Since the availability of the plant is restricted by geographic location, season, and weather conditions, plant cell and tissue cultures are being investigated as an alternative and steady source of these medicinal compounds. Greater understanding of the regulatory mechanisms leading to TIA biosynthesis would enable genetic engineering of plant cell and tissue cultures for the economical production of these compounds.

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

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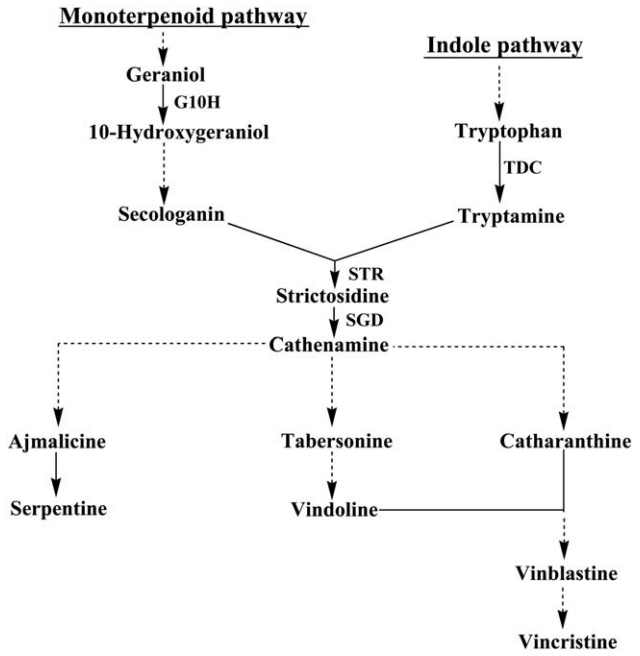

Figure 1. The biosynthesis of TIAs in *Catharanthus roseus*.

G10H, geraniol 10-hydroxylase; TDC, tryptophan decarboxylase; STR, strictosidine synthase; SGD, strictosidine β-d-glucosidase.

The addition of elicitors such as fungal extracts and jasmonates, a class of plant stress hormones, increases secondary metabolite production in whole plants and plant cell and tissue cultures.[5–7] This production-enhancement strategy mimics the plant's natural response to pathogen attack or wounding by activating the octadecanoid pathway, which leads to endogenous jasmonic acid production.[8–11] In *C. roseus*, jasmonates (jasmonic acid, JA, and its methyl ester, methyl jasmonate, MJ) activate the expression of key enzymes in the TIA biosynthetic pathway. We have previously shown that MJ elicits TIA production in a dose-dependent manner.[12] Our current study suggests that this dosage dependency is partly mediated by the relative levels of specific transcription factors (Figure 2), which in turn regulate the expression of key enzymes in the TIA biosynthetic pathway.[13,14]

In the absence of JA, JAZ (Jasmonate ZIM-domain) proteins bind to the MYC2 (basic Helix-Loop-Helix, bHLH) proteins and inhibit their action. However, in the presence of JA (specifically, JA-Isoleucine), JAZ proteins complex with COI1 (CORONATINE-INSENSITIVE1), which leads to rapid degradation of JAZ.[15,16] The newly freed MYC2 proteins then activate the expression of transcription factor genes including *Jaz* and *Orca3* (Octadecanoid-responsive *Catharanthus* AP2). This induction of *Jaz* genes leads to *de novo* synthesis of JAZ proteins and therefore re-repression of MYC2 proteins.[15,16] In addition, MYC2 activates *Orca3* gene expression by binding to the qualitative sequence or "on–off switch" of the *Orca3* promoter. This interaction is necessary for MJ responsiveness.[17] AT-hook proteins also bind specifically to the quantitative region of the *Orca3* promoter.[18] These proteins are thought to determine the level of *Orca3* gene expression.

Increases in ORCA activate the expression of several TIA biosynthetic genes. *Tdc* and *Str* genes are the most well-characterized targets of ORCA.[19,20] In transiently transformed *C. roseus* cells, constitutive expression of *Orca2* or *Orca3* results in direct activation of the *Str* promoter.[19,20] Similarly, overexpression of *Orca3* in stably transformed *C. roseus* cell cultures leads to increased expression of several TIA biosynthetic genes including *Tdc*, *Cpr*, *Str*, *D4h*, but not *G10h* and *Dat*.[20] These studies indicate that ORCA3 is indeed an activator of TIA biosynthetic genes in *C. roseus* as part of the JA-responsive transcriptional cascade.

While ORCA is an activator of TIA biosynthetic genes, the ZCT (Zinc finger *Catharanthus* transcription factor) family proteins act as transcriptional repressors of *Tdc* and *Str*. Transient expression of *Zct1, Zct2*, and *Zct3* in *C. roseus* cell suspensions represses the activity of the *Tdc* and *Str* promoters by at least 2 and 5-fold, respectively.[21] Although the addition of MJ induces both *Orca* and *Zct* genes in *C. roseus* suspension cultures,[19–21] the mechanism by which *Zct* gene expression is regulated remains less understood.

Recently, a new transcription factor, WRKY1, has been discovered that regulates both *Orca* and *Zct* gene expression in *C. roseus*.[22] Overexpression of WRKY1 in stably transformed *C. roseus* hairy root lines increases expression of *Zct1*, *Zct2*, and *Zct3*, and decreases expression of the transcriptional activators *Orca2*, *Orca3*, and *Myc2*. Conversely, overexpression of a dominant-repressive form of WRKY1 (from fusing the SRDX repressor domain to WRKY1) results in increased expression of *Orca3* and *Myc2* and decreased expression of *Zct1*, *Zct2*, and *Zct3*.[22] In addition to regulating *Zct* and *Orca*, WRKY1 also binds directly to the W-box elements of the *Tdc* promoter, increasing its expression.[22]

In this study, we demonstrate that the dose-dependent response of TIA production to MJ is correlated with the levels of ORCA and ZCT transcription factors. We also explore the role of early transcription factors such as MYC2, AT-hook, and WRKY1, in regulating the levels of *Orca* and *Zct*. The integration of these gene expression and TIA profiles suggests a mechanism by which TIA production is controlled at the transcriptional level with jasmonate dosage.

Materials and Methods

Maintenance of C. roseus *hairy root cultures*

C. roseus hairy root cultures were obtained from Prof. Jacqueline Shanks (Iowa State University, Ames, IA) and Prof. Ka-Yiu San (Rice University, Houston, TX). Hairy roots were subcultured every 4 weeks by transferring five 3–4 cm long white and young roots to 50 mL of growth media. Growth media was composed 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) adjusted to a pH of 5.7 (adapted from Rijhwani et al.[23]). These roots were maintained at 26°C and agitated at 100 rpm in the dark.

Elicitation with methyl jasmonate (MJ)

C. roseus hairy root cultures were elicited with 0, 50, 250, or 1,000 μM MJ (assay $\geq$ 95+%, SAFC®, St. Louis, MO) on day 21 (mid-exponential growth phase). Stock solutions of MJ were prepared in ethanol (200 proof, USP grade, Decon Laboratories, Inc., King of Prussia, PA) and aliquots (20–30 μL) were added to 50 mL of media to achieve the desired final concentrations. Following MJ treatment, cultures were harvested at 0, 1, 4, and 8 h for expression

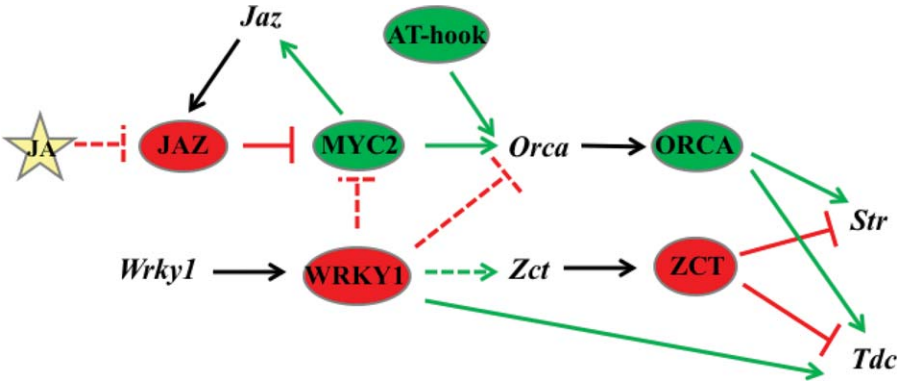

Figure 2. Transcriptional regulation of TIA biosynthesis in *C. roseus*.

JA, jasmonate; JAZ, jasmonate ZIM-domain proteins; MYC2, bHLH proteins; AT-hook, AT-hook DNA-binding motif proteins; WRKY1, WRKY domain proteins; ORCA, octadecanoid-responsive *Catharanthus* AP2 proteins; ZCT, zinc finger *Catharanthus* transcription factor proteins. Red and –| indicate repression, green and arrows indicate activation, and black arrows indicate translation. Dotted lines indicate multiple steps, uncharacterized steps, or indirect interactions.

analysis of early-response transcription factor genes (*Myc2*, *AT hook*, and *Wrky1*) and 0, 12, 24, and 48 h for expression analysis of both TIA pathway genes (*G10h*, *Tdc*, *Str*, and *Sgd*) and downstream transcription factor genes (*Orca*, *Zct*, and *Gbf*). Cultures were also harvested 0, 1, 3, and 5 days following MJ-elicitation for alkaloid analysis. All statistical significance tests for alkaloid production analysis were performed using the Student's *t*-test in Excel.

Extraction and analysis of alkaloids from hairy root cultures using HPLC

Frozen hairy root cultures were freeze-dried using a Flexi-Dry MP Freeze-Dryer (Kinetics Thermal Systems, Stone Ridge, NY), extracted, and analyzed using protocols previously described by Goklany et al.[24] Extracted alkaloids were analyzed using a HPLC system consisting of Waters 2,695 Separations Module, a Waters 996 Photodiode Array Detector, and Millennium 32 Software (Waters Corporation, Milford, MA). Separation of TIAs (strictosidine and tabersonine) was performed using a Luna C18 column (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 45 min, (3) gradient to 100% organic over 15 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 et al.[25]). 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 an organic phase consisting of acetonitrile with 0.1% formic acid. This HPLC protocol consisted of the following steps: (1) 100% aqueous as the initial condition, (2) isocratic at 100% aqueous over 60 min at 1.2 mL/min, (3) gradient to 100% organic over 10 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.[26]).

Strictosidine (a gift from Prof. Sarah O' Connor, John Innes Centre, Norwich, UK), secologanin (Sigma-Aldrich), and tabersonine (a gift from Prof. Martin Kuehne, University of Vermont, Burlington, VT) were monitored at 274, 254, and 329 nm, respectively. The retention times for strictosidine, secologanin, and tabersonine were approximately 22.0, 24.4, and 30.3 min, respectively; the peaks were collected and identified by comparing retention time, UV absorbance spectra, and MS analysis with standards.[24]

Gene expression analysis by qRT-PCR

Hairy root cultures were flash-frozen in liquid nitrogen upon harvesting, and RNA was extracted using the method described by Chang et al.[27] The extracted RNA was quantified using a NanoDrop ND-1,000 Spectrophotometer (ThermoScientific, Wilmington, DE) and treated with DNase to remove genomic DNA (Deoxyribonuclease I Amplification Grade, Invitrogen, Carlsbad, CA). cDNA was synthesized from the RNA (200 ng) using a kit available from Invitrogen (SuperScript First-Strand Synthesis System for RT-PCR) for quantitative reverse transcriptase PCR (qPCR) as described previously.[24]

Primer sequences for *Orca2*, *Orca3*, *Zct1*, *Zct2*, *Zct3*, *Gbf1*, *Gbf2*, *Myc2*, *AT-hook*, and *Wrky1* were designed using the database available from the National Center for Biotechnology Information (www.pubmed.com) and Integrated DNA Technologies (www.idtdna.com) (Table 1). Primer sequences for *G10h*, *Tdc*, *Str*, *Sgd*, and *Rps9* (the housekeeping gene) were described previously.[24] Preparation for qPCR was performed using the RT^2 Real-Time™ SYBR Green/Rox PCR master mix (SABiosciences, Frederick, MD). The forward and reverse primer concentrations were optimized for all transcription factors and genes monitored (Supporting Information Table S1).

qPCR was performed using the ABI Prism 7,700 Sequence Detection System (Applied Biosystems, Foster City, CA)

Table 1. Forward and reverse primer sequences of transcription factors (*Orca2*, *Orca3*, *Zct1*, *Zct2*, *Zct3*, *Gbf1*, *Gbf2*, *Myc2*, *AT-hook*, and *Wrky1*) and their respective product sizes

Transcription Factor	Primer Sequence
Orca2	
Forward primer	GAAATTCGCTGCGGAAATCAGGGA
Reverse primer	AGATGACACGATGAAGATCGGCGT
Product size	213 bp
Orca3	
Forward primer	TGTCAGGAGGATTCTGTTGTGGGA
Reverse primer	CGCATATTAAACGCGGCTGCATCA
Product size	221 bp
Zct1	
Forward primer	AATCTTTAGCGGTGACGAAGCCGA
Reverse primer	CGTTGTCCTCAGGCGTCAAATTCA
Product size	252 bp
Zct2	
Forward primer	TTTCCATCGTTTCAAGCTCTCGGC
Reverse primer	ATTACCGGACGCCGAATCACTCAT
Product size	213 bp
Zct3	
Forward primer	CAGCAACAACAACCCACCGAAGAA
Reverse primer	TTGCCTTATGTCCTCCGAGTGCTT
Product size	220 bp
Gbf1	
Forward primer	TGCACCGGAGCAGAGTAATGTTCA
Reverse primer	CATGAGCATTGGCATGTGAACCCA
Product size	251 bp
Gbf2	
Forward primer	GCTCAGCGAGCTGAAGCATTGAAA
Reverse primer	GATCATTCTGCATCAACGCTGCGA
Product size	237 bp
Myc2	
Forward primer	TCTCCAACAGCGCCTTCAAACTCT
Reverse primer	CCTTGTGGCCCAGCAATCAAAGAA
Product size	246 bp
AThook (2D173)	
Forward primer	ATCTCCCGGTGCGGTTGTTACATT
Reverse primer	AAACGACGCCGCCATAATCACAAC
Product size	199 bp
Wrky1	
Forward primer	GATGGCTTGGCATGATCGGAAGAA
Reverse primer	TCTGACGAATATCGTCGGGACCAA
Product size	203 bp

Product size was obtained from integrated DNA technologies (www.idtdna.com); product size and sequence were confirmed by agarose gel electrophoresis and DNA sequencing.

using the thermocycler protocol as previously described.[24] The RT-PCR product size was verified by running the products on an agarose gel (Table 1); the product was extracted from the gel using a QIAquick Gel Extraction kit (QIAgen, Valencia, CA) and sequenced to confirm the formation of the correct product (GENEWIZ Boston, Cambridge, MA). The amplification efficiency for the transcription factors monitored was calculated using Ct values for different cDNA dilutions and was ~100% for each gene monitored.

Viability staining and confocal laser scanning microscopy

C. roseus hairy root cultures were elicited with 0, 250, or 1,000 μM MJ on day 21 (mid-exponential growth phase). Cultures were harvested 3 d after MJ treatment for viability analysis using propidium iodide (PI) staining.[28] Root tips (~5 mm) were cut from these cultures and PI (BD Biosciences, San Jose, CA) was added to a final concentration of 10 μg/mL in deionized water for ~0.5 h. Root tips were rinsed with deionized water then placed on a microscope slide with a 1% agarose gel cushion bed to avoid damaging the roots. Images were obtained with a laser scanning confocal microscope (LSM 710, Carl Zeiss Microscopy GmbH, Jena, Germany) using an inverted microscope (Observer.Z1, Zeiss) and a 10× (NA 0.3) EC Plan-Neofluor objective. PI was excited at a wavelength of 561 nm using a DPSS laser, and detected at 620 nm.

Results

Effect of MJ dosage on secologanin and TIA profiles

To determine the effect of MJ dosage on the production of TIAs, *C. roseus* hairy root cultures were induced with 0, 50, 250, or 1,000 μM MJ on day 21 (mid-exponential growth phase) and harvested for secologanin and TIA analysis 0, 1, 3, and 5 days later (Figure 3 and Supporting Information Figure S1). Strictosidine synthase (STR, Figure 1) is limiting in these MJ-induced hairy root cultures, which leads to the accumulation of secologanin.[24] The production of strictosidine and tabersonine was monitored to assess downstream TIA accumulation. The production of ajmalicine and serpentine is not shown here since their production was low (<4 mg/L) even at the optimal MJ dosage (250 μM).[24]

We found that lower MJ dosages (50 or 250 μM) enhance secologanin, strictosidine, and tabersonine production, while high dosage (1,000 μM) inhibits production (Figure 3 and Supporting Information Figure S1). Maximum secologanin levels (30 mg/L) are observed in cultures elicited with 50 or 250 μM MJ on day 3, which represents a 370% increase compared with non-induced cultures. Maximum strictosidine (16 mg/L) and tabersonine levels (33 mg/L) occur in cultures elicited with 250 μM MJ on day 5, which represents a 150% and 270% increase, respectively. In contrast, the addition of 1,000 μM MJ to hairy root cultures does not increase specific secologanin, strictosidine, or tabersonine production (Supporting Information Figure S1) and even decreases total production (Figure 3) compared with non-induced controls.

To determine whether high MJ dosage is toxic, hairy roots were stained with PI, a membrane impermeant dye that is excluded from viable cells. Root viability is not affected at either 250 or 1,000 μM MJ (Supporting Information Figure S2). These results suggest that the low specific TIA production observed at high MJ dosage is not attributed to low viability but to repressed TIA biosynthesis. High MJ dosage was also found to reduce overall growth, which contributes to a decrease in the total TIA production compared with untreated controls. Three days after elicitation, dry weight decreased by 36% in cultures treated with 1,000 μM MJ compared with untreated cultures but this decrease was not statistically significant (Supporting Information Figure S3). This is consistent with earlier work reporting that exogenous JA reduces growth of roots in *Arabidopsis* and *C. roseus*.[29–31]

Previous results demonstrate that TIA production is MJ dosage-dependent in *C. roseus* cell suspensions.[12,32] This study explores this observation further by investigating the MJ dosage-dependent response of transcription factors involved in regulating TIA biosynthesis. Maximum ajmalicine and serpentine production occurs in *C. roseus* cell suspensions induced with 10–400 μM MJ (up to a 300% increase) whereas higher dosages (500–1,000 μM MJ) either slightly enhance or have no effect on alkaloid levels compared with non-induced cultures.[12,32] In our *C. roseus* hairy root cultures, alkaloid production is enhanced with lower MJ dosages (50 and 250 μM), optimal at 250 μM, and inhibited by higher MJ dosage (1,000 μM). To investigate how alkaloid production is transcriptionally regulated with MJ dosage,

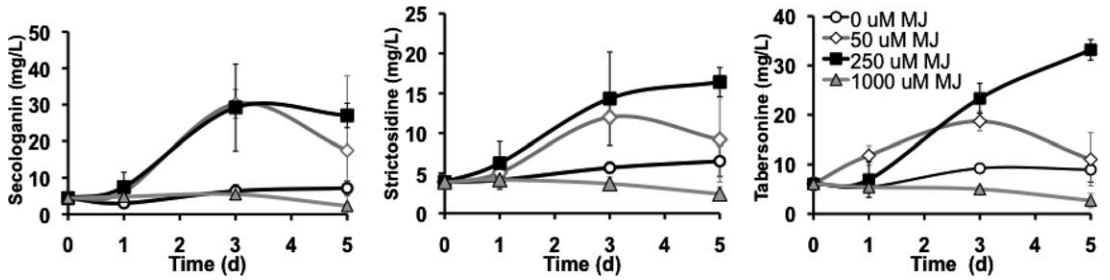

Figure 3. Secologanin, strictosidine, and tabersonine levels (mg/L) in *C. roseus* hairy root cultures elicited with 0, 50, 250, or 1,000 μM MJ on day 21 (mid-exponential growth phase) and harvested 0, 1, 3, and 5 days following treatment.

Alkaloid levels are based on averages of two biological replicates and the error bars denote standard deviations. Secologanin and strictosidine levels were significantly higher (**) in cultures elicited with 50 μM MJ harvested on day 3 and in cultures elicited with 250 μM MJ harvested on day 5 compared with the non-induced cultures. Tabersonine levels were significantly higher (*) in cultures elicited with 50 and 250 μM MJ and significantly lower (**) in cultures elicited with 1,000 μM MJ harvested on day 3; tabersonine levels were significantly higher (**) in cultures elicited with 250 μM MJ harvested on day 5. All significance tests were performed using the Student's *t*-test in Excel (* denotes $0.05 < P < 0.1$; ** denotes $P < 0.05$).

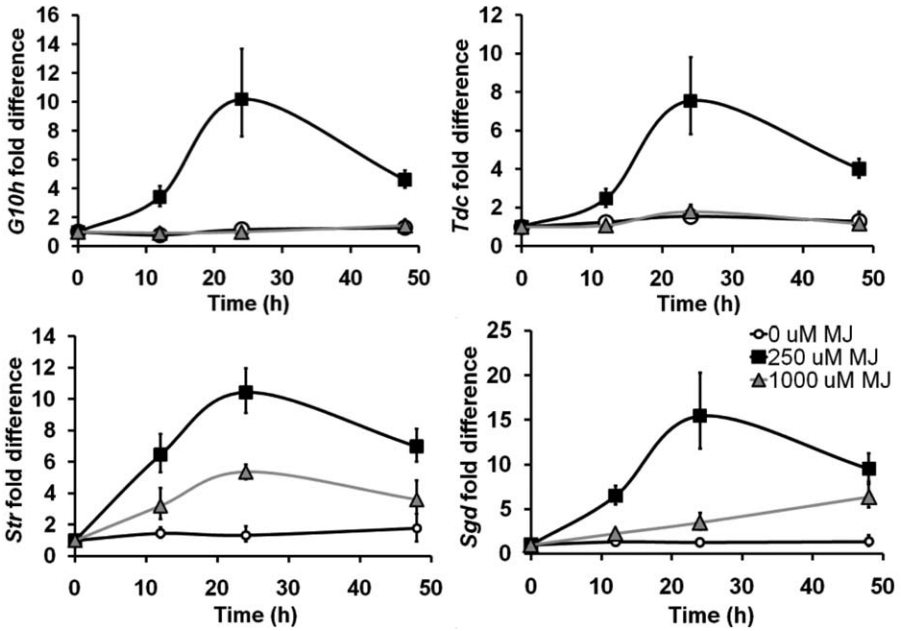

Figure 4. *G10h*, *Tdc*, *Str*, and *Sgd* expression in *C. roseus* hairy root cultures elicited with 0, 250, and 1,000 μM MJ on day 21 (mid-exponential growth phase) and harvested 0, 12, 24, and 48 h following elicitation.

Transcript levels were normalized relative to *Rps9* as the housekeeping gene. Fold differences are based on a single biological replicate and error bars denote standard deviations from qPCR triplicates.

we monitored the expression levels of TIA pathway genes and TIA transcription factor genes following treatment with this range of MJ dosages.

Effect of MJ dosage on expression profiles of TIA pathway genes

To monitor the expression profiles of TIA pathway genes, cDNA was prepared from hairy roots treated with the optimal and inhibitory MJ dosages (250 and 1,000 μM) on day 21 and harvested after 0, 12, 24, or 48 h. Since expression of the biosynthetic enzymes is required to produce the TIAs, gene expression was monitored at time points prior to maximum TIA accumulation (occurring between day 3 and 5). *G10h*, *Tdc*, *Str*, and *Sgd* were monitored since they control the overall flux to TIA production (Figure 1). *G10h* and *Tdc* encode the enzymes that catalyze the first committed step of the respective precursor branches, and *Str* and *Sgd* encode enzymes responsible for the condensation reactions leading to the backbone of all TIAs, strictosidine, and cathenamine (the aglucon of strictosidine). To investigate the MJ dosage-dependent response of transcription factors, *Tdc* and *Str* genes were also chosen because they are the most well-characterized targets of the TIA transcription factors, ORCA and ZCT.

We found the MJ dosage that promotes alkaloid accumulation also enhances TIA gene expression. For instance, all TIA pathway genes monitored (*G10h*, *Tdc*, *Str*, and *Sgd*) are upregulated in cultures induced with 250 μM MJ (Figure 4). Maximum TIA gene expression occurs at 24 h where *G10h*, *Tdc*, *Str*, and *Sgd* are upregulated by 10.2, 7.6, 10.4, and 15.5-fold, respectively.

The MJ dosage that inhibits alkaloid accumulation also depresses TIA gene expression. For example, *G10h* and *Tdc* genes are not induced in hairy roots treated with 1,000 μM MJ, and *Str* and *Sgd* levels increase by a lesser extent (<6-fold) compared with cultures treated with 250 μM MJ. These gene expression profiles are consistent with the TIA profiles

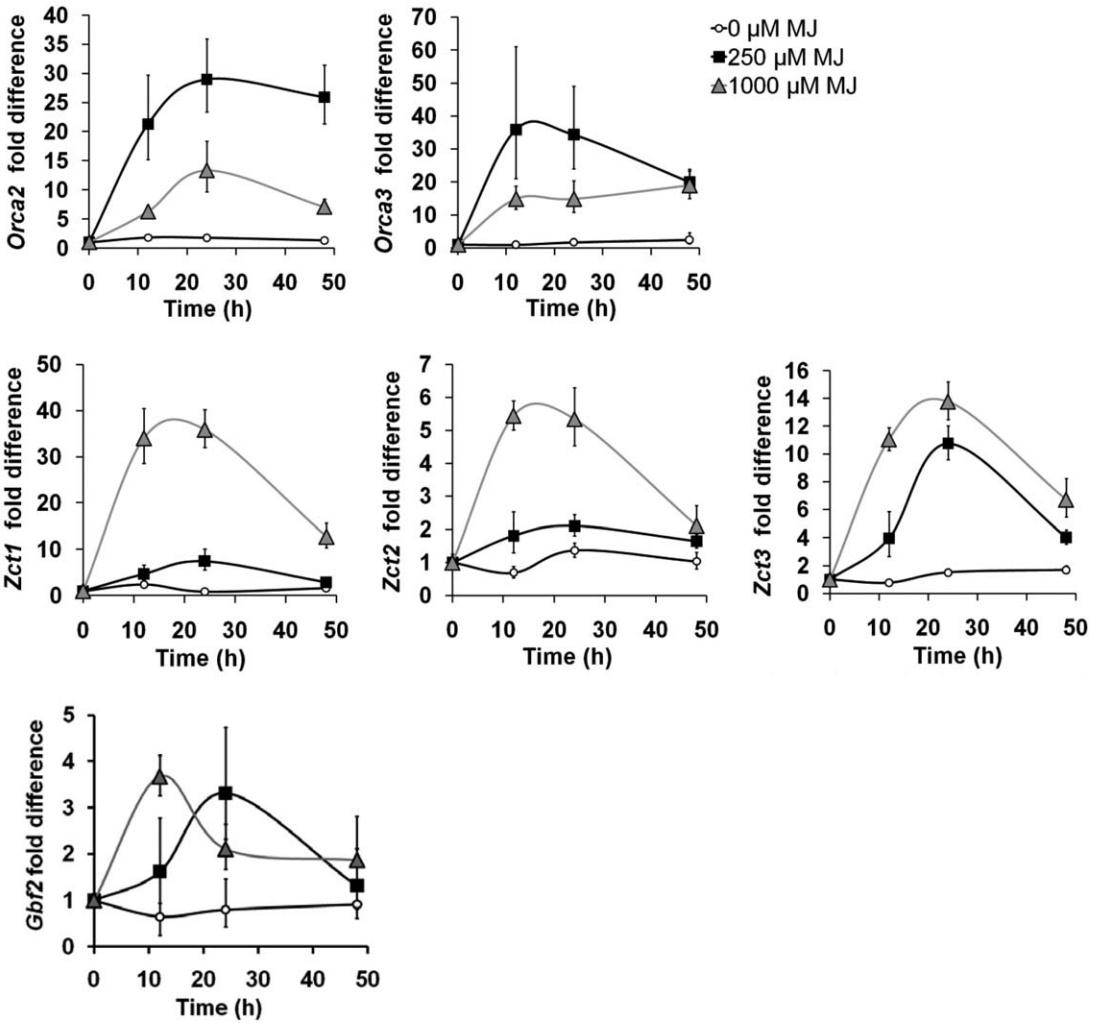

Figure 5. Expression levels of transcriptional activators (*Orca2*, *Orca3*) and repressors (*Zct1*, *Zct2*, *Zct3*, and *Gbf2*) in *C. roseus* hairy root cultures elicited with 0, 250, and 1,000 μM MJ on day 21 (mid-exponential growth phase) and harvested 0, 12, 24, and 48 h following elicitation.

Transcript levels were normalized relative to *Rps9* as the housekeeping gene. Fold differences are based on a single biological replicate and error bars denote standard deviations from qPCR triplicates. *Gbf1* transcript levels were very low in the treated and untreated cultures as indicated by the high C_t values and are not shown here.

observed at inhibitory levels of MJ (Figure 3). If *G10h* and *Tdc* genes are not induced (representing the first committed steps of the respective precursor branches), neither secologanin (the terpenoid precursor) nor TIAs would be expected to increase. These results demonstrate that optimal expression of these TIA biosynthetic genes is correlated with high TIA levels. Conversely, repressed expression of these TIA biosynthetic genes is consistent with low TIA levels.

Effect of MJ dosage on expression profiles of downstream transcription factors

To monitor the expression profiles of JA-regulated transcription factors, cDNA was prepared from hairy roots treated with the optimal and inhibitory MJ dosages (250 and 1,000 μM) on day 21 and harvested 0, 12, 24, or 48 h later. The transcription factors monitored included activators (*Orca2* and *Orca3*) and repressors (*Zct1*, *Zct2*, *Zct3*, *Gbf1*, and *Gbf2*) of the TIA pathway genes.[19–21,33–35]

We found that *Orca2* and *Orca3* levels are strongly elevated by both 250 and 1,000 μM MJ; however, *Orca2* and *Orca3* levels are most pronounced in cultures induced with the optimal MJ dosage of 250 μM (Figure 5). For instance, *Orca2* and *Orca3* are induced by 29 and 40-fold, in cultures elicited with 250 μM MJ compared with 13 and 19-fold, in cultures elicited with 1,000 μM MJ.

In contrast, the levels of *Zct* repressors are most strongly induced in cultures treated with the inhibitory MJ dosage of 1,000 μM. In particular, *Zct1* and *Zct2* levels increase by 36 and 5-fold in cultures induced with 1,000 μM MJ compared with 7 and 2-fold, in cultures induced with 250 μM MJ (Figure 5). The levels of *Zct3* are similar between the two MJ dosages (i.e., 11–14 fold increases). *Gbf* expression does not respond significantly to MJ treatment and does not correlate with the response of alkaloid production with jasmonate (Figure 5). For example, *Gbf2* levels increase similarly with 250 or 1,000 μM MJ by 3–4-fold. *Gbf1* transcript levels are very low in both controls and MJ-induced cultures (as indicated by the high Ct values, not shown).

The role of ORCAs and ZCTs as a transcriptional activators and repressors, respectively, has previously been demonstrated,[20,21,34] but our results suggest that their levels are strongly modulated by jasmonate dosage and that their relative levels are correlated with either an induction or

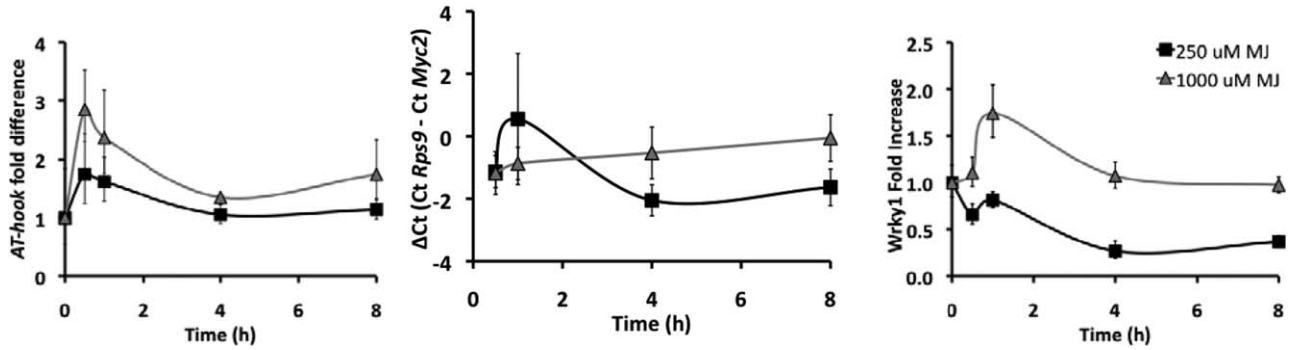

Figure 6. Expression levels of early-response transcription factors *AT-hook*, *Myc2*, and *Wrky1* in *C. roseus* hairy root cultures elicited with 0, 250, and 1,000 μM MJ on day 21 (mid-exponential growth phase) and harvested 0, 1, 4, and 8 h following elicitation.

Transcript levels were normalized relative to *Rps9* as the housekeeping gene. Fold differences or ΔC_t values are based on a single biological replicate and error bars denote standard deviations from qPCR triplicates.

repression of TIA biosynthesis. To investigate how *Orca* and *Zct* levels are transcriptionally regulated with varying MJ dosage, the expression levels of early-response TIA transcription factors thought to regulate *Orca* and *Zct* were monitored.

Effect of MJ dosage on expression profiles of upstream early-response transcription factors

AT-hook, MYC2, and WRKY1 are early-response transcription factors thought to regulate the expression of *Orca* and *Zct*. AT-hook and MYC2 bind directly to the quantitative and qualitative sequences of the *Orca3* promoter, respectively.[17,18] Several clones encoding proteins with DNA-binding AT-hook motifs (2D238, 2D173, 2D499, 2D38M, and 2D7) were identified but only 2D173 and 2D7 showed a weak increase in *Gus* reporter expression levels during transactivation studies.[18] Therefore, clone 2D173 was monitored in our studies (hereafter referred to as *AT-hook*) to investigate its involvement in affecting *Orca3* expression levels in response to jasmonate. In addition, *Wrky1* levels were monitored since increased WRKY expression is correlated with downregulated *Orca2, Orca3*, and *Myc2* and upregulated *Zct1, Zct2*, and *Zct3* expression.[22]

C. roseus hairy roots were treated with the optimal and inhibitory MJ dosages (250 and 1,000 μM) on day 21 and harvested after 0, 1, 4, and 8 h to monitor the expression profiles of *AT-hook, Myc2*, and *Wrky1*; these time points preceded the changes in *Orca* and *Zct* levels shown in Figure 5. We found that the levels of *AT-hook* are not strongly induced with jasmonate or significantly different between 250 and 1,000 μM MJ. The maximum level of *AT-hook* induced is between 1.8 and 2.8-fold with MJ (Figure 6). Similarly, previous studies show little or no temporal variation of *AT-hook* levels in *C. roseus* suspensions after treatment with 10 μM MJ within a 24 h time course.[18] The lack of dosage dependency is surprising since *AT-hook* is thought to control the quantitative expression of *Orca3* whose expression levels appear to be MJ dosage dependent.

In contrast, *Myc2* levels are strongly elevated with MJ treatment. Since *Myc2* levels are undetectable before MJ treatment (0 h time point), fold-differences could not be calculated. Figure 6 illustrates the difference between threshold cycle of *Rps9* and *Myc2* (ΔC_t) as a function of each time point. Maximum *Myc2* expression occurs after 1 h with 250 μM MJ treatment followed by a decrease over the next 3 h, whereas *Myc2* expression steadily increases with 1,000 μM MJ treatment (Figure 6). MYC2 is believed to act as an "on–off" switch of *Orca3* expression, suggesting that a threshold value of *Myc2* may be all that is required and that its induction with both optimal and inhibitory MJ dosages is sufficient for the induction of *Orca*.

Although not strongly induced nor repressed, the levels of *Wrky1* did show a significant difference with MJ dosage (Figure 6). *Wrky1* levels steadily decrease with 250 μM MJ, dropping and remaining below uninduced cultures even 8 h after treatment. In contrast, higher *Wrky1* levels are observed with 1,000 μM MJ. Cultures treated with 1,000 μM MJ show peak *Wrky1* levels 1 h after treatment (i.e., 1.7-fold increase) before returning to basal levels 4 h after treatment. Previous studies in *C. roseus* seedlings have reported that *Wrky1* expression increases within 30 min after exposure to 100 μM MJ, gradually decreases during the next 8 h, and dips below basal levels after 8 h.[22] Although, the response of *Wrky1* expression with MJ dosage are not as significant as those observed with *Orca* and *Zct* expression, our *Wrky1* results are consistent with the MJ dosage-dependency of *Orca* and *Zct* expression profiles. For instance, at optimal MJ concentrations, elevated *Orca* and low *Zct* levels are associated with low *Wrky1* levels while at inhibitory MJ concentrations, reduced *Orca* and elevated *Zct* levels are associated with higher *Wrky1* levels.

Discussion

Our results suggest that the dependence of TIA production on MJ dosage may be partly mediated at the transcriptional level through the relative expression of *Orca* and *Zct* family transcription factors. At the optimal MJ dosage (250 μM) for TIA production, the high ratio of the transcriptional activator *Orca* to repressor *Zct* is consistent with increased expression levels of TIA biosynthetic genes such as *Tdc* and *Str* (Figure 4), genes regulated by ORCA.[19,20,34] However, *G10h* is not regulated by ORCA3,[20] suggesting that another transcriptional activator is induced with the optimal MJ dosage. In contrast, the condition that inhibited alkaloid production (1,000 μM MJ) is correlated with elevated expression levels of *Zct* relative to *Orca*. The elevated *Zct* to *Orca* levels are consistent with the depressed expression of TIA biosynthetic genes such as *Tdc* and *Str*, genes also regulated by ZCT.[21] *G10h* was also strongly depressed, suggesting ZCT may also play a role in regulating this gene.

The simultaneous induction of transcriptional activators and repressors to adjust the plant's response to stress is observed in other plant systems as well. For instance, in *Arabidopsis thaliana*, two transcription factors from the APETALA2/ethylene response factor (AP2/ERF) family, AtERF2 and AtERF4, are induced following treatment with MJ and the fungus *Fusarium oxysporum*.[36] AtERF2 activates the expression of the defense response gene *PDF1.2* and promotes the plant's resistance to *F. oxysporum*. In contrast, AtERF4 acts as a repressor of *PDF1.2* and reduces the plant's resistance to *F. oxysporum*. In *Arabidopsis thaliana* cell suspensions, the transcriptional activators (MYC2 and AP2/ERF factor ORA47) and repressors (C2H2 zinc finger proteins STZ/ZAT10 and AZF2) are induced rapidly with MJ addition and are believed to modulate the effects of MJ on JA biosynthesis (*Lox3* gene), the cell cycle, and monolignol biosynthesis.[37] In addition, our results suggest that MJ dosage can be used to tune *Orca* and *Zct* levels and alkaloid biosynthesis in *C. roseus*. This strategy may also be efficacious in other plant systems with homologous transcription factors.

Although 1,000 μM MJ increases the expression of both *Zct* and *Orca* levels, *Zct* is more strongly upregulated and appears to dominate with regard to *Tdc* and *Str* expression. Despite moderately increased *Orca* levels at the inhibitory MJ dosage (13–19-fold, Figure 5), *Str* and *Sgd* expression are reduced and *G10h*, *Tdc*, and TIA production are completely repressed (Figures 3 and 4). These results are consistent with previous findings of the dominant role of ZCT in repressing *C. roseus* alkaloid biosynthesis.[21,38,39] For instance, the constitutive and transient co-expression of *Orca* and *Zct* resulted in the overall repression of the *Str* promoter.[21] Also *Orca3*-overexpressed *C. roseus* hairy root cultures do not increase TIA production despite high *Orca3* levels, probably due to the strong activation of *Zct1* and *Zct2* expression observed in these transgenic cultures.[38] Although the *G10h* promoter has been sequenced,[40] the regulation of *G10h* promoter by ZCT through promoter binding or transactivation studies has not been performed. Our results suggest that ZCT can strongly repress *Tdc* expression and that either ZCT or an undiscovered transcriptional repressor induced with high MJ dosage may be repressing *G10h* expression.

To investigate the dosage-dependent expression of *Orca* and *Zct*, the expression of upstream transcription factors known to regulate *Orca* and *Zct* expression were monitored: *AT-hook, Myc2*, and *Wrky1*.[17,18,22] Our results show *AT-hook* may not, in fact, be involved in mediating the MJ-dosage dependent effects on *Orca3* expression, similar to previous literature findings.[18] In contrast, we observed significantly elevated *Myc2* levels in response to both MJ treatments, supporting the idea that MYC2 acts as an "on–off" switch for *Orca3* expression. bHLH transcription factors similar to the *C. roseus* MYC2 play a central regulatory role in JA-signaling in many plant species.[41] For example, the JA-signaling cascade that activates nicotine biosynthesis in tobacco also involves JA-mediated degradation of JAZ repressor proteins and the subsequent release of bHLH transcription factors.[42] The *Nicotiana tabacum* bHLH transcription factors like NtMYC2 increase nicotine biosynthesis by inducing both the expression of NIC2-locus ERF genes like ORC1 (a close homologue of *C. roseus* ORCA3) and several genes encoding nicotine biosynthetic enzymes.[42,43]

Besides transcriptional regulation by MYC2, *Orca* expression levels may also be responsive to mitogen activated protein kinase (MAPK) signaling. Recently, a MAPK was discovered in *C. roseus*, MPK3, which is JA-responsive and upregulates *Orca3, Tdc*, and *Str* expression between 1 and 3 fold.[44] These results suggest that post-translational modifications, in addition to changes in gene expression of key regulatory factors, may play an important role in the activation of *Orca* gene expression.

WRKY transcription factors are implicated in regulating alkaloid biosynthesis and JA-responsive genes in other plant species.[45–47] For example, the transcription factor WRKY70 in *Arabidopsis* is thought to be involved in the repression of JA-responsive genes.[46,48] However, in our study, *Wrky1* expression is not as strongly affected by MJ as *Zct* and *Orca* are, suggesting other factors may play a more important regulatory role. A recent transcriptomic sequencing and profiling effort identified numerous other induced transcription factors in MJ-treated *C. roseus* cultures, including other WRKY family transcription factors, that may be involved in the transcriptional regulation of *Orca* and *Zct*.[49]

Conclusion

Our study shows that TIA biosynthesis in *C. roseus* hairy root cultures is regulated by MJ in a dose-dependent manner, which is correlated at the transcriptional level by the relative expression levels of *Orca* and *Zct* factors. Lower MJ dosages enhance the level of *Orca* relative to *Zct*, which is associated with elevated levels of TIA pathway genes and alkaloids, whereas higher MJ dosages instead enhance the level of *Zct* relative to *Orca*, which is associated with repressed levels of TIA pathway genes and alkaloids. Furthermore, changes in the expression levels of upstream early-response transcription factors (*AT-hook*, *Myc2*, and *Wrky1*) do not correspond to the significant changes in the expression of *Orca* or *Zct* with MJ dosage. The dosage-dependent regulation of *Orca* and *Zct* warrants further investigation since understanding the regulation of transcription factors involved in secondary metabolism is important towards the goal of improving secondary metabolite production from plant cell and tissue systems.

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Notation

bHLH = basic helix-loop-helix
ERF = ethylene response factor
LSM = laser scanning confocal microscope
MAPK = mitogen activated protein kinase
MJ = methyl jasmonate
PI = propidium iodide
qPCR = quantitative reverse transcriptase PCR
STR = Strictosidine synthase
TIA = terpenoid indole alkaloid

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