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EXPRESSION OF TABERSONINE 16-HYDROXYLASE AND 16-HYDROXYTABERSONINE-O-METHYLTRANSFERASE IN *CATHARANTHUS ROSEUS* HAIRY ROOTS [†]

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Abstract

The monoterpene indole alkaloids vindoline and catharanthine, which are exclusively synthesized in the medicinal plant *Catharanthus roseus*, are the two important precursors for the production of pharmaceutically important anti-cancer medicines vinblastine and vincristine. Hairy root culture is an ideal platform for alkaloids production due to its industrial scalability, genetic and chemical stability, and availability of genetic engineering tools. However, *C. roseus* hairy roots do not produce vindoline due to the lack of expression of the seven-step pathway from tabersonine to vindoline (Murata and De Luca, 2005). The present study describes the genetic engineering of the first two genes tabersonine 16-hydroxylase (*T16H*) and 16-O-methyltransferase (*16OMT*) in the missing vindoline pathway under the control of a glucocorticoid-inducible promoter to direct tabersonine toward vindoline biosynthesis in *C. roseus* hairy roots.

In two transgenic hairy roots, the induced overexpression of *T16H* and *16OMT* resulted in the accumulation of vindoline pathway metabolites 16-hydroxytabersonine and 16-methoxytabersonine. The levels of root-specific alkaloids, including lochnericine, 19-hydroxytabersonine and hörhammericine, significantly decreased in the induced hairy roots in comparison to the uninduced control lines. This suggests tabersonine was successfully channeled to the vindoline pathway away from the roots competing pathway based on the overexpression. Interestingly, another two new metabolites were detected in the induced hairy roots and proposed to be the epoxidized-16-hydroxytabersonine and lochnerinine. Thus the introduction of vindoline pathway genes in hairy roots can cause unexpected terpenoid indole alkaloids (TIA) profile alterations. Furthermore, we observed complex transcriptional changes in TIA genes and regulators detected by RT-qPCR which highlight the tight regulation of the TIA pathway in response to *T16H* and *16OMT* engineering in *C. roseus* hairy roots. This article is protected by copyright. All rights reserved

Key words: Terpenoid indole alkaloid, vindoline pathway, metabolic engineering, Madagascar periwinkle, plant secondary metabolites

1. Introduction

Vinca alkaloids vinblastine and vincristine are powerful antineoplastic drugs that are widely used in many chemotherapy regimens to treat a variety of cancers (Nobili et al., 2009). They act by binding to intracellular tubulin which results in the inhibition of cell division by blocking mitosis (Chi et al., 2015). The chirality and complexity of these chemicals make the chemical synthesis of these drugs economically infeasible (Sears and Boger, 2015). *Catharanthus roseus* (Madagascar periwinkle) continues to be the exclusive source for the industrial production of vinblastine and vincristine. The low yields of these dimeric monoterpenoid indole alkaloids (TIAs) have motivated extensive effort to genetically engineer *C. roseus* for overproduction. Currently, the lack of tools for whole plant engineering, the genetic instability of the cell cultures, and the high accumulation of alkaloids and genetic stability of engineered gene in highly differentiated hairy roots have made hairy roots a promising system to engineer (Georgiev et al., 2007; Pasquali et al., 2006; Sun et al., 2017).

The biosynthesis of TIAs in *C. roseus* requires two precursors, secologanin from terpenoid pathway and tryptamine from indole pathway (Figure 1). The first alkaloid strictosidine synthesized by strictosidine synthase (STR) (McKnight et al., 1990) is converted to strictosidine aglycone via strictosidine glucosidase (SGD) (Luijendijk et al., 1998). The highly active aglycone is catalyzed to corynanthe-type alkaloids (serpentine, ajmalicine), iboga-type alkaloids (catharanthine), and aspidosperma-type alkaloids (tabersonine, vindoline, hörhammericine) via several branched pathways (O'Connor and Maresh, 2006). However, the genes involved in these branched pathways are still largely unknown (Kellner et al., 2015). The coupling of vindoline and catharanthine produces the two pharmaceutically important bisindole alkaloids vinblastine and vincristine (Figure 1) (Costa et al., 2008). In addition, the complex compartmentalization of the TIA pathway (Courdavault et al., 2014) spatially separates catharanthine and vindoline in different locations, which is one reason for the low yield of bisindole alkaloids within *C. roseus*. Catharanthine is synthesized in leaf epidermis and stored

in upper cuticle, and vindoline is produced in laticifers and idioblast (Roepke et al., 2010). Moreover, these downstream alkaloid pathways are tissue specific. In *C. roseus* cell cultures and hairy roots cultures, the absence of vindoline accumulation is the major challenge to produce the desired bisindole alkaloids in these biological systems, but the vindoline precursor tabersonine does accumulate (Shanks et al., 1998a). Tabersonine in un-differentiated cells and hairy roots is directed to 19-O-acetylhornhamericine instead of vindoline (Giddings et al., 2011). Recently the full seven steps catalyzing the conversions from tabersonine to vindoline were elucidated (Qu et al., 2015). Engineering of this missing vindoline pathway in hairy roots or cell culture is now possible.

To demonstrate the potential of the vindoline pathway to be expressed in hairy roots, we introduced the first two enzymes in the vindoline pathway, tabersonine 16-hydroxylase (T16H) and 16-O-methyl transferase (16OMT), into *C. roseus* hairy roots under the control of an inducible promoter system. The metabolic profile changes were measured after overexpressing *T16H* and *16OMT*. Unsurprisingly no vindoline accumulation was detected, since we did not express the last five steps of the vindoline pathway in hairy roots. The catalytic products of T16H and 16OMT were identified as expected in the engineered hairy roots. This is the first report of producing vindoline pathway intermediates in hairy roots. Furthermore, previous studies have pointed out the tight regulation of the TIA pathway based on the overexpression of TIA genes and regulators in *C. roseus* hairy roots (Peebles et al., 2009; Sun and Peebles, 2015). To better understand how the transcription of alkaloid pathway genes and regulators respond to the metabolic alterations at this branch point, the relative mRNA levels of TIA genes including tabersonine 19-hydroxylase (T19H), minovincinine 19-hydroxy-O-acetyltransferase (MAT), strictosidine synthase (STR), strictosidine beta-glucosidase (SGD), and TIA regulators including AP2-domain DNA-binding proteins (ORCAs) and zinc finger proteins (ZCTs) were examined by RT-qPCR analysis in this study.

2. Materials and Methods

2.1 Plasmid construction and clone generation

Plasmid *pTA7002/T16H* and *pTA7002/16OMT* were obtained from Dr. Ka-Yiu San. These two plasmids were generated by constructing the *T16H* or *16OMT* gene into *XhoI* and *SpeI* sites in *pTA7002* (Aoyama and Chua, 1997; Hong et al., 2006). *T16H* and *16OMT* sequences were verified by sequencing. The sequencing results of these two genes matched the published sequence for *T16H* (GenBank: FJ647194.1) and *16OMT* (GenBank: EF444544.1). *16OMT* was cut from *pTA7002/16OMT* and moved to the intermediate plasmid *pUCGALA* (Hughes et al., 2004) at the *XhoI/SpeI* site to construct *pUCGALA/16OMT*. *16OMT* along with the promoter sequence *GAL4-UAS* was cut from *pUCGALA/16OMT* with restriction enzyme *SbfI*, and was constructed next to the right border in the T-DNA region of *pTA7002/T16H* (Figure 2). The *cis* orientation and sequence of *16OMT* in *pTA7002/T16H/16OMT* was further verified by sequencing. Both *T16H* and *16OMT* genes are under the control of a glucocorticoid-inducible promoter (Hughes et al., 2002).

Plasmid *pTA7002/T16H/16OMT* were electroporated into *Agrobacterium rhizogenes* ATCC 15834. The presence of the plasmids was confirmed by sequencing. The generation of transgenic *C. roseus* hairy roots was previously described (Bhadra et al., 1993). Briefly, *A. rhizogenes* containing the plasmid was cultured in 6ml YEM media at 28 °C and 225 rpm for 36 h. Forceps dipped into the agrobacteria were used to pinch the stem of the *C. roseus* seedlings. After 6 weeks, hairy roots protruding from the wounding sites were harvested and grown on selection plates (hairy roots media supplemented with 350 mg/L cefotaximine and 30 mg/L hygromycin). Hairy roots growing on selection media were transferred to new hairy roots plates and then adapted to liquid culture.

2.2 Hairy roots culture and induction

Hairy roots were sub-cultured every three weeks in 50 ml hairy roots media consisting of 30 g/L sucrose, half strength Gamborg salt (Sigma-Aldrich), full strength Gamborg vitamin (Sigma-

Aldrich) as previously described (Peebles et al., 2005). Triplicates of the transgenic hairy roots at late exponential growth stage (18 days after sub-culture) were treated with 3 μ M inducer dexamethasone to induce overexpression of the genes or with an equal volume of ethanol as a negative control. After 72 h of induction, 300 mg fresh hairy root tissue was grinded with liquid nitrogen in a mortar and was frozen at -80 °C for RNA extraction. The remaining hairy root sample was stored at -80 °C for further alkaloid extraction.

2.3 Alkaloids extraction

The cultured hairy root samples were lyophilized and ground with a mortar and pestle. About 100 mg of the powdered hairy root was weighed. Each of the samples was spiked with 150 μ g vincamine (Sigma-Aldrich) as an internal standard. The extraction protocol was adapted from a previous report (Liu et al., 2012). Metabolites were extracted in 4 ml of methanol with vortexing for 2 min and sonicating for 10 min. The extracts were centrifuged at 3000 rpm for 3 min. The supernatant was removed and the biomass was extracted twice with 2 ml of methanol in each time. The combined supernatants were dried on a nitrogen evaporator. To further isolate TIAs from the methanol extracted mixture, the dried extract was partitioned between 2 ml of ethyl acetate and 2 ml of 1% HCl solution. TIAs stayed in the water phase. The ethyl acetate upper phase was discarded, and the pH of water phase was adjusted to 10 with ammonium hydroxide. When the water phase became alkaline, the solubility of TIAs in water decreased. So 2 ml of ethyl acetate was used to extract TIAs from the water phase. The ethyl acetate extract was dried on a nitrogen evaporator and re-dissolved in 500 μ L of methanol.

2.4 Metabolite standard from yeast fermentation

A *T16H* gene encoding tabersonine 16-hydroxylase and a *16OMT* gene encoding 16-hydroxytabersonine-O-methyltransferase were cloned into a yeast plasmid *pESC-Leu*. The promoter and terminator for *T16H* ORF were *PGAL1* and *TCYC1*. And the promoter and terminator for *16OMT* ORF were *PGAL10* and *TADH1*. The *pESC-Leu-T16H-16OMT* construct was transformed into *Saccharomyces cerevisiae* WAT11. *S. cerevisiae* harboring the desired

plasmid were firstly cultured in Synthetic Defined Medium without leucine (SD-Leu) to grow biomass. After 48 hours, the collected *S. cerevisiae* WAT11 cells were inoculated into 1 L SD-Leu with the glucose replaced by raffinose and galactose to induce the GAL1 and GAL10 promoters, keeping an initial OD as 0.2. And the medium was supplemented with 150 μ M tabersonine. The medium was then extracted after another 48 hours with 1 L of ethyl acetate three times and concentrated with a rotary evaporator. The TIAs in the extract were isolated using same water and ethyl acetate partition method described in previous section.

2.5 Metabolites identification using LC/MS or LC/MS/MS

The extract from *S. cerevisiae* WAT11 and hairy roots was analyzed with the Agilent Technologies 1100 series liquid chromatography (LC) system with a binary pump, a temperature-controlled autosampler, a photo diode array detector (PDA) coupled to an Agilent Technologies Mass Selective Trap SL detector equipped with a nanoflow electrospray. The column used to separate alkaloids was a Phenomenex Kinetex 5 μ m C18 (150 $\times$ 2.1 mm). The flow rate was kept at 0.2 ml/min. The mobile phase was a 30:70 mixture of acetonitrile: 10mM ammonium acetate (pH=5) during the first 6 min. The mobile phase was linearly ramped to 64:36 from the 6th min to the 18th min and maintained at that ratio till the 25th min. The ratio was then further increased to 85:15 within 5min and maintained for 20 min. In the next 5 min, the ratio was returned to 30:70 and the column was allowed to re-equilibrate for another 5 min.

The mass spectrometer was operated in positive mode. Nebulizer pressure was set to 25 psi and the dry gas (nitrogen) was heated up to 350 $^{\circ}$ C with a flow rate at 10 l/min. The m/z values were obtained with a full scan from 50 to 600 to detect the molecular weight of different TIA compounds. Most of the peaks in the spectra were identified with the comparison of calculated molecular weight and the comparison of retention time from available standard compounds. In the MS/MS mode, fragmentation is induced by collision-induced dissociation (CID) technique. Helium was used as the collision gas, and manual MS/MS programs were set up for specific time segments, which were the retention times of some TIAs. The m/z of the

target compound was chosen in the time segment with 4 m/z window width, and 1 voltage was applied as the amplitude of the excitation, with a 27% cutoff of the m/z of the precursor ion.

2.6 Metabolites quantification with HPLC

Five microliters of the alkaloid extract was injected into the Waters HPLC system consisted of two 510 pumps, a 717plus Autosampler, and a 996 Photo Diode Array (PDA) detector. And we used a Phenomenex Luna 3 μ m C18(2) LC column (250 $\times$ 4.6 mm) to separate the alkaloids. The HPLC protocol was almost the same as the LC-MS protocol, except the flow rate was kept at 0.5 ml/min. Ajmalicine, serpentine, catharanthine were identified and quantified at 254 nm with their own standard compounds (Sigma-Aldrich). Hörhammericine, lochnericine, tabersonine, 16-hydroxytabersonine, 16-methoxytabersonine were identified at 329 nm with their own standard compounds (Sigma-Aldrich or house-made), and quantified with tabersonine standard compound (Morgan et al., 2000). The two putative compounds, epoxidized-16-hydroxytabersonine and lochnerinine, were also quantified with tabersonine standard compound.

2.7 RT-qPCR

Total RNA was isolated from the frozen hairy root powder using TRIzol reagent according to manufacturer's instructions (Ambion RNA by Life Technologies). DNA was removed from the sample with TURBO DNA-free according to the manufacturer's instructions (Ambion RNA by Life Technologies). cDNA was synthesized from 500 ng RNA using random primers and GoScript reverse transcriptase according to manufacturer's instructions (Promega). A 'no-amplification control' (without reverse transcriptase) was performed for each sample. cDNA was diluted 10 times to 200 μ L with nuclease-free water. Each q-PCR reaction (20 μ L) contained 1 μ L diluted cDNA, 1.25 pmol/mL mixed primers, 10 μ L SsoAdvanced SYBR green super mix (BIO-RAD) and nuclease-free water. The primers used for qPCR of *T16H* are 5'-GCGGAACCTAACATTGCAGA-3' and 5'-GCACATCAACAAGGTCCTCC-3'. The qPCR primer

pairs for 16OMT are 5'-CTTGTTTGAGGGCTTGGCTT-3' and 5'-TCAAACATGTCACCTGCAACA-3'. The primers used for the other genes were previously described (Sun and Peebles, 2015). The q-PCR amplifications were carried out in BIO-RAD CFX Connect™ Real-Time PCR Detection System with the program: 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 1 min at 60 °C. The relative gene expression was quantified by using the comparative threshold cycle CT method as previously described (Shalel-Levanon et al., 2005). The 40S ribosomal protein S9 (RPS9) was used for the control gene (Menke et al., 1999).

2.8 Statistical analysis

Data were analyzed using the Student's t-test.

3. Results

3.1 Genetic engineering of T16H and 16OMT in *C. roseus* hairy root

Stems of *C. roseus* seedlings were infected with *Agrobacterium rhizogenes* ATCC 15834 carrying the plasmid *pTA7002/T16H/16OMT*. After selection on solid media containing hygromycin followed by six sub-culture cycles in liquid hairy root media, two stable transgenic hairy root lines #S3 and #S8 were obtained and used for further analysis. The expression of *T16H* and *16OMT* were controlled by the same glucocorticoid-inducible promoter (Hughes et al., 2002). Both genes showed increased expression in the two induced hairy roots compared to the uninduced ones by RT-qPCR. In the induced #S3 hairy roots, *T16H* mRNA was increased 452±64 times, and *16OMT* mRNA was increased 27±6 times compared to the uninduced control. #S8 hairy roots demonstrated a 107±19 fold increase in *T16H* mRNA level and 150±30 fold increase in *16OMT* after 72 h induction. Noticeably, #S3 hairy roots showed a much higher overexpression fold change in *T16H* than in *16OMT*, while #S8 hairy roots revealed a lower overexpression fold change in *T16H* than in *16OMT*. These differences based on mRNA analysis by qPCR could be due to the differences in the background level of transcripts in the two different hairy roots lines. The significant clonal variation between these two transgenic

hairy roots may also result from the random T-DNA insertion into the nuclear chromosome mediated by *Agrobacterium* transformation. Nevertheless, the use of a glucocorticoid inducible promoter system in this study allows us to investigate the effects of increased expression to that of the control within the same background.

3.2 Identification of new metabolites in *C. roseus* hairy roots overexpressing *T16H* and *16OMT*

Tabersonine is an important branch point in vindoline production. In order to divert tabersonine into the vindoline pathway, the first two enzymes in vindoline pathway *T16H* and *16OMT* (Figure 3) were engineered into *C. roseus* hairy roots under the control of an inducible promoter system (Aoyama and Chua, 1997). Due to the inaccessibility of TIAs intermediates 16-hydroxytabersonine and 16-methoxytabersonine on market, we chose to use *S. cerevisiae* as a production host to prepare the standard molecules. *S. cerevisiae* does not naturally produce TIAs due to the lack of the terpenoid pathway derived from geranyl pyrophosphate (GPP) (Brown et al., 2015), therefore the separation of TIAs from other yeast native metabolites is much easier since the chemical structures of these contaminants are very different from TIAs. Functional expression of microsomal plant P450s has precedence in yeast (Pompon et al., 1996). In order to obtain 16-hydroxytabersonine and 16-methoxytabersonine standards for LC/MS, a *S. cerevisiae* strain *WAT11* transformed with the plasmid *pESC-Leu-T16H-16OMT* expressing *T16H* and *16OMT* was fed with tabersonine. The two alkaloids, 16-hydroxytabersonine and 16-methoxytabersonine, together with the substrate, tabersonine sharing the α -methyleneindoline structure, were identified in yeast extracts by LC/MS at 329 nm according to their molecular weights (Figure 4). The yeast extracted alkaloids were subsequently used for analyzing hairy root samples.

By comparing the chromatograms of the induced and uninduced hairy roots extracts, four new peaks were detected in the induced hairy roots compared to the uninduced control (Figure 4a and 4b). Peak 1 and 2 in the induced roots showed the same retention time as 16-hydroxytabersonine and 16-methoxytabersonine from the yeast extracts. Mass to charge ratio

(m/z) of peak 1 and 2 further confirmed that peak 1 is the T16H-catalyzed product 16-hydroxytabersonine, and peak 2 is the 16OMT-catalyzed product 16-methoxytabersonine. Interestingly, two unexpected peaks (Figure 4a and 4b, peak 3 and 4) showed up in the induced hairy roots. Based on the m/z value of each peak, the molecular weight is 16 higher than that of 16-hydroxytabersonine and 16-methoxytabersonine respectively, suggesting the new metabolites could likely be the oxidized forms of 16-hydroxytabersonine and 16-methoxytabersonine.

The extremely low abundance of the two new TIA metabolites in the hairy roots made purification infeasible. In order to collect more evidences to confirm their chemical structures, MS/MS was performed for the two new TIAs, as well as other five related TIAs, including tabersonine, lochnericine, 16-hydroxytabersonine, 16-methoxytabersonine, and 19-hydroxytabersonine (Figure 4c). Comparing the fragment patterns of tabersonine, 16-hydroxytabersonine, and 16-methoxytabersonine, we could see that the modifications at the 16 position in the indole moiety did not change the fragment patterns. There was only single abundant fragment ion $[M-31]^+$ in each of their fragment spectra. On the other hand, the modifications in the terpene moiety changed the fragment patterns. For example, by adding a hydroxyl group at the 19 position (comparing the chromatograms of tabersonine and 19-hydroxytabersonine in Figure 4c), besides the most abundant $[M-31]^+$ peak, a second abundant $[M-17]^+$ appeared; by forming an epoxide ring between the 6 and 7 positions (comparing the chromatograms of tabersonine and lochnericine in Figure 4c), the fragment pattern changed dramatically, with four abundant peaks appeared ($[M-17]^+$, $[M-31]^+$, $[M-59]^+$, $[M-124]^+$). The fragment patterns of the two putative compounds were similar to the one of lochnericine, they all contained abundant $[M-17]^+$, $[M-31]^+$, $[M-59]^+$, $[M-124]^+$ peaks, so the two unknown TIAs may have an epoxide ring occurring at the 6 and 7 positions. Meanwhile, since the modifications at the 16 position in the indole moiety kept the most abundant fragment ion $[M-31]^+$, the putative 6,7-epoxidized-16-hydroxytabersonine and 6,7-epoxidized-16-methoxytabersonine contained

more [M-31]⁺ fragment ion than lochnericine. Altogether, these pieces of evidence provided strong supporting information to indicate that the two unknown metabolites could be 6,7-epoxidized-16-hydroxytabersonine (peak 3 in Figure 4a and 4b) and 6,7-epoxidized-16-methoxytabersonine (peak 4 in Figure 4a and 4b). In addition, T6,7-epoxidase (T6,7E) activity has been demonstrated in *C. roseus* hairy roots, and this enzyme is responsible for generating the epoxidized derivatives of tabersonine (Figure 3) (Rodriguez et al., 2003). We hypothesize that T6,7E can convert 16-hydroxytabersonine and 16-methoxytabersonine to epoxidized-16-hydroxytabersonine and epoxidized-16-methoxytabersonine (also named as lochnerinine). In addition, we proposed the chemical bond breaking models for lochnericine and 19-hydroxytabersonine in Figure 4d. It showed the two main fragment ions, [M-17]⁺ and [M-31]⁺, for 19-hydroxytabersonine, and three in the four main fragment ions, [M-17]⁺, [M-31]⁺, [M-59]⁺, for lochnericine. The [M-17]⁺ and [M-59]⁺ ions of lochnericine are contributed by the epoxide ring.

3.3 Quantification of alkaloids in the induced and uninduced hairy roots expressing *T16H* and *16OMT*

After identifying the four new metabolites in the transgenic hairy roots expressing *T16H* and *16OMT*, the yields of all the detected alkaloids in the induced and uninduced hairy roots were quantified by HPLC (Figure 5). 16-hydroxytabersonine increased from the undetectable level in the uninduced hairy roots to 0.15 ± 0.88 mg/g dry weight (DW) in the 72 h induced #S3 hairy root line and to 0.05 ± 0.00 mg/g DW in the induced #S8 hairy root line. The concentration of 16-methoxytabersonine was also increased to 0.15 ± 0.06 and 0.42 ± 0.04 mg/g DW after 72 h induction of *T16H* and *16OMT* in #S3 and #S8 hairy roots, respectively, while the uninduced control lines barely showed accumulation of 16-methoxytabersonine (Figure 5a). The induced #S3 hairy roots produced more 16-hydroxytabersonine but less 16-methoxytabersonine than the induced #S8 hairy roots. In #S3 hairy roots, the fold increase of *T16H* transcripts was much higher than that of *16OMT* after 72 h induction. Therefore we hypothesize that the intermediate

16-hydroxytabersonine accumulated due to the relatively low expression of *16OMT*. Similarly we hypothesize that 16-hydroxytabersonine could be rapidly converted to 16-methoxytabersonine in the induced #S8 due to the higher-level expression of *16OMT* in this hairy root line. The proposed epoxidized-16-hydroxytabersonine and lochnerinine showed very similar accumulation trends with 16-hydroxytabersonine and 16-methoxytabersonine, which further demonstrated that these two metabolites are most likely the derivatives of 16-hydroxytabersonine and 16-methoxytabersonine.

The metabolite levels in the 19-O-acetylhörhammericine pathway including lochnericine, hörhammericine and 19-hydroxytabersonine (Figure 3) were significantly decreased in the two induced hairy root lines compared to the uninduced ones (Figure 5b). Tabersonine concentration did not change in the #S3 hairy root line after induction, while a 26% decrease in tabersonine was observed in the induced #S8 hairy root line. Figure 6 shows the obvious accumulation of the total measured vindoline pathway metabolites in the induced roots and the great decrease in the total 19-O-acetylhörhammericine pathway metabolites after overexpressing *T16H* and *16OMT*. Such changes were expected, because it indicates tabersonine has been converted into the desired intermediates in the vindoline pathway and less metabolites were channeled to the root-specific 19-O-acetylhörhammericine pathway. However, the aspidosperma alkaloid pool consisting of tabersonine and its derivatives (including vindoline pathway intermediates) did not show significant changes after 72 h induction in both root lines (Figure 6). This indicates that no additional carbon was pulled into tabersonine biosynthesis.

Interestingly, catharanthine and ajmalicine were increased by 23% and 21% in #S8 hairy roots after induction, while serpentine showed 43% increase after induction in #S3 hairy roots (Figure 5c). Small but significant increases in total alkaloid pool (all measured alkaloids), corynanthe alkaloid pool (serpentine and ajmalicine), and iboga alkaloid (catharanthine) were noted in #S8 hairy roots after 72 h induction, while in #S3 lines the total alkaloid level was

slightly reduced, and corynanthe alkaloid and iboga alkaloid levels remained unchanged after induction (Figure 6). The changes outside the tabersonine metabolic pathway may result from the complex regulations involved in the TIA pathway. Thus the transcriptional changes of the TIA genes and regulators were further analyzed by RT-qPCR.

3.4 Transcriptional changes of the TIA pathway genes and regulators after inducing T16H and 16OMT in C. roseus hairy roots

Transcriptional alteration of TIA pathway genes and transcriptional factors was assessed in the 72 h induced and uninduced hairy root lines using RT-qPCR (Figure 7). Interestingly, the 19-O-acetylhörhammericine pathway genes *T19H* and *MAT* were significantly down regulated in both #S3 and #S8 induced hairy roots compared to the uninduced control. Similarly, it was reported that the overexpression of the last gene *DAT* from vindoline pathway in *C. roseus* hairy roots can inhibit the activity of roots native *MAT* (Magnotta et al., 2007).

From previous studies, genetic perturbation of TIA genes or transcription factors in *C. roseus* hairy roots caused complex transcriptional changes of the TIA pathway genes and the associated regulators (Peebles et al., 2009; Li et al., 2013; Sun et al., 2016; Sun and Peebles, 2015). The mRNA levels of the two alkaloid pathway genes *STR* and *SGD* upstream of tabersonine did not show any changes after overexpressing *T16H* and *16OMT* in #S8 hairy roots, while *SGD* were up-regulated in #S3 hairy roots after induction (Figure 7). The transcription of two positive regulators *ORCA2* and *ORCA3* were not changed in #S3 hairy roots after induction, while *ORCA2* was up-regulated and *ORCA3* was down-regulated in the induced #S8 hairy roots compared to the uninduced control. In addition, overexpression of *T16H* and *16OMT* also triggered the negative regulation response of TIA pathway. Notably, the negative TIA regulators *ZCTs* were greatly up regulated in both root lines after 72 h induction, with the only exception that *ZCT3* was not changed in the induced #S3 hairy roots compared to the control (Figure 7).

4. Discussion

In this study, the first two genes of the vindoline pathway *T16H* and *16OMT* were successfully introduced into *C. roseus* hairy roots with the aim of initiating production of metabolite intermediates of the vindoline pathway. The induced overexpression of *T16H* and *16OMT* resulted in the synthesis of 16-hydroxytabersonine and 16-methoxytabersonine which are the first two intermediates in the vindoline pathway. Previous efforts have not identified any metabolite along the pathway from tabersonine to vindoline in other hairy root lines (Shanks et al., 1998b). The production of these alkaloids in this study is the first report of these metabolites in hairy roots. Interestingly, two unknown alkaloids were detected in the *T16H* and *16OMT* induced hairy roots. The presence of unexpected metabolites after introducing the leaf-specific genes into hairy roots might be caused by two reasons: the naturally occurring metabolites were catalyzed by the newly introduced enzymes due to the promiscuous substrate specificity, or the catalytic products of the new enzymes were converted by the native enzymes. The molecular weight of the two new compounds is 16 higher than that of 16-hydroxytabersonine and 16-methoxytabersonine individually. Moreover, these two previously unknown metabolites showed similar accumulation trends with 16-hydroxytabersonine and 16-methoxytabersonine. Thus they are likely the oxidized forms of 16-hydroxytabersonine and 16-methoxytabersonine. In hairy roots, the tabersonine metabolic pathway involves four enzymes including T19H, MAT, T6,7E, and T6,7R, among which, T19H (Giddings et al., 2011) and T6,7E (Rodriguez et al., 2003) oxidize tabersonine and its derivatives at different positions (Figure 3). The two unknown metabolites showed similar fragmentation patterns with the tabersonine derivative with an epoxide ring on the 6,7 position based on MS/MS analysis (Figure 4c and 4d). In addition, feeding 16-hydroxytabersonine and 16-methoxytabersonine to *S. cerevisiae* WAT11 overexpressing T19H did not result in the conversion of these two metabolites (data not shown here). It excluded the possibility that the oxidation occurred at the 19 position. The function of T6,7E in crude protein from hairy roots was characterized even though the sequence of T6,7E

remains unknown. Lochnericine, hörhammericine, and 19-O-acetyl hörhammericine are all oxygenated derivatives of tabersonine at position 6,7 which results from T6,7E enzyme activity (Rodriguez et al., 2003). Thus this root-dominant enzyme T6,7E is proposed to have a broader substrate specificity and contributes to the production of the two possible epoxidized-16-hydroxytabersonine and 16-methoxytabersonine in the induced hairy roots.

Lochnericine and hörhammericine both had significant lower concentrations in the induced hairy roots compared to the uninduced controls. This suggests that the induced *T16H* is competing for the precursor tabersonine with the root-specific enzymes T6,7E or T19H. Moreover, the down regulation of *T19H* and *MAT* mRNA levels after induction may be responsible for the decrease in the metabolites in this pathway branch. However, it is unclear whether these down regulations resulted from transcription-level control. Noticeably, the vindoline pathway and the 19-O-acetylhörhammericine pathway not only share the same precursor tabersonine, some enzymes in these two pathways have certain homology in sequence or similar function. For example, MAT in the 19-O-acetylhörhammericine pathway shares 63% nucleic acid and 78% amino acid identities with DAT in the vindoline pathway (Laflamme et al., 2001). They catalyze acetyl transfer reactions at different hydroxyl positions (Figure 3). T16H and T19H can hydrolyze the substrate tabersonine at 16 and 19 positions, respectively. T3O in vindoline pathway and T6,7E from 19-O-acetylhörhammericine pathway both catalyze oxygenate reaction. T6,7E epoxides the double bound at 6,7 position of tabersonine and tabersonine derivatives. T3O oxygenates the double bond at position 3 of tabersonine and its derivatives. The engineering of the terminal step of vindoline biosynthesis *DAT* inhibited the activity of MAT in *C. roseus* roots. These results demonstrate a competitive relationship between the vindoline pathway and 19-O-acetylhörhammericine pathway. The total concentration of aspidosperma alkaloids did not change significantly after overexpressing *T16H* and *16OMT*, because the increase in metabolites in the vindoline pathway is compensated for the decrease in lochnericine and hörhammericine. Nevertheless, it indicates tabersonine flux

was successfully diverted to the desired vindoline pathway from the root native 19-O-acetylhornhamericine pathway.

Moreover, engineering of *T16H* and *16OMT* also caused metabolite level changes beyond tabersonine metabolic pathway. The level of corynanthe and iboga alkaloids, which are alkaloids that diverge from a common precursor (strictosidine aglycone), increased in the induced #S8 hairy roots, while the induced hairy roots of #S3 did not show significant changes in these two alkaloid pools. In addition, the total measured alkaloids showed opposite accumulating trends in two induced hairy roots #S3 and #S8. It is hypothesized that a much higher overexpression level of *T16H* in the induced #S3 hairy roots than that in the induced #S8 hairy roots caused a higher metabolic burden or a negative effect on total alkaloids accumulation.

Many previous *C. roseus* engineering studies showed that the modification of TIA genes usually led to complex transcriptional changes in other pathway genes and transcription factors (Li et al., 2015; Peebles et al., 2009; Sun and Peebles, 2015). Similarly, complex mRNA changes in the TIA pathway regulators were triggered after inducing two vindoline pathway genes, *T16H* and *16OMT*. The relative expression of the positive regulators, ORCAs, and the negative regulators, ZCTs, have been correlated with the jasmonate-dependent alkaloid biosynthesis (Goklany et al., 2013). In this study, ZCTs showed significant down-regulation in the induced hairy roots compared to the uninduced control, while ORCAs revealed complex changes. The tight control of TIA biosynthesis by transcription factors is a strategy used by plants to balance growth and defense. In the future, a better understanding of how to bypass negative regulation is necessary to successfully engineer the TIA pathway for increased alkaloid accumulation.

5. Conclusion

The vindoline pathway derived from the precursor tabersonine is spatially regulated in *C. roseus*. The inactivity of this pathway in hairy roots and cell suspension cultures is the biggest

barrier for the production of vindoline, vinblastine and vincristine. In the present study, we describe the expression of the first two genes, *T16H* and *16OMT*, in the vindoline pathway that initiates the channeling of tabersonine to vindoline pathway intermediates in *C. roseus* hairy root cultures. It is not surprising that expressing only the first two genes of the vindoline pathway is not sufficient to make vindoline in hairy roots. Besides seeing the accumulation of the expected products of *T16H* and *16OMT*, two additional alkaloids were produced, and the concentrations of other root specific metabolites were significantly changed. This study also illustrates how introduction of *T16H* and *16OMT* triggered complex transcriptional responses, especially the up-regulation of the negative transcription factors. This study provides insights regarding how to express the full vindoline biosynthesis pathway to generate vindoline accumulating hairy roots. In the future, eliminating reactions competing for tabersonine, removing root dominant enzymes such as T6,7E, and silencing the negative regulators in combination with overexpressing the vindoline pathway will be necessary to reach industrial relevant yields of vindoline production in hairy roots.

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List of Figure Legends

Figure 1. Monoterpenoid indole alkaloid pathway in *C. roseus*. STR, strictosidine synthase; SGD, strictosidine beta-glucosidase; THAS, tetrahydroalstonine synthase; T19H, tabersonine 19-hydroxylase; MAT, minovincinine 19-hydroxy-O-acetyltransferase; T16H2, tabersonine 16-hydroxylase in leaves; 16OMT, 16-O-methyltransferase; T3O, tabersonine 3-oxygenase; T3R, tabersonine 3-reductase; NMT, N-methyltransferase; D4H, desacetoxyvindoline 4-hydroxylase; DAT, deacetylvindoline-4-O-acetyltransferase; Prx1, Peroxidase 1.

Figure 2. Plasmid construct. RB, right border of T-DNA; LB, left border of T-DNA; p35S, cauliflower mosaic virus 35S promoter; GVG, chimeric transcription factor containing GAL4 DNA-binding domain and VP16 transactivating domain and rat-GR (Glucocorticoid receptor) HBD (hormone binding domain); E9, pea rbcS-E9 polyadenylation terminator sequence; pNOS, nopaline synthase promoter; HPT, hygromycin phosphotransferase gene; tNOS, nopaline synthetase polyadenylation sequence terminator; p6xUAS, GVG-regulated promoter; 3A, pea rbcS-3A polyadenylation terminator sequence.

Figure 3. Tabersonine metabolism in roots and leaves. Metabolite and enzyme names showed in black indicate that they are naturally present in hairy roots. Blue ones represent the engineered enzymes and the detected metabolites after overexpressing the engineered genes in hairy roots. Red represents the hypothesized enzyme and metabolites after overexpressing *T16H* and *16OMT*. Green represents the enzymes or metabolites which are not present in the transgenic hairy roots.

Figure 4. The selected LC chromatograms of alkaloid extracted from the hairy roots cell lines

#S3 (a) and #S8 (b) induced (I) or un-induced (UI) for 72 h, the selected LC-MS/MS fragment ion chromatograms of the extracted alkaloids (c), and the proposed chemical bond breaking model for lochnericine and 19-hydroxytabersonine (d). In (a) and (b), YE represents alkaloids extracts from the medium of tabersonine-supplemented yeast cell cultures expressing *pESC-Leu-T16H-16OMT*. Peak 1, 16-hydroxytabersonine. Peak 2, 16-methoxytabersonine. Peak 3, putative epoxidized-16-hydroxytabersonine. Peak 4, putative epoxidized-16-methoxytabersonine. In (c), the y-axis in all the graphs represents ion intensity, the x-axis in all the graphs represents fragment ion $[m/z]^+$.

Figure 5. The alkaloid levels in the transgenic *Catharanthus roseus* hairy roots expressing T16H and 16OMT. The two hairy roots overexpressing T16H and 16OMT (#S3-I and #S8-I) were induced with 3 μ M dexamethasone during exponential growth phase and harvested 72 h later. The controls (#S3-UI and #S8-UI) were the same root line fed with an equivalent volume of ethanol. HPLC analysis was used to determine the concentrations of the new metabolites: (a) metabolites appearing after overexpressing T16H and 16OMT (16-hydroxytabersonine, 16-methoxytabersonine, proposed epoxidized-16-hydroxytabersonine and epoxidized-16-methoxytabersonine/lochnericine), (b) root aspidosperma metabolites (tabersonine, lochnericine, hörhammericine, and 19-hydroxytabersonine) and, (c) other TIAs (serpentine, ajmalicine, catharanthine, and tetrahydroalstonine). “*”($p < 0.1$) and “**”($p < 0.05$) indicates significant results between the UI and I cultures. Error bars represent the standard deviation of triplicate cultures.

Figure 6. The terpenoid indole alkaloid pools in the transgenic *Catharanthus roseus* hairy roots expressing T16H and 16OMT. The hairy roots overexpressing T16H and 16OMT (#S3-I and #S8-I) were induced with 3 μ M dexamethasone during exponential growth phase and harvested 72 h later. The controls (#S3-UI and #S8-UI) were the same root line fed with an equivalent

volume of ethanol. HPLC analysis was used to determine the TIA pools belong to aspidosperma family (tabersonine, lochnericine, hörhammericine, 16-hydroxytabersonine, 16-methoxytabersonine, putative epoxidized-16-hydroxytabersonine and putative epoxidized-16-methoxytabersonine/lochnerinine), vindoline pathway (16-hydroxytabersonine, 16-methoxytabersonine, putative epoxidized-16-hydroxytabersonine and putative epoxidized-16-methoxytabersonine/lochnerinine), 19-acetylhorhammericine pathway (lochnericine and hörhammericine), the total alkaloids (all alkaloids measured), corynanthe family (serpentine and ajmalicine), and iboga family (catharanthine). Error bars represent the standard deviation of triplicate cultures. “*” ($p < 0.1$) and “**” ($p < 0.05$) indicates significant results between the UI and I cultures. Error bars represent the standard deviation of triplicate cultures.

Figure 7. RT-qPCR of the TIA pathway genes and transcription factors mRNA transcripts fold change in the 72 h induced root line compared to the uninduced root line. Data represents the mean of triplicate $\pm$ standard deviation. “*” indicates significant results ($p < 0.1$ and two fold change) between the UI and I cultures. Error bars represent the standard deviation of triplicate cultures.

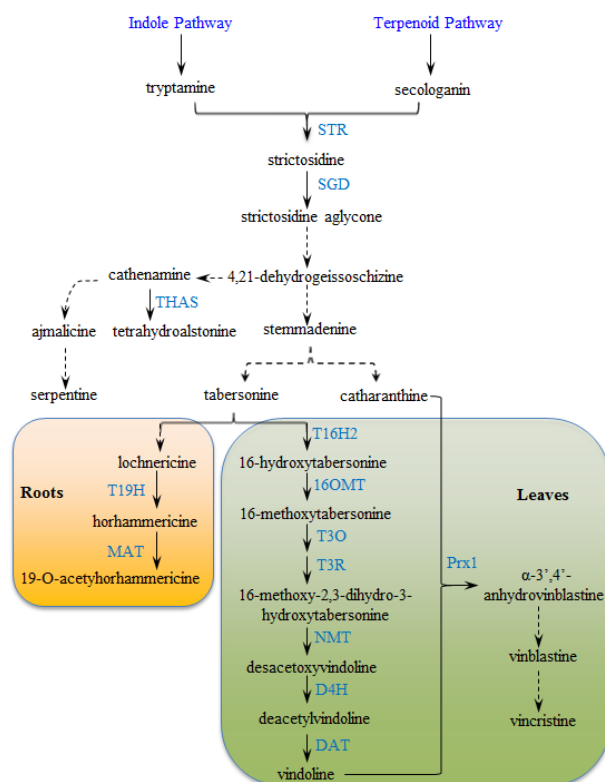


Figure 1

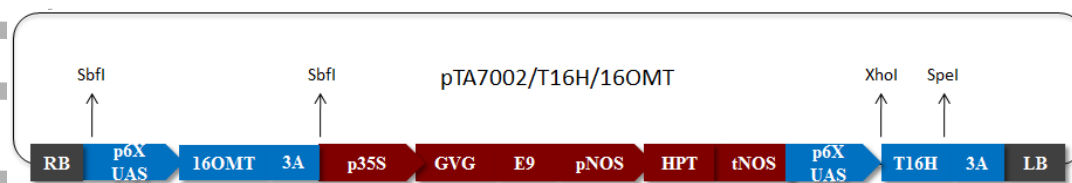


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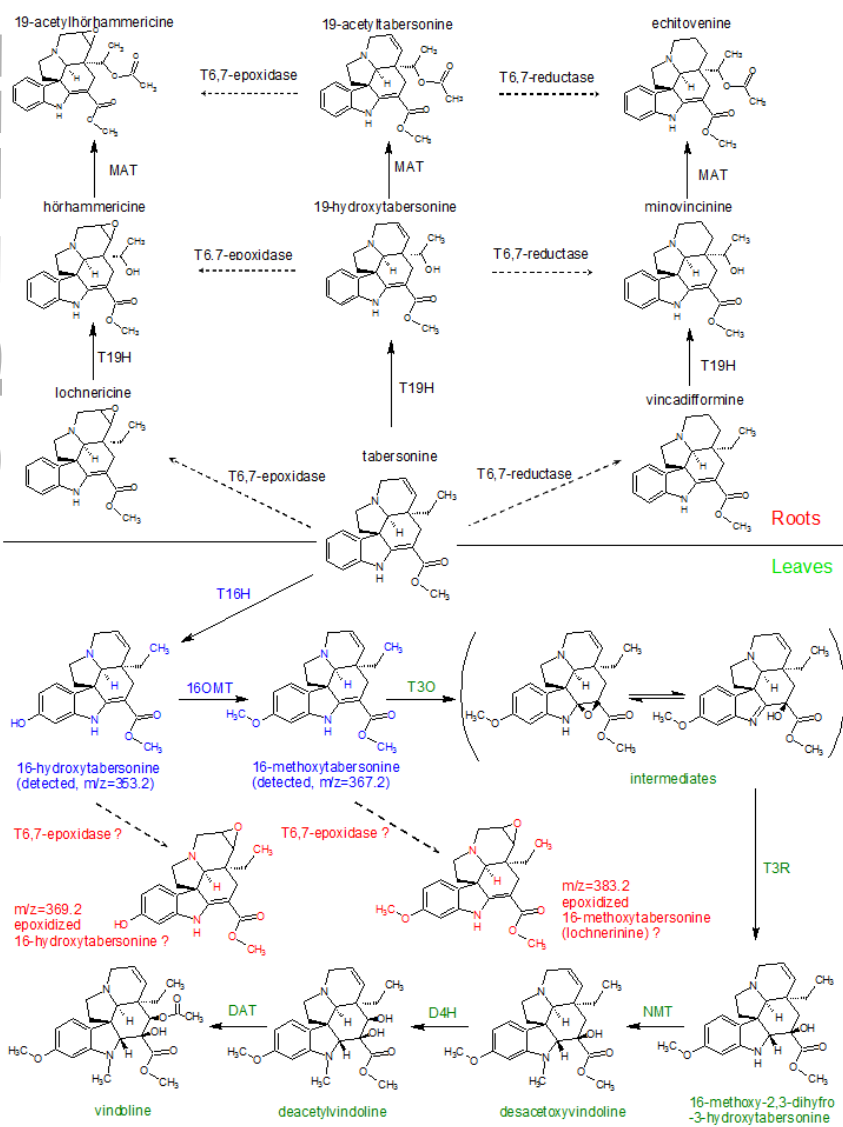


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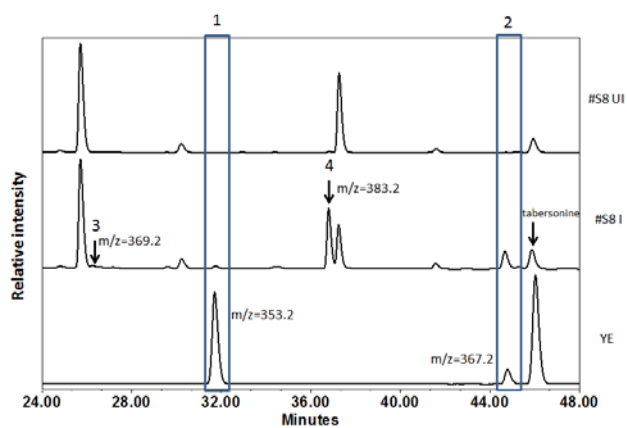


Figure 4(a)

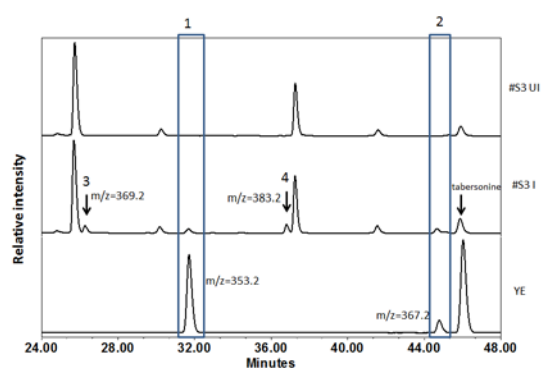


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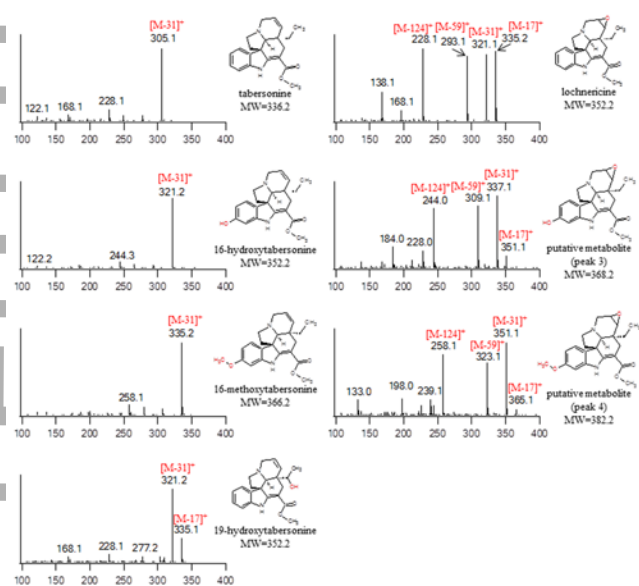
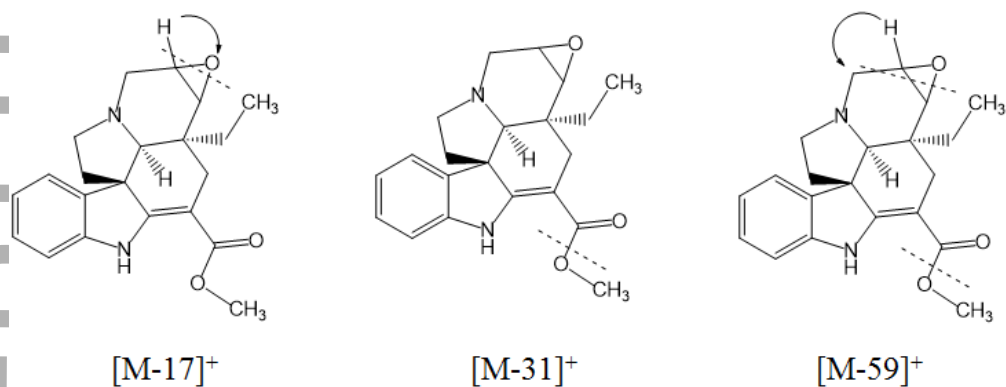


Figure 4(c)

Chemical bond breaking model for lochnericine



Chemical bond breaking model for 19-hydroxytabersonine

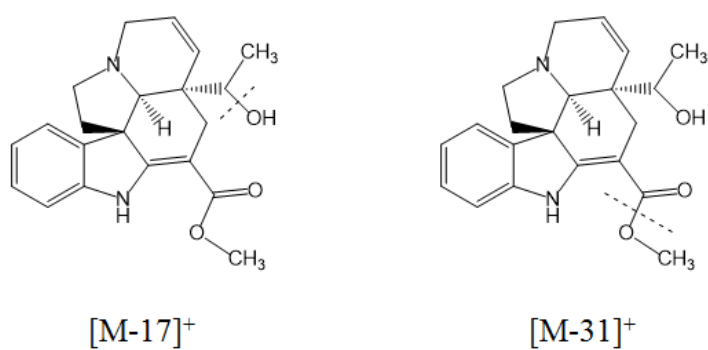


Figure 4(d)

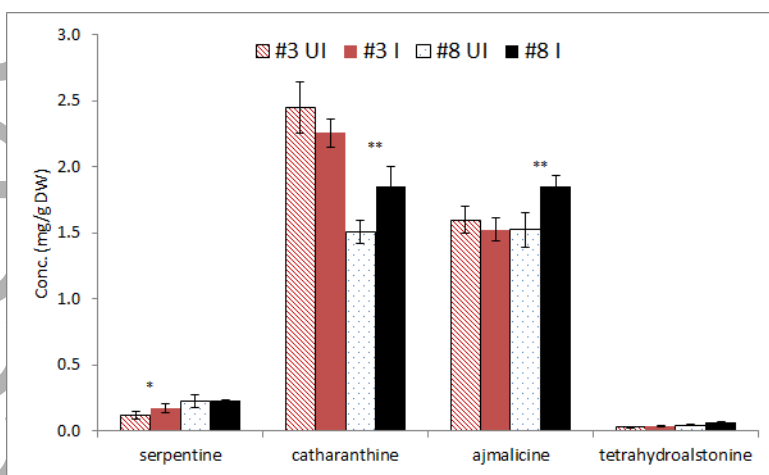
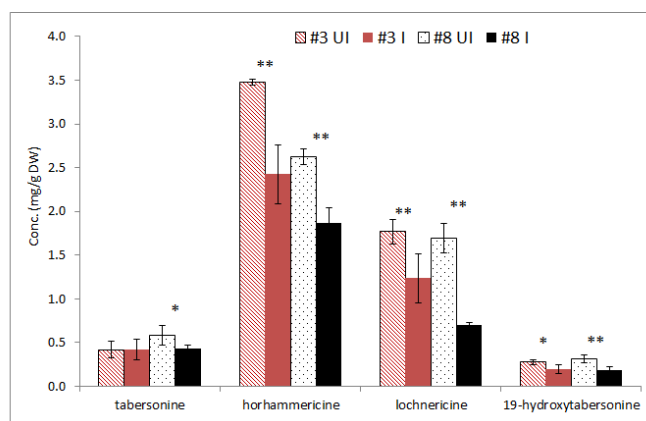
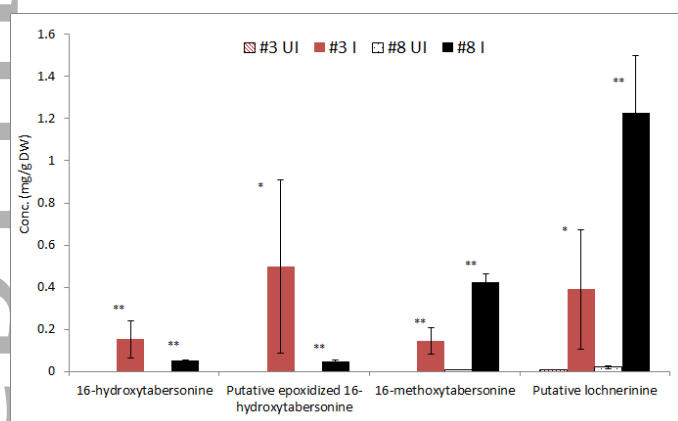


Figure 5

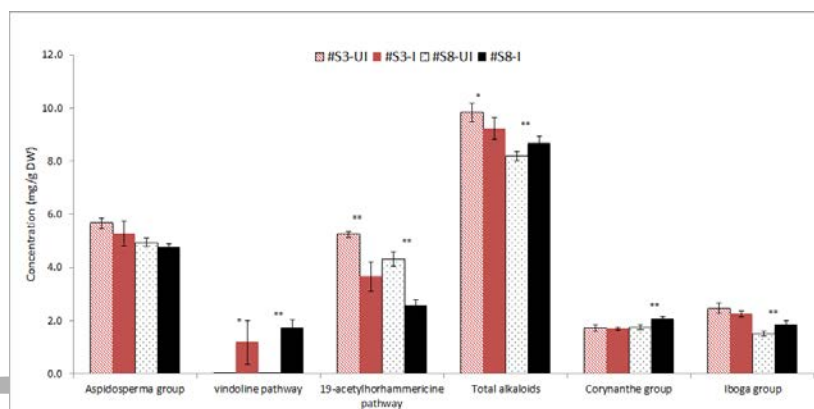


Figure 6

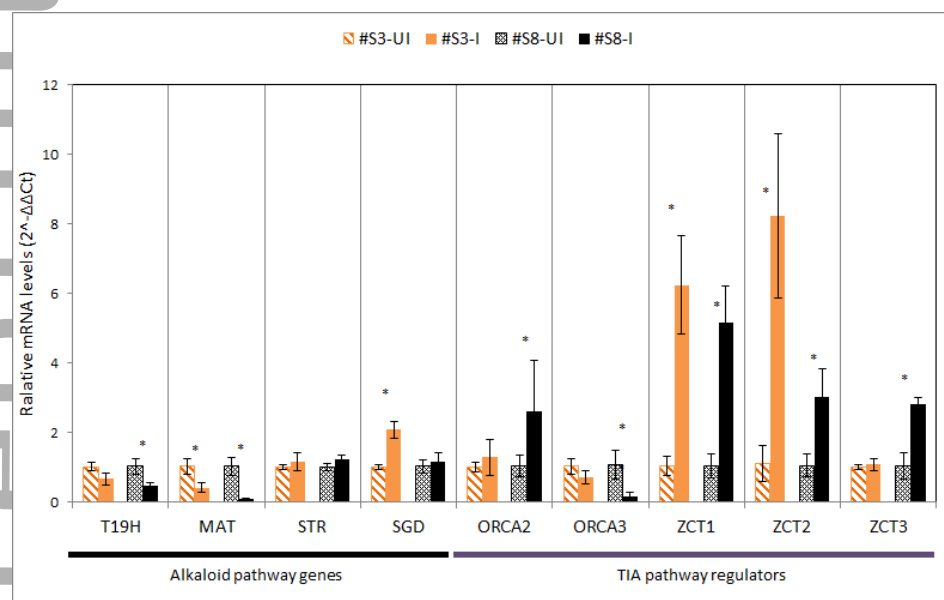


Figure 7