

Limitation of Mitragynine Biosynthesis in *Mitragyna speciosa* (Roxb.) Korth. through Tryptamine Availability

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Metabolite profiles of *Mitragyna speciosa* were determined by means of ¹H NMR-based and HPLC-based analyses. The results indicated that high contents of secologanin, caffeic acid, gallic acid, epigallocatechin, and mitragynine were accumulated in leaves. In *M. speciosa*, feedings of tryptamine, tryptophan, phenylalanine or tyrosine significantly increased the mitragynine contents. Feedings of tryptamine and loganin also enhanced the mitragynine accumulation, but feeding of loganin only did not affect the mitragynine level. The mRNA levels of anthranilate synthase alpha subunit (*ASA*), tryptophan decarboxylase (*TDC*), and strictosidine synthase (*STR*) were measured by quantitative real-time polymerase chain reaction (RT-qPCR) in control plants and those exposed to methyl jasmonate (MJ; 10 μM). All genes responded to MJ after a 24-h treatment. The mitragynine contents were also enhanced and corresponded to the transcript levels. From the present results we conclude that a high content of secologanin together with a undetectable level of tryptamine in *M. speciosa* feature the limitation of mitragynine biosynthesis. Additionally, expression of all the genes limits production of an essential precursor for mitragynine production.

Key words: *Mitragyna speciosa*, Mitragynine, Tryptamine

Introduction

Mitragyna speciosa (Roxb.) Korth. (Rubiaceae) is a tropical tree distributed in Southeast Asia, mainly in Thailand and Malaysia. The plant is generally known as ‘kratom’ in Thailand and ‘ketum’ in Malaysia (Singh, 1932). *M. speciosa* produces various kinds of monoterpenoid indole alkaloids (MIAs). Valuable MIAs are mitragynine and 7-hydroxymitragynine. Mitragynine, which accumulates in *M. speciosa* leaves, has been traditionally used as an opium substitute, and for treating diarrhea and cough in Thailand (Suwanlert, 1975; Thongpraditchote *et al.*, 1998). Owing to its antinociceptive activity as well as its low addictive properties, mitragynine has attracted much scientific interest. In addition, 7-hydroxymitragynine was found to be 10-fold more potent than morphine, the classical opiate (Kikura-Hanajiri *et al.*, 2009). As with other metabolites found in plants, mitragynine is produced only in low yields of about 0.81% (w/w) of dried leaves (Ponglux *et al.*, 1994). Therefore, the principle aim of the

experiments described here was to study the regulation of the mitragynine biosynthetic pathway. This knowledge might be further used to develop strategies for the metabolic engineering of *M. speciosa* to obtain a more efficient production of mitragynine or 7-hydroxymitragynine. Both metabolites and transcription profiles have been used as tools to obtain insight into the mitragynine biosynthesis. Metabolite profiling may provide the most useful information about the state and dynamics of the metabolic pathways of an organism at a given time point. Moreover, a metabolite profile represents the endpoint of gene expression at a particular time, and changes in the metabolite concentrations are expected to correlate with changes in the transcript levels (Sumner *et al.*, 2011).

The biosynthesis of MIAs involves several enzymatic steps that are distributed among several subcellular compartments. In *M. speciosa*, mitragynine and 7-hydroxymitragynine originate from strictosidine, which is the condensation product

of secologanin from the terpenoid pathway and tryptamine from the shikimate pathway (Rueffer *et al.*, 1978; Nagakura *et al.*, 1979). Recently, the regulation of *M. speciosa* strictosidine synthase has been investigated and found to respond to high concentrations of salicylic acid (Jumali *et al.*, 2011). Application of methyl jasmonate and yeast extract to *M. speciosa* shoot cultures increased the transcript levels of tryptophan decarboxylase and strictosidine synthase (Wungsintaweekul *et al.*, 2012). Since mitragynine is a compound of medicinal importance, the regulation of the mitragynine biosynthesis is an attractive area of research for advances in biotechnological applications. Thus, in the present study, we report the transcript profiles of the genes involved in the early steps of mitragynine biosynthesis. The limitation of mitragynine production was assessed by establishing metabolite profiles and by precursor feeding.

Experimental

Chemicals

Loganin, secologanin, tryptamine, and tryptophan were from Sigma-Aldrich (Singapore). Lloyd & McCown woody plant medium (WPM; L449) was from PhytoTechnology Laboratories (Lenexa, KS, USA). *N*-Benzyladenine (BA) was from Fluka Chemie (Buchs, Switzerland). Thidiazuron (TDZ) was from Supelco (Bellefonte, PA, USA). Authentic mitragynine was isolated from the leaves of *M. speciosa* as described previously (Janchawee *et al.*, 2007). Its structure was confirmed by ^{13}C NMR spectrometry. The purity was approximately 98%, as determined by high-performance liquid chromatography (HPLC).

Plant materials

M. speciosa seeds were collected from Hat Yai district, Songkhla, Thailand. The seeds were surface-sterilized by rinsing with 70% (v/v) ethanol for 5 min, rinsing with 20% (v/v) Clorox[®] for 5 min, and finally rinsing with sterile distilled water. Sterilized seeds were germinated on hormone-free WPM medium and incubated at 25 °C under long-day conditions (16 h light/8 h dark). After 2 months, the plantlets were ready for analysis of metabolites, feeding precursors, and treatment with methyl jasmonate (MJ).

The shoot cultures of *M. speciosa* were initiated from axillary buds of the two-month-old *M.*

speciosa plantlets as described previously (Wungsintaweekul *et al.*, 2012). The axillary buds were cut off and placed on WPM medium supplemented with 2 mg/L TDZ and 1 mg/L BA. The shoot culture was maintained at 25 °C under light for 16 h/d. These shoot cultures were used as starting materials for feeding precursors.

NMR-based metabolite analysis

Leaves of *M. speciosa* were harvested, immediately ground in liquid nitrogen, lyophilized, and divided into 50-mg lots to be ready for ^1H NMR analysis. A sample was transferred to a 2-mL microtube, to which 0.75 mL CD_3OD and 0.75 mL D_2O (KH_2PO_4 buffer, pH 6.0) were added. The mixture was vortexed at room temperature for 1 min, ultrasonicated for 20 min, and then centrifuged at $15,500 \times g$ at room temperature for 20 min. The supernatant was then transferred to a 5-mm NMR tube and used for the NMR analyses. ^1H NMR and 2D *J*-resolved spectra were recorded at 25 °C on a 500-MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany). CD_3OD was used as the internal lock. The resulting spectra were manually phased and baseline-corrected, and referenced to the internal standard [0.01% (w/w) trimethylsilanepropionic acid (TMSP)] at 0.0 ppm, using Topspin (version 2.1; Bruker). The 2D *J*-resolved NMR spectra were acquired using 8 scans per 128 increments of F_1 and 8k increments for F_2 using spectral widths of 500 Hz in F_2 (chemical shift axis) and 66 Hz in F_1 (spin-spin coupling constant axis). The *J*-resolved spectra were tilted by 45°, symmetrized about F_1 , and then calibrated using XWIN NMR software. The obtained NMR spectra were compared to the NMR spectra database of the Natural Products Laboratory, Institute of Biology, Leiden University, Leiden, The Netherlands. Chemical shifts (δ) are reported in ppm and coupling constants (*J*) are given in Hz.

HPLC-based metabolite analysis

Freeze-dried *M. speciosa* leaves, roots, and stems (50 mg) were separately extracted by reflux boiling with methanol at 70 °C for 1 h. The extracts were filtered, then washed twice with petroleum ether, and concentrated under reduced pressure. Samples were analysed immediately after extraction in order to avoid possible chemical degradation.

HPLC analysis was carried out using an Agilent 1100 series system equipped with an Agilent 1100 series photodiode-array detector (San Francisco, CA, USA) and a fluorescence detector; data analysis was performed using Agilent software. Chromatographic separation was performed on a reverse phase VertiSep™ UPS C18 column (250 mm × 4.6 mm i.d., 5 μm particle size) (Vertical, Bangkok, Thailand) with a binary gradient mobile phase profile [1.5 mL/min; acetonitrile/100 mM H₃PO₄ in water, pH 2.4 (10:90 to 90:10, v/v, within 15 min)]. The identification of metabolites, including secologanin, tryptophan, tryptamine, and mitragynine, was based on the retention times and comparison of the absorption spectra with those of authentic standards. The wavelength for quantitative determination was set at 225 nm and 238 nm for mitragynine and secologanin, respectively. Tryptophan and tryptamine were quantified by fluorescence measurements with the excitation wavelength at 270 nm and the emission wavelength at 370 nm. Quantification was repeated three times for each culture. The parameters of linearity, reproducibility, accuracy, and specificity of the method were evaluated.

Precursor feeding

Solutions of 1 mg/mL of tryptamine, tryptophan, tyrosine, and phenylalanine were separately prepared in 50% (v/v) aqueous methanol, filter-sterilized, and individually added to liquid WPM to 100 μg/mL final concentration. The 2-month-old plantlets were grown in the media under long-day conditions for 1 month, the control samples were cultivated in WPM supplemented only with the corresponding amount of 50% (v/v) aqueous methanol. After feeding for 1 month, the newly formed shoots of plantlets were collected in order to determine the amounts of metabolites by HPLC as mentioned above. Three samples of each experiment were separately prepared for analysis.

A feeding study of tryptamine, loganin, and a combination of both compounds on mitragynine production was performed in *M. speciosa* shoot cultures. A cluster of shoots (4–5 shoots/explant) was inoculated into 30 mL of liquid WPM supplemented with the same plant growth regulators. Stock solutions of tryptamine were prepared in ethanol at concentrations of 250 mM and 500 mM. Stock solutions (24 μL each) were added to the

shoot cultures to obtain final concentrations of 0.2 mM and 0.4 mM, respectively. A stock solution of loganin was prepared in sterilized water (100 mM). Volumes of 60 μL and 120 μL of the solutions were added to achieve final concentrations of 0.2 mM and 0.4 mM. Applications of tryptamine, loganin, or combinations of both were performed on the day of inoculation. The shoot cultures were incubated under culture conditions and shaking at 60 rpm. After 14 and 21 d of feeding, the shoot cultures were harvested, thoroughly washed with water, and the excess water absorbed by layers of tissue paper. The shoots were lyophilized and ground to powder. Samples were prepared for quantification of their mitragynine content.

Methyl jasmonate (MJ) treatment

Two-month-old plantlets were removed to liquid WPM. A stock solution of 1 mM MJ was prepared in 50% (v/v) aqueous methanol. The MJ solution was added to obtain a final concentration of 10 μM MJ. Plantlets were incubated for 12 h and 24 h. Leaves and stems were excised. Samples were lyophilized and prepared for HPLC analysis. For quantitative real-time polymerase chain reaction (RT-qPCR) analysis, samples were immediately frozen and kept at –80 °C until used.

Transcription profile analysis by RT-qPCR

The plant tissues were ground in liquid nitrogen. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) by following the manufacturer's protocols, including the DNA elimination step. The RNA pattern was evaluated for intactness by electrophoresis in 1.2% (w/v) agarose gel. RNA amounts were determined by the absorbance at 260 nm (A_{260}). The A_{260}/A_{280} ratios of the purified RNA samples were in the range of 1.8 to 2.1. The first-strand DNA was then synthesized by reverse transcriptase. The reverse transcription was carried out on 1.0 μg of total RNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) following the user's manual.

The RT-qPCR was used to determine the transcription of *M. speciosa* anthranilate synthase alpha subunit isoform 1 and isoform 2 (*ASA1* and *ASA2*; GenBank ID: JQ775867 and JQ775866), tryptophan decarboxylase (*TDC*; GenBank ID: JN643922.1), and strictosidine synthase (*STR*; GenBank ID: EU288197.1) genes from the non-

Table I. List of primers used in RT-qPCR.

Primer name	Primer efficiency ^a	Nucleotide sequence (5' → 3')	Amplicon size [bp]
18S rRNAf	0.88	CAAAGCAAGCCTACGCTCTG	530
18S rRNAr		CGTCCACCACTAAGAACG	
EF1af	0.97	CTGGTGGAACAAGATGGATG	270
EF1ar		CCAATACCACCAATCTTGTA	
TUAf	0.84	ACGCTGTTGGTGGTGGTAC	106
TUAr		GAGAGGGGTAAACAGTGAATC	
UBQ10f	0.89	ATCACCTTGAAGTGGA	194
UBQ10r		GAAACCACCACGAAGAC	
UBQ5f	0.80	GACGCTTCATCTCGTCC	186
UBQ5r		CCACAGGTTGCGTTAG	
TEF2f	0.81	GGTGTGACGATGAAGAGTGATG	129
TEF2r		TGAAGGAGAGGGGAAGGTGAAAG	
ASA1f	0.97	CTCTCTCCAGTCTTCATCTCC	316
ASA1r		CACAACCATCTCCTTCAACTTCC	
ASA2f	0.97	CCACAGTTTCTCCTCCGTCA	311
ASA2r		CTGTTCCACTCTTCGTCCTTG	
TDC147S	0.98	GCTTTTTTCGTC AAGACTTGCAAATTGGCTG	361
TDC508A		GTAACAAAGTAGCCACTTATGAGGGCTC	
STR808S	0.92	GGAATTACTGTTACGCCTAGAGC	245
STR1053A		AGAAGAAGCCACTCCATTCAAAG	

^a Primer efficiency was calculated from REST 2009 software V.2.0.13 (Qiagen).

induced and MJ-induced samples. The RT-qPCR was performed using specific primers (Table I) on an ABI PRISM[®] 7300 Sequence Detector (Applied Biosystems, Foster City, CA, USA) with SYBR[®] Green to monitor the synthesis of double-stranded DNA. The software program Vector NTI[®] (Invitrogen) was used to design and determine the propensity of primers to form dimers and to determine the optimal annealing temperature. The PCR reactions were carried out in a 96-well plate, and the final PCR reaction volume was 20 μ L, containing 1x SYBR[®] GreenER[™] qPCR Supermix for ABI PRISM[®], 300 nM ROX reference dye, 0.2 μ M forward primer, 0.2 μ M reverse primer, 15 ng/ μ L cDNA, and DEPC-treated water in a total volume of 20 μ L. Amplifications involved 40 cycles of PCR reaction and the conditions were: 1 cycle at 95 °C for 10 min; 40 cycles at 95 °C for 30 s and 60 °C for 1 min; and finally a dissociation stage at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The dissociation curve was observed to detect possible primer dimers. Samples were run in triplicate, and a negative control of the Master Mix with primers was performed in all runs.

The Normfinder software (Andersen *et al.*, 2004) was used to identify stable reference genes,

and six candidate reference genes including 18S ribosomal RNA (*18S rRNA*), elongation factor-1 α (*EF1a*), translation elongation factor 2 (*TEF2*), α -tubulin (*TUA*), ubiquitin extension protein (*UBQ5*), and polyubiquitin (*UBQ10*) were optimized for their stability. For validation of the PCR efficiency, the dilution series of different input amounts for cDNA were performed equivalent to 60, 30, 15, and 7.5 ng/ μ L of total RNA. Efficiency analysis was performed using the REST 2009 software V.2.0.13 (Qiagen) (Pfaffl *et al.*, 2002). The crossing point (*Cq*) cycles versus the cDNA concentration input were plotted to calculate the slope (mean \pm SD; $n = 3$). The corresponding real-time efficiencies (*E*) as shown in Table I were calculated according to the equation $E = 10^{(-1/\text{slope})} - 1$.

The relative expression levels were also calculated by the REST 2009 software V.2.0.13. When estimating a sample's expression ratio, an intermediate concentration value was calculated using the following equation:

$$\text{concentration} = \frac{\text{efficiency}^{\text{average } Cq(\text{control}) - \text{average } Cq(\text{sample})}}{\text{efficiency}^{\text{average } Cq(\text{control}) - \text{average } Cq(\text{sample})}} \quad (1)$$

To calculate the relative expression, the geometric means of all reference gene concentrations were used according to

$$\text{relative expression} = \frac{\text{concentration of gene of interest}}{\text{geometric mean of the concentration of all reference genes}} \quad (2)$$

The bootstrapping technique was used to provide 95% confidence intervals for the expression ratios. The relative expression data are presented using Whisker-box plots.

Statistical analysis

Values are expressed as means \pm standard deviation (SD). Data were analysed by the paired *t*-test using Microsoft Excel. The levels of 99% and 95% statistical significance were taken at $P < 0.01$ and $P < 0.05$, respectively.

Results

Metabolite profile of *M. speciosa*

¹H NMR spectroscopy was used as a tool for viewing the profile of abundant metabolites of *M. speciosa* leaves, in which mitragynine accumulates (Fig. 1). The metabolites were identified based on a database comparing the unique signals of each compound (Kim *et al.*, 2010). A total of 15 metabolites were identified including amino acids, sugars, organic acids, phenolic acids, flavonoids, triterpenes, and iridoids (Table II). In the aliphatic region, almost all the proton signals (δ_{H} 0.5–3.0 ppm) represented amino ac-

ids such as alanine, arginine, leucine, isoleucine, and valine. The exception were the unique four singlet signals between δ_{H} 0.8–1.0 ppm that were assigned to ursolic acid. The proton signals in the sugar region around δ_{H} 3.0–5.5 ppm overlapped. Nevertheless, glucose, sucrose, and xylose were identified among the signals in this region. In the region around δ_{H} 5.5–9.0 ppm, protons were detected and identified as being from formic acid, gallic acid, caffeic acid, and epigallocatechin. These signals were present in relatively high intensity. A triplet signal at δ_{H} 9.66 ppm (H-aldehyde) and a doublet signal at δ_{H} 7.48 ppm (H-9 proton in the pyran ring) were interpreted to represent secologanin. From the ¹H NMR spectrum, no signals for tryptophan, tryptamine, and mitragynine were detected. The limit of detection and specificity caused some difficulty in identifying all metabolites. The HPLC metabolite analysis thus was performed to detect metabolites involved in mitragynine biosynthesis. Methanol extracts of leaves, roots, and stems of *M. speciosa* cultures were prepared for HPLC analysis.

The optimal conditions for the simultaneous quantitative determination of secologanin, tryptophan, tryptamine, and mitragynine using a gradient reverse phase HPLC system were investigated for the first time in this plant system. All compounds were eluted within 10 min with a satisfactory resolution. The retention times for tryptophan, tryptamine, secologanin, and mitragynine

Table II. ¹H NMR chemical shifts and coupling constants of metabolites found in *M. speciosa* leaves, identified by references and using 1D and 2D NMR spectra^a.

Metabolite	Selected characteristic NMR signals
Alanine	1.58 (d, $J = 7.2$ Hz)
Arginine	1.75 (m), 3.75 (t, $J = 5.5$ Hz)
Leucine and isoleucine	0.96 (d, $J = 7.5$ Hz), 0.98 (d, $J = 7.5$ Hz)
Valine	1.00 (d, $J = 7.0$ Hz), 1.06 (d, $J = 7.0$ Hz)
α -Glucose	5.18 (d, $J = 3.73$ Hz)
β -Glucose	4.57 (d, $J = 7.9$ Hz)
Sucrose	5.40 (d, $J = 3.82$ Hz), 4.17 (d, $J = 8.5$ Hz)
Xylose	3.20 (s), 3.90 (m), 5.18 (d, $J = 3.6$ Hz)
Formic acid	8.45 (s)
Gallic acid	7.01 (s)
Caffeic acid	6.35 (d, $J = 15.9$ Hz), 6.89 (d, $J = 8.2$ Hz), 7.15 (d, $J = 1.9$ Hz), 7.61 (d, $J = 15.9$ Hz)
Ursolic acid	0.80, 0.86, 0.90, 1.00 (s)
Epigallocatechin	4.26 (brs), 2.89 (dd, $J = 17.0, 4.5$ Hz), 2.73 (dd, $J = 17.0, 2.5$ Hz), 6.02 (d, $J = 2.0$ Hz), 6.05 (d, $J = 2.0$ Hz), 6.59 (s)
Secologanin	9.66 (t, $J = 1.4$ Hz)

^a The NMR spectra were processed and compared to the standards in the database of the Natural Product Laboratory, Leiden, The Netherlands.

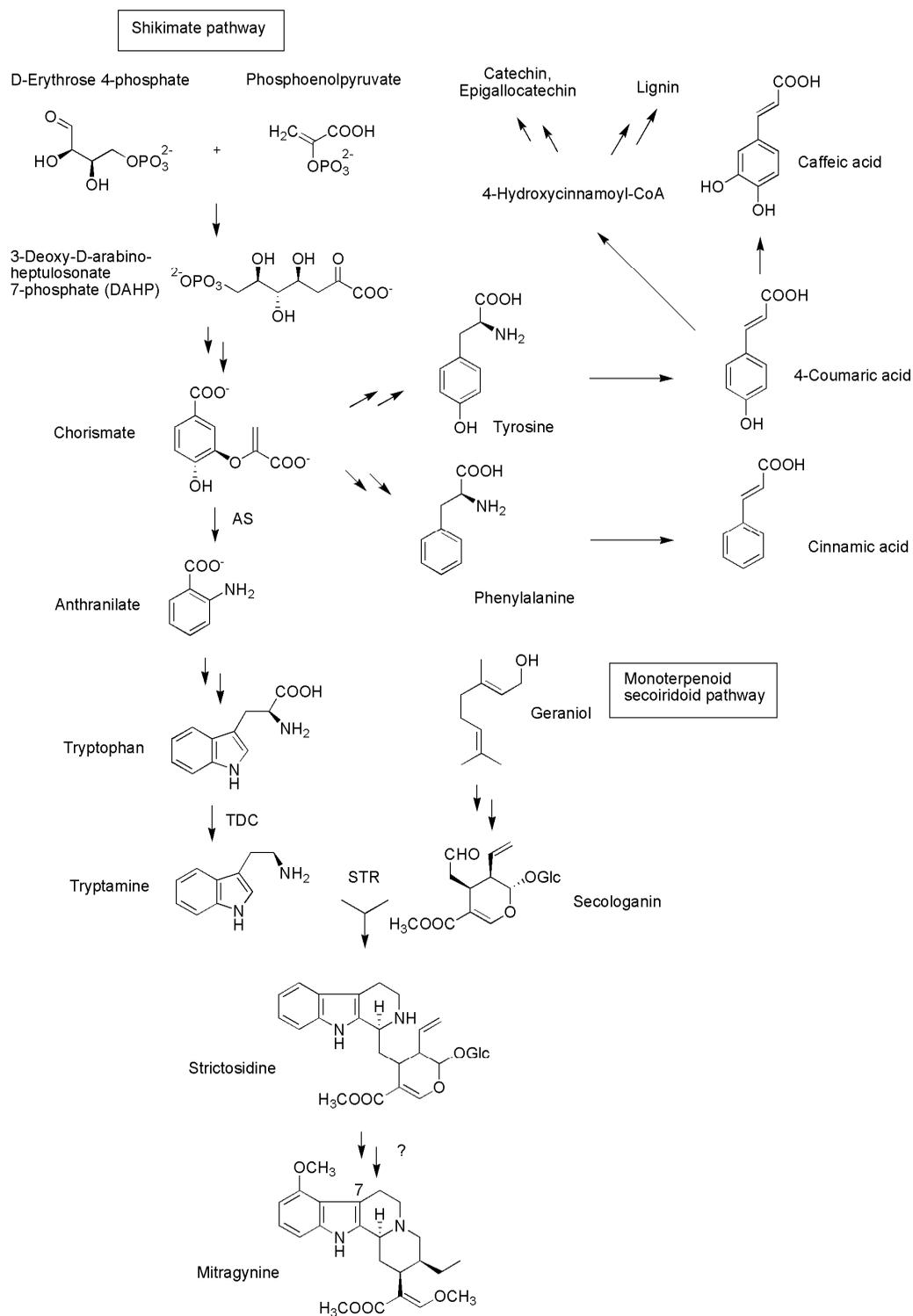


Fig. 1. Proposed mitragynine biosynthetic pathway and of its related metabolites. AS, anthranilate synthase; TDC, tryptophan decarboxylase; STR, strictosidine synthase.

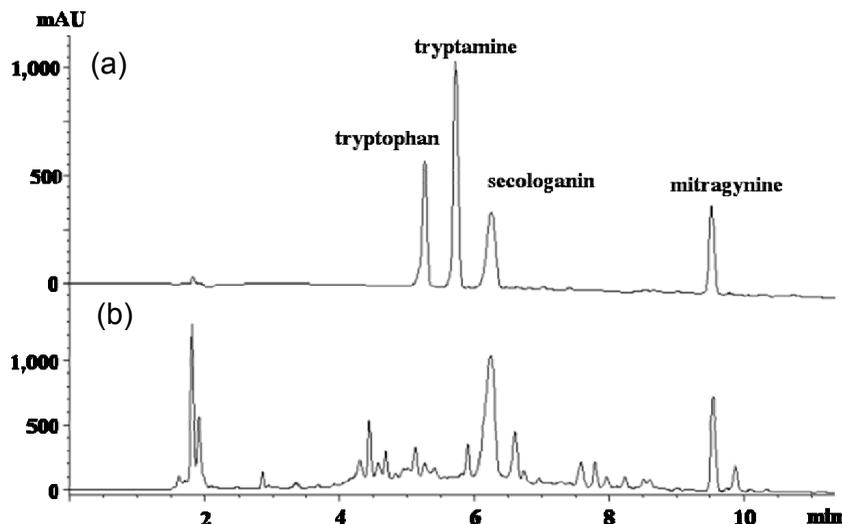


Fig. 2. HPLC chromatograms of (a) authentic compounds and (b) the methanol extract from *M. speciosa* leaves.

were 5.2 min, 5.7 min, 6.2 min, and 9.5 min, respectively (Fig. 2). Linearity was examined using authentic standards over five calibration points with six measurements for each calibration point. Secologanin, tryptophan, tryptamine, and mitragynine exhibited good linearity over the evaluated ranges with correlation coefficients of 0.9997, 0.9999, 0.9998, and 0.9999, respectively. The precision of the method was assessed by determining the relative standard deviation (% R.S.D.) of intra- and interday analysis. The method was shown to be reproducible and reliable as both the intraday and interday precisions were lower than 5%. The accuracy of the method was evaluated by analysing the *M. speciosa* leaf extract spiked with known concentrations of the standards. Prior to spiking, the background levels of metabolites were determined so as to calculate actual recoveries. Mean recoveries were in the range

of 95–100% for all compounds. The established HPLC method was found to be very sensitive for tryptophan and tryptamine with a limit of detection (LOD) and limit of quantification (LOQ) of 0.6 and 2.4 $\mu\text{g}/\text{mL}$, respectively. The LOD and LOQ for secologanin and mitragynine were 0.2 and 1.2 $\mu\text{g}/\text{mL}$, respectively.

The established HPLC procedure was used to determine the content of tryptophan, tryptamine, secologanin, and mitragynine in leaves, roots, and stems of *M. speciosa*. As can be seen from Table III, tryptophan and tryptamine were not detectable in any part of *M. speciosa*. In contrast, secologanin accumulated in considerable amounts in the leaves, followed by smaller amounts in the roots and stems. On the other hand, mitragynine accumulated only in the aerial parts, mainly in the leaves, and was not detected in the roots.

Table III. Metabolite contents in 2-month-old *M. speciosa* determined by HPLC.

Metabolite	Content (mg/g DW \pm SD) ^a		
	Leaves	Roots	Stems
Tryptophan	n.d. ^b	n.d.	n.d.
Tryptamine	n.d.	n.d.	n.d.
Secologanin	9.36 \pm 0.23	4.11 \pm 0.29	4.11 \pm 0.24
Mitragynine	3.96 \pm 0.21	n.d.	0.78 \pm 0.07

^a Mean values \pm standard deviation ($n = 3$).

^b n.d., not detected.

Table IV. Metabolite contents in 2-month-old *M. speciosa*, supplemented with precursors.

Metabolite	Content (mg/g DW \pm SD) ^a				
	Control	Fed precursor ^b			
		Tryptamine	Tryptophan	Tyrosine	Phenylalanine
Tryptophan	n.d. ^c	n.d.	0.15 \pm 0.03 ^d	0.10 \pm 0.01 ^d	0.09 \pm 0.01 ^d
Tryptamine	n.d.	n.d.	n.d.	n.d.	n.d.
Secologanin	9.59 \pm 0.12	9.31 \pm 0.20	9.74 \pm 0.09	9.54 \pm 0.10	9.33 \pm 0.15
Mitragynine	3.22 \pm 0.18	6.38 \pm 0.23 ^d	4.66 \pm 0.15 ^d	4.20 \pm 0.18 ^d	4.47 \pm 0.20 ^d

^a Mean values \pm standard deviation ($n = 3$).

^b Each precursor was fed at the concentration of 1 mg/mL.

^c n.d., not detected.

^d Significance at $P < 0.05$ compared with the control group.

Effect of precursor feeding on mitragynine production

Data from the ¹H NMR-based and HPLC-based metabolite analyses, respectively, indicated that there was an excess of the amount of secologanin and no detectable tryptamine in the *M. speciosa* plant model. To assess the limitation on mitragynine biosynthesis, tryptophan and tryptamine were selected, whereas tyrosine and phenylalanine were chosen since they act as precursors for caffeic acid and epigallocatechin (Fig. 1).

After feeding of the amino acids for one month, the newly formed shoots were dissected and prepared for HPLC metabolite analysis. As shown in Table IV, tryptamine was utilized and significantly increased the amount of mitragynine that accumulated by about 2-fold. In addition, tryptophan was also utilized, but to a lesser extent, and the mitragynine contents were enhanced by about 1.4-fold ($P < 0.05$). It can be noted that secologanin was found in comparatively large amounts in all parts.

An additional precursor feeding experiment was performed in *M. speciosa* shoot cultures. Feeding of tryptamine caused an increment of mitragynine production, that was observed after 21 days ($P = 0.08$) (Fig. 3a). In contrast, addition of loganin had no effect on mitragynine production. However, feeding the combination of tryptamine and loganin significantly stimulated mitragynine production within 14 days of feeding ($P < 0.01$). It can be noted that the shoot cultures, fed with this combination for 21 days, contained lower amounts of mitragynine than after 14 days of treatment.

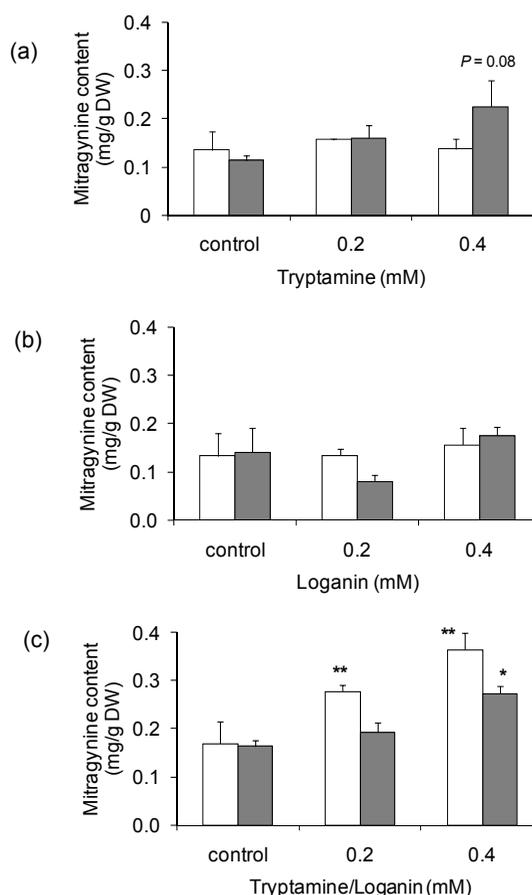


Fig. 3. Mitragynine production in 2-month-old *M. speciosa* shoot cultures fed with (a) tryptamine, (b) loganin, or (c) their combinations for 14 days (\square) and 21 days (\blacksquare). * and ** indicate $P < 0.05$ and $P < 0.01$, respectively.

Transcription profile of the shikimate pathway genes in *M. speciosa*

The mRNA levels of shikimate pathway genes, including *ASA1*, *ASA2*, *TDC*, and *STR*, of *M. speciosa* were measured under non-induced and MJ-induced (for 12 h and 24 h) conditions, respectively. The transcript levels were determined by RT-qPCR for which a method validation was required. RT-qPCR runs with no-template were included in the control mixture in order to check for the purity of the PCR components and non-specific amplification. For the design of primers, the Vector NTI® program provided the oligonucleotides with good specificity and efficiency. The sizes of the PCR products were confirmed by agarose gel electrophoresis. Only one derivative was observed in each PCR reaction, and this had a unique melting temperature profile. Thus formation of primer dimers did not occur.

The PCR efficiency was determined using an external standard curve with a serial dilution series of a cDNA template in separate wells. The efficiency values for each well were calculated by the REST 2009 software V.2.0.13, as shown

in Table I. In order to find an appropriate normalized gene for the RT-qPCR analysis, the expression stability of the normalized gene was analysed using Normfinder algorithms (Andersen *et al.*, 2004). This method identified the optimum reference gene among groups of candidate genes by taking the data from the estimate of their intra- and intergroup variations. An internal control gene was needed for the normalization strategy for the RT-qPCR (Huggett *et al.*, 2005). Six internal candidate genes were selected. Based on the Normfinder data, a combination of *18S rRNA* and *UBQ10* was selected as the reference genes with stability values of 0.586, which was considered to be medium reference target stability.

According to the validated RT-qPCR method, transcripts of all genes were detected in all samples. The *ASA2*, *TDC*, and *STR* were expressed in almost equal abundances among stems and leaves, but the expression of *ASA1* in the stems was higher than that in the leaves (Fig. 4a). After application of MJ (10 μ M) to the plant, levels of the transcripts of all genes increased. The increment of the expression appeared to be most prevalent after 12 h of treatment in the stems and 24 h

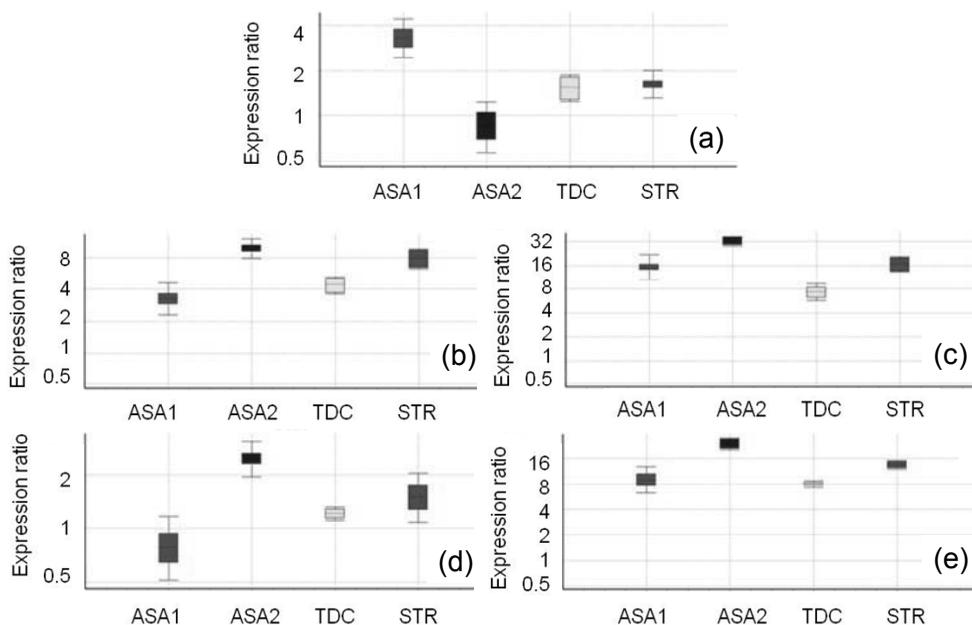


Fig. 4. Whisker-box plot of the relative expressions calculated by the REST 2009 Software of the *ASA1*, *ASA2*, *TDC*, and *STR* genes from (a) the stems relative to the leaves of a non-induced group, (b) 12-h MJ-induced stems relative to the non-induced stems, (c) 24-h MJ-induced stems relative to the non-induced stems, (d) 12-h MJ-induced leaves relative to the non-induced leaves, (e) 24-h MJ-induced leaves relative to the non-induced leaves. *ASA1*, anthranilate synthase alpha isoform 1; *ASA2*, anthranilate synthase alpha isoform 2; *TDC*, tryptophan decarboxylase; *STR*, strictosidine synthase.

in the leaves. After 12 h of treatment with MJ, transcript levels of *ASA1* and *TDC* were about 4-fold and those of *ASA2* and *STR* were about 8-fold higher than in the non-induced stems. All three genes in the stems were significantly expressed (about 8- to 32-fold) after MJ treatment for 24 h (Figs. 4b, c). As expected, the mitragynine content of the MJ-induced stems was increased significantly ($P < 0.05$) (Table V). This was also apparent in the leaves. MJ induced the expression of all genes by about 8- to 16-fold after 24 h of treatment (Figs. 4d, e), which was consistent with the enhanced production of mitragynine (Table V). It was noted that the amount of mitragynine in the leaves increased in the first 12 h and then declined after 24 h of MJ treatment.

Discussion

An NMR- and HPLC-based metabolite profiling study was used to identify the biosynthetic profile of mitragynine and related compounds in *M. speciosa*. The most remarkable outcome, confirmed by both techniques, was the undetectable amount of tryptamine in contrast to the excess amounts of secologanin found in all tissues of *M. speciosa*. In addition, gallic acid, caffeic acid, epigallocatechin, and ursolic acid were present in relatively high amounts in *M. speciosa* leaves. Caffeic acid is known to be involved in the lignin biosynthesis, whereas gallic acid is a constituent of tannins (Dewick, 2002). Since the biosyntheses of caffeic acid and epigallocatechin utilize chorismate from the shikimate pathway, this means that the flux of primary metabolites to a great

extent goes through tyrosine and phenylalanine (Fig. 1). Thus, they compete with the biosynthesis of tryptophan. In order to overcome the possible limitation of substrate availability, the growth medium was supplemented with the mitragynine precursors tryptophan or tryptamine. Tyrosine and phenylalanine were also fed. This revealed that a major limiting factor in mitragynine biosynthesis is the provision of tryptamine, the immediate substrate of strictosidine synthase (*STR*). The amount of mitragynine was increased significantly in all precursor-supplemented samples, and the most influential precursor was tryptamine. While the actual control point is not known, the feedback inhibition of tyrosine and phenylalanine on chorismate utilization and of tryptophan on anthranilate synthase can be proposed. The existence of ursolic acid indicated that there was a channeling of isoprene units into terpenoid biosynthesis. The accumulation of high amounts of secologanin made the monoterpenoid pathway only a second priority for investigation of the potential of metabolic engineering in *M. speciosa*. This conclusion was confirmed by the results of feeding tryptamine or loganin. Although the metabolite profile of *M. speciosa* shoot culture is different from that of the intact plant, the cultures have the capacity to produce mitragynine (Wungsintaweekul *et al.*, 2012). At 0.2 mM and 0.4 mM of tryptamine or loganin, mitragynine production in the shoot culture reacted more sensitively to tryptamine than to loganin, and even more to the combination of both (Fig. 3). The determining factor appeared to be tryptamine.

The role of the shikimate pathway in mitragynine biosynthesis is summarized in Fig. 1. Based on the metabolite profiles and the results of precursor feeding, it is clear that the availability of tryptamine is an important target for further studies on the enhancement of mitragynine yields. Tryptophan decarboxylase (*TDC*) expression probably plays a crucial role in the rate of the conversion of tryptophan to tryptamine, a substrate of *STR*. Evidence from the literature indicates that expression of *TDC* affects MIA accumulation. For instance, induction of ajmalicine formation was observed in low-density *Catharanthus roseus* cell cultures with concomitant enhancement of *TDC* activity (Moreno *et al.*, 1993). In *Agrobacterium rhizogenes* transformed hairy roots from *C. roseus*, the production of MIAs oc-

Table V. Mitragynine contents in stems and leaves of control and MJ-induced plants.

Sample	Mitragynine (mg/g DW \pm SD) ^a
<i>Stems</i>	
Control	1.22 \pm 0.05
12 h MJ	1.46 \pm 0.03 ^b
24 h MJ	1.59 \pm 0.04 ^b
<i>Leaves</i>	
Control	4.01 \pm 0.10
12 h MJ	4.48 \pm 0.13 ^b
24 h MJ	3.90 \pm 0.09

^a Mean values \pm standard deviation ($n = 3$).

^b Significance at $P < 0.05$ compared with the control group.

curred only upon induction by MJ, which caused an elevation of *TDC* transcript levels.

In this study, MJ was also applied to the plants to potentially improve the alkaloid production in *M. speciosa*. It was previously found that MJ induces genes related to MIA biosynthesis. The involvement of an octadecanoid-responsive *Catharanthus* AP2 domain (ORCA)-like transcription factor in *M. speciosa* can be proposed, since in *C. roseus*, MJ has been found to induce ORCA transcription factors that bind to the promoters of *TDC* and other MIA biosynthetic genes, thereby enhancing their transcription and eventually MIA production (Memelink and Gantet, 2007). Recently, CrWRKY1 was identified as another MJ-induced transcription factor in *C. roseus* hairy roots, and its overexpression resulted in an up to 3-fold increase of the serpentine content in these roots (Suttipanta *et al.*, 2011).

In this study, the mitragynine content increased in stems, which was consistent with the higher expression levels of *ASA* and *TDC*. The observation that the mitragynine content of leaves declined after 24-h MJ treatment, as well as after feeding tryptamine and loganin for 21 days, suggests that mitragynine may be not the endpoint of al-

kaloid biosynthesis in *M. speciosa*. Many MIAs are found at much higher concentrations in leaves as compared to stems of this plant (Takayama, 2004). Mitragynine could be metabolized to other alkaloids or be degraded, but the later steps of mitragynine biosynthesis in *M. speciosa* and its turnover still need to be unraveled.

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