Functional genomic analysis for overproduction of key terpenoid indole alkaloids in mature leaf of *Catharanthus roseus*

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Abstract: Metabolic engineering strategies are mandatory in order to alter the production of important secondary metabolites or terpenoid indole alkaloids (TIAs), e.g., vinblastine and vincristine in the medicinal plant *Catharanthus roseus*. In the present study, we have detected high levels of these two bisindoles along with their precursors, vindoline, catharanthine and taberosinine in response to treatment with chemical elicitors. We also detected the expression levels of nine genes in the TIA biosynthetic pathway. There were slight positive effects of 6-Benzylaminopurine (BA) and kinetin on the percentages of catharanthine. Treatments with indole acetic acid (IAA) at low and moderate concentrations (128.51 and 123.57, respectively), and tryptophan as well as kinetin at moderate concentration (162.94 and 185.27, respectively) resulted in higher percentages of taberosinine. Similar results were found when vindoline was treated with different amounts of IAA. Percentages of vindoline increased at moderate concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D), tryptophan and kinetin. Vinblastine and vincristine levels increased with the five different treatments. The study also involved the detection of expression levels of three genes, e.g., *tdc*, *sls* and *str*, directly responsible for the biosynthesis of strictosidine, five genes, e.g., *t16h*, *omt*, *nmt*, *d4h* and *dat*, acting in the six-step conversion of taberosinine to vindoline and *per 1* gene, catalyzing the condensation of catharanthine with vindoline. The results indicated the upregulation of genes *tdc*, *str* and *per 1* due to the effect of 2,4-D. Treatment with tryptophan resulted in increased expression of *tdc*, *str*, *t16h*, *d4h* and *dat* genes. The *d4h* and *dat* genes were upregulated due to the effect of IAA. No influence of BA and kinetin on the expression of genes in the TIA biosynthetic pathway was observed. The results also indicate that other genes related to the biosynthesis of both bisindoles vinblastine and vincristine might be involved and induced by either BA or kinetin. For industry, we recommend overexpressing the genes *tdc*, *str*, *d4h*, *dat* and/or *per 1* through metabolic engineering approaches for large-scale production of vinblastine and vincristine, as well as other key compounds in the pathway, from leaves of *C. roseus* three-month-old plants.
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1. Introduction

The exploration of new metabolic engineering strategies is required in order to alter the production of economically important secondary metabolites or terpenoid indole alkaloids (TIAs) in medicinal plants, e.g., *Catharanthus roseus* (Madagascar periwinkle). This plant is known for the production of the two anticancer bisindoles, namely vinblastine and vincristine (Verma *et al*., 2012; Van Moerkercke *et al*., 2013). All TIA compounds originate from the central compound strictosidine, a condensation product of the monoterpenoid compound, secologanin and the indole compound tryptamine, due to the action of strictosidine synthase encoded by *str* gene (Facchini and De Luca, 2008). Secologanin and tryptamine compounds are synthesized by the two key enzymes, tryptophan decarboxylase and secologanin synthase, which are encoded by *tdc* and *sls* (or *CYP72A1*) genes, respectively. In general, the production of TIAs relies mainly on the action of the three genes *tdc*, *sls* and *str*. TIAs biosynthesis requires the involvement of at least seven intracellular compartments including plastids and extensive transport of intermediates (Facchini and St-Pierre, 2005; Rischer *et al*., 2006; Guirimand *et al*., 2011). Although undifferentiated *C.*

roseus cells and immature leaves produce high levels of several alkaloids in the early phases of TIA biosynthensis, vindoline which represents one of the building blocks for the formation of bisindole alkaloids is synthesized only in mature leaves (Rischer *et al*., 2006). These results explain the failure to significantly increase the production of TIAs in cell cultures or hairy root systems that lack the required complex cellular organization (Shanks and Morgan, 1999; Rischer *et al*., 2006; Ruiz-May *et al*., 2008). Besides, our knowledge of all the enzymes involved in a number of key steps in the TIAs pathway (Zhou *et al*., 2009) is incomplete, except for the six-step conversion of tabersonine to vindoline (Loyola-Vargas *et al*., 2007; Facchini and De Luca, 2008).

The supply of biosynthetic precursors of the shikimate and non-mevalonate pathways (e.g., tryptophan and loganin, respectively) in *C. roseus* plants was found potentially useful in the biosynthesis of TIAs (Van der Heijden *et al*., 2004; Rischer *et al*., 2006). Exogenous tryptophan was reported to cause ~3-fold increase in alkaloid production in *C. roseus* cells (Facchini and Dicosmo, 1991). Supply of the substrate loganin also resulted in higher rate of strictosidine synthesis (Moreno *et al*., 1993). In addition, cytokinins and auxins have been found to induce the regulation of genes in TIA biosynthetic pathway of *C. roseus* cells (Gantet *et al*., 1998; Van der Heijden *et al*., 2004).

In the present study, we utilized 3-month-old *C. roseus* plants to detect levels of the bisindoles, vinblastine and vincristine, along with their precursors, vindoline, catharanthine and taberosinine in response to treatment with chemical elicitors. Besides, expression levels of nine genes in the TIA biosynthetic pathway were also detected in order to obtain a better understanding of the genes influencing high levels of the two important bisindoles.

2. Materials and Methods

Seeds of *Catharanthus roseus* (L.) cv. "Experimental Rose Pink", whose rose has pale pink petals, red radiating eye and red center (El-Domyati *et al*., 2012), were germinated in trays filled with potting mix consisting of vermiculite : perlite (1:1). Seedlings were grown in greenhouse at 14 h/day photoperiod, 80% humidity and 25/22°C day/night temperature regime and watered with half-strength Hoagland solution for one month, then full-length Hoagland solution for two months. Plants at blooming period were treated with five different chemical elicitors for 24 h, and leaves of individual plants were harvested in three replicates. Chemical treatments included indole acetic acid (IAA), 2,4-D, 6-Benzylaminopurine (BA) or kinetin (Kin) at 0.5, 1 and 2 mg/L. Separately, tryptophan was used as an exogenous substrate or precursor of Shikimate pathway at 0.25, 0.5 and 1 mg/L. Leaves of non-treated plants were used as controls. Leaves obtained were instantly surface sterilized using ethanol and sodium hypochlorite to remove all microorganisms.

Sample preparation

Leaves of *C. roseus* under different treatments were dried at 70°C until the weight is constant. Then, leaves were homogenized and 1 g was taken for alkaloids extraction in 2 mL of methanol: water (1:1) for 24 h in screw-capped tubes as previously described (Ferreres *et al*., 2010). The extract was centrifuged at 6000 rpm for 5 min followed by filtration through a 0.2 µm pore size syringe filter and 3 µL were injected into the LC-MS/MS system.

LC-MS/MS analysis

The *C. roseus* alkaloids were analyzed by Acquity UPLC-MS/MS (Waters) system, equipped with Xevo triple quadrupole mass spectrometer with an ESI source. Data acquisition was performed with Masslynx V.4.1 software. The separation was performed with BEH C18 column (50 mm × 2.1 mm, 1.7 µm). The mobile phase was a mixture of water with 0.1% formic acid (A) and acetonitrile (B) in a gradient elution as shown in Table 1. The flow rate was 0.5 mL/min. The column temperature was maintained at 30°C. The mass spectrometer was operated in the positive mode and quantification was performed using selected reaction monitoring (SRM) of the transitions of m/z as shown in Table 2. The heated capillary and voltage were maintained at 350°C and 2.6 KV and nitrogen was used for de-solvation with a flow of 650 L/h. The collision energy was adjusted at 3 V, while the cone voltage was adjusted at 20 V**.** The LC/MS/MS analysis was performed at the Chemical Analysis laboratory, National Gene Bank, Agriculture Research Center, Giza, Egypt.

Table 1. The gradient elution of *C. roseus* alkaloids for UPLC-MS-MS analysis.

Time	% A	% B
0	80	20
12	32	68
13	80	20

Table 2. $(M+H)^+$ values and daughter ions for the five *C. roseus* alkaloids.

Alkaloide	$(M+H)^+$	Daughter ions
Catharanthine	337	173, 144
Taberosinine	337	305, 180
Vindoline	457	427
Vinblastine	811	793, 751, 733, 680, 649, 542, 522, 366, 337
Vincristine	825	807, 765, 747 (13), 723, 705, 687

Calibration curve preparation of vincristine

Vinracine (vincristin sulfate, 1mg/ml) was obtained from EIMC United Pharmaceuticals (EUP), Egypt. Three different concentrations were made from the vincristine solution (0.25, 0.5 and 1 mg/mL). Three microliters were injected into the LC-MS system and the calibration curve was drawn. The quantitative analysis of both vincristine and vinblastine were determined as "vincristine" from the same calibration curve. The other three alkaloids were determined qualitatively (percentages) from their peak areas as compared with the control plants.

RNA isolation and qRT-PCR

Harvested leaves were flash-frozen in liquid nitrogen and stored at -80^{o}C. Then, leaves from individual plants were crushed into a fine powder in a microcentrifuge tube. Total RNAs were extracted from similar-sized leaf samples (0.5 g) of individual plants under different treatments and concentrations using Trizol (Invitrogen, Life Tech, Grand Island, NY, USA). The samples were treated with RNase-free DNase (Promega Corporation, Madison, WI, USA) in the presence of 1 U/uL of RNasin® Plus RNase Inhibitor (Promega) for 2 h at 37^{o}C. Extraction was done in three replicates and RNAs from each treatment were then bulked. RNAs were quantified and diluted at 400 ng/uL. To test for the presence of DNA contamination in RNA samples, the *actin* gene was amplified by PCR of the original RNA samples. Expression levels of ten genes were detected by real time PCR using the Agilent Mx3000P QPCR Systems (Agilent technology, USA). First-strand cDNA was synthesized using 1 ug of total RNA, 0.5 ug of reverse primers of each gene (Table 3) and Superscript II reverse transcriptase (Invitrogen). All cDNA-synthesized samples were diluted (1:10) prior to amplification. The reaction (25 µL) components were 12.5 µL Maxima™ SYBR Green/ROX qPCR master mix, 0.2 µM of each gene forward and reverse primers (Table 3), and PCR-grade water was added up to 22.5 µL. Primers were designed using Netprimer software (http://www.premierbiosoft.com/netprimer/index.html) with the following criteria: length ~20 bases, GC content ~50%, minimal secondary structures, comparable annealing temperatures (55^{o}C) of the primer pairs, and PCR products of ~300 bp. Finally, 2.5 µL of diluted cDNA template were added to the reaction mix. Forty PCR cycles for each gene product included denaturation at 94^{o}C for 15 sec, annealing at 55^{o}C for 30 sec, and extension at 72^{o}C for 45 sec. Amplification for each sample was carried out in triplicate along with a no-template control (NTC, PCR-grade water). Data was collected and amplification plots of ΔRn versus cycle number were generated for analysis. Calculations were made to detect the expression level of each gene under a given treatment relative to its expression under control condition (or fold change). *Actin* gene was used as a reference to show that equal amounts of RNA were used in the analysis.

3. Results and Discussion

LC-MS/MS analysis

The ESI/MS spectrum of the direct injection of the *C. roseus* extract of control plants showed presence of most alkaloids m/z values as shown in Figure 1. The detected $(M+H)^{+}$values were 337.1 (catharanthine and taberosinine), and 457.1 (vindoline). The vincristine ($(M+H)^{+}$ 811) and vinblastine ($(M+H)^{+}$ 825) were not detected in this scan due to their low concentration in the control plants extract, however, both of them were detected in the LC/MS analysis and showed distinct signals in the obtained chromatogram (Figure 2). This scanning is compatible with that performed by Zhou *et al*. (2005), in which both vincristine and vinblastine showed very small signals. The mass spectra of the identified five alkaloids are displayed in Figure 3. The two alkaloids with the same m/z (337.1) were differentiated based on their daughter ions as shown in Table 2 in agreement with the findings of Ferreres *et al*. (2010).

Levels of alkaloids under different treatments

The results of the levels of the five alkaloids under different treatments and concentrations are shown in Table 4. These alkaloids include taberosinine that represents the substrate required for the six-step conversion to vindoline. The latter compound acts together with catharanthine as the two building blocks for the biosynthesis of the two bisindole alkaloids, e.g., vinblastine and vincristine. In industry, vindoline and catharanthine have important industrial implications considering the fact that they are chemically coupled to produce vinblastine and vincristine. The results of the effects of chemical elicitors on the percentages of catharanthine in *C. roseus* plants were either negative or not existent, except for BA, where catharanthine percentage relative to that in the untreated plants increased at 2 mg/L, and kinetin, where its percentage increased at moderate concentration (1 mg/L). However, taberosinine percentages were reduced at different concentrations of 2,4-D or BA, at low (0.5 mg/L) and high concentrations of kinetin, at high concentration of IAA and at low (0.25 mg/L) concentration of tryptophan. Treatments with IAA at low and moderate concentrations, and tryptophan as well as kinetin at moderate concentration resulted in higher percentages of taberosinine. Similar trends of results to those for taberosinine were found when studying vindoline at different IAA treatments.

Percentages of vindoline increased at moderate concentrations of 2,4-D, tryptophan and kinetin, but were reduced at low and high concentrations of 2,4-D (Table 4). Interestingly, vinblastine and vincristine levels increased in similar trends under the five different treatments. None of the chemical elicitor treatments at different concentrations resulted in the reduction of either alkaloid. The influence of chemical elicitor was higher at low concentrations of IAA and BA, while at high concentration of 2,4-D. Levels of the two bisindoles increased in the presence of tryptophan regardless of its concentration. The overall results indicated that treating three-month-old plants with low concentration of BA might be ideal to obtain the highest levels of both bisindoles on a commercial scale.

Differential expression of genes in the TIA biosynthetic pathway under different treatments

This study was done in order to shade some light on the genes whose expression is regulated by a given chemical elicitor and/or related to the levels of the five alkaloids under study. Expression levels of nine genes related to the TIA biosynthetic pathway were detected by quantitative PCR or qPCR (Figure 4). Three genes, e.g., *tdc*, *sls* and *str*, are directly responsible for the biosynthesis of strictosidine, the compound plays a central role in the onset of TIA biosynthetic pathway and the production of the two bisindoles, vinblastine and vincristine (Facchini and De Luca, 2008). The other five genes, e.g., *t16h*, *omt*, *nmt*, *d4h* and *dat*, act in the six-step conversion of taberosinine to vindoline (Rischer *et al*., 2006). The ninth gene, i.e., *per 1*, catalyzes the condensation of catharanthine with vindoline (Costa *et al*., 2008) towards the synthesis of vinblastine and vincristine. The results indicated the upregulation of genes *tdc*, *str* and *per 1* due to the effect of 2,4-D, where expression reached about 4x that of the control plants. In other words, 2,4-D induced expression of genes in the two genes acting upstream the TIA biosynthetic pathway and the gene acting at the last step(s) of vinblastine and vincristine biosynthesis (Figure 4). Treatment with tryptophan resulted in increased expression of *tdc*, *str*, *t16h*, *d4h* and *dat* genes. The results indicated no pattern of expression across tryptophan concentrations used, except for *d4h* gene that showed upregulation in mature leaves. The latter gene along with *dat* gene, act at the last two steps in the pathway of vindoline biosynthesis, were upregulated due to the effect of IAA. No influence of the other chemical elicitors, e.g., BA and kinetin, used in the study on the expression of genes in the TIA biosynthetic pathway (Figure 4) was observed. However, these data unaligned with those of metabolite accumulations in Table 4, where high levels of vinblastine and vincristine were obtained due to the effects of BA or kinetin. This indicates that other genes related to the biosynthesis of both bisindoles vinblastine and vincristine might be involved and induced by either BA or kinetin.

Table 3. Primer names, sequences, expected sizes (nt) in the coding regions of the nine genes related to TIA biosynthetic pathway in *C. roseus* along with the house-keeping gene *actin*.

Gene	Forward (5`-3`)	Reverse (5`-3`)	nt
tdc	ATACCTACGACCGTCGAAAC	GAGTAGCCATTTGTGTGG	300
sls	AGATCCCTTCCACCAAACAG	ATCACCATCAACCCTCCAAC	300
str	CATGATGACAGTCCCGAAGG	TGAAGACACCCAAAAATG	300
t16h	GGCACTGAGACATCGTCAA	TTCCGATAGCCCATGCATTG	295
omt	TTCTTGTTTGAGGGCTTGGC	ATCACCTTTCCACCCTTCGC	299
nmt	CCAACGAGAACAGATCACAC	AACGGAAGCAAATCGGAGAG	300
d4h	GGAATTGCTTGCAATTGGAG	TGTCCTGCATGTCATCAATG	296
dat	TTTCTCAGTCGATGTGCCAC	ACGAACTCTTCCATTTGTGC	300
per 1	AACAACTCGGCCACCAACAG	ATCTACTATGCACGCGCCTC	300
actin	TCCTCTTCCAGCCTTCTCTG	GCTTTGCAATCCACATCTGC	300

Table 4. Percentages of three compounds in the TIA biosynthetic pathway along with levels of vinblastine and vincristine (µg/g DW) in *C. roseus* leaves treated with different chemical elicitors at different concentrations in three replicates as compared to the control untreated leaves.

Treatment	Control	IAA (mg/L)			2,4-D (mg/L)			Trp (mg/L)			BA (mg/L)			Kin (mg/L)		
Concentration	0	0.5	1	2	0.5	1	2	0.25	0.5	1	0.5	1	2	0.5	1	2
Catharanthine (%)	100	107.9	88.3	61.1	54.6	84.2	58.1	98.2	126.3	101.1	93.3	82.9	132.2	123.7	159.0	125.8
Taberosinine (%)	100	128.5	123.6	44.4	39.5	71.6	30.8	69.1	162.5	113.0	61.3	51.4	63.4	46.2	185.3	61.8
Vindoline (%)	100	196.6	154.4	98.9	66.4	147.0	79.9	128.1	237.2	158.8	104.7	124.1	113.2	121.8	162.4	127.5
Vinblastine (µg/g DW)	83.33	687.9	558.2	362.3	255.1	487.9	593.6	579.4	592.9	594.0	716.7	693.7	449.8	380.1	414.8	149.2
Vincristine (µg/g DW)	15.33	422.8	445.7	254.5	242.6	332.1	409.0	392.0	344.1	312.5	425.2	373.1	268.1	350.3	248.0	246.4

Those genes might be related to the steps in the conversion of stemmadinine to catharanthine, stemmadinine to taberosinine, or those downstream in the condensation step towards the synthesis of vinblastine and vincristine.

The role of plant growth regulators in regulating TIA biosynthetic pathway and in improving levels of important alkaloids of *C. roseus* was intensively studied in earlier works (El-Sayed and Verpoorte, 2005; El-Sayed and Verpoorte, 2007a,b; Zhao and Verpoorte, 2007; Roytrakul and Verpoorte, 2007; Ruiz-May *et al*., 2008). More recently, Pan *et al*. (2010) indicated that salicylic acid (SA) and ethylene treatments significantly increased the accumulation of vinblastine, vindoline and catharanthine. However, chlormequat chloride increased the accumulation of vinblastine, only. Overall, our observations indicated that three-month-old *C. roseus* plants, where blooming period started, possessed high yields of alkaloids under different treatments and are ideal targets for commercial use in the production of bisindole alkaloids. Contradicting results were reported for the influence of methyl jasmonate (MeJA) on genes in the TIA pathway, hence, the production of corresponding TIA compounds. While Pan *et al*. (2010) showed no great effect of MeJA on the production of these valuable alkaloids, Rischer *et al*. (2006) and Van Moerkercke *et al*. (2013) indicated the importance of this chemical elicitor in inducing almost all the genes in the TIA pathway and the accumulation of alkaloids with commercial value. Moghazee *et al*. (2014) indicated that treatment with benzyl adenine resulted in higher expression of *str* (1.9x) and *sls* (4.6x) (or *cyp72A1*) genes, while treatment with jasmonic acid resulted in higher expression of *tdc* gene (2x). In the present study, five genes including *tdc* and *str* related to the TIA biosynthetic pathway induced by 2,4-D and IAA were detected.

Based on the results of the present study, we recommend overexpressing the genes *tdc*, *str*, *d4h*, *dat* and/or *per 1* through metabolic engineering approaches (genetic transformation and/or treatment with chemical elicitors) for the large-scale production of vinblastine and vincristine, as well as the other key compounds in the pathway, from leaves of *C. roseus* plants by the industry.

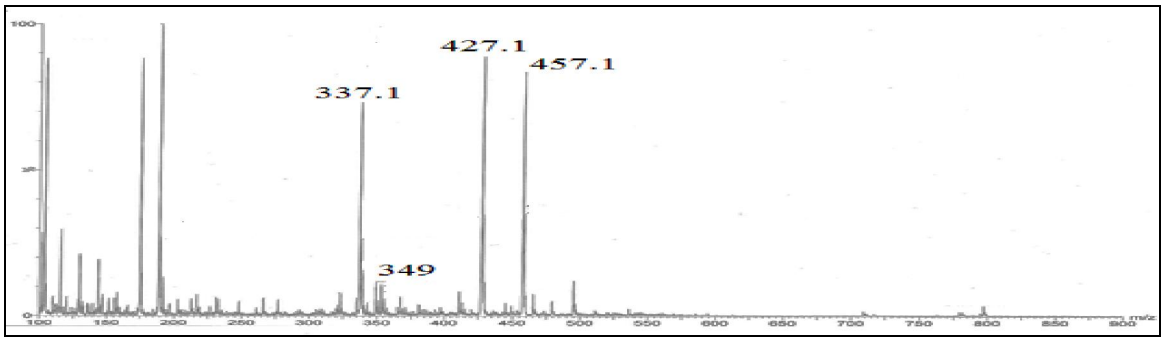

Figure 1. The positive direct-injection ESI/MS spectrum of the *C. roseus* extracts (m/z) showing presence of most alkaloids $(M+H)^+$ values.

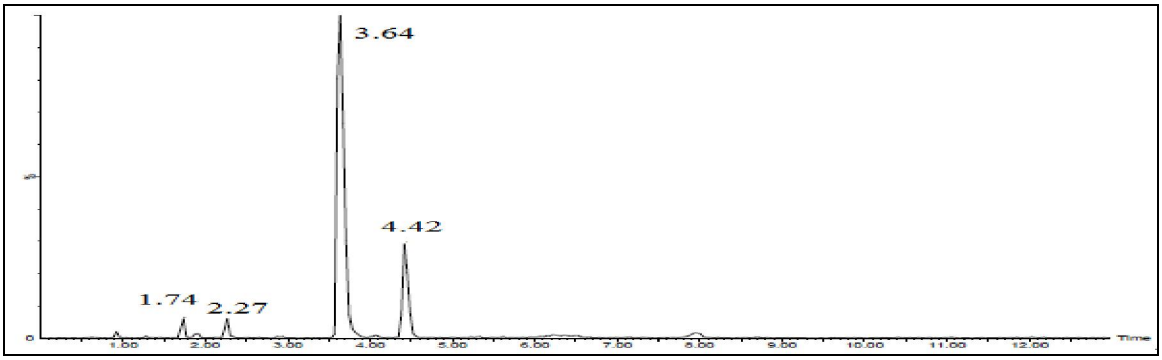

Figure 2. LC/MS chromatogram for separation of *C. roseus* alkaloids. Rt (min) was as follows: 1.74, catharanthine; 2.27, 19-S-vindolinine; 3.64, vindolinine; 3.64, Serpentine, 3.61, Vindoline; 3.69, Vinblastine, 3.76, Vincristine; 4.41, Taberosinine and 4.47, Vindolidine.

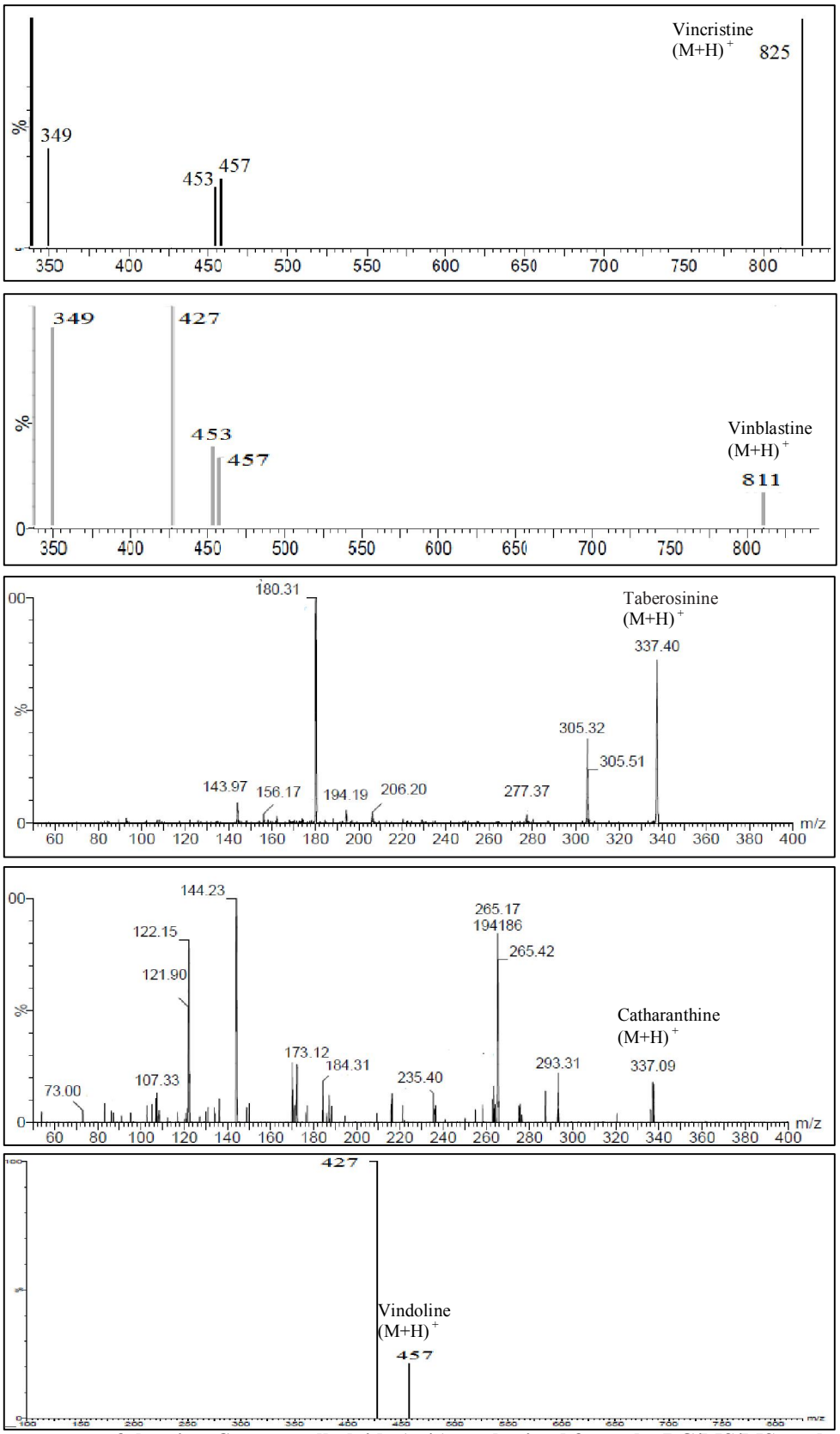

Figure 3. The mass spectra of the nine *C. roseus* alkaloids (m/z) as obtained from the LC/MS/MS analysis.

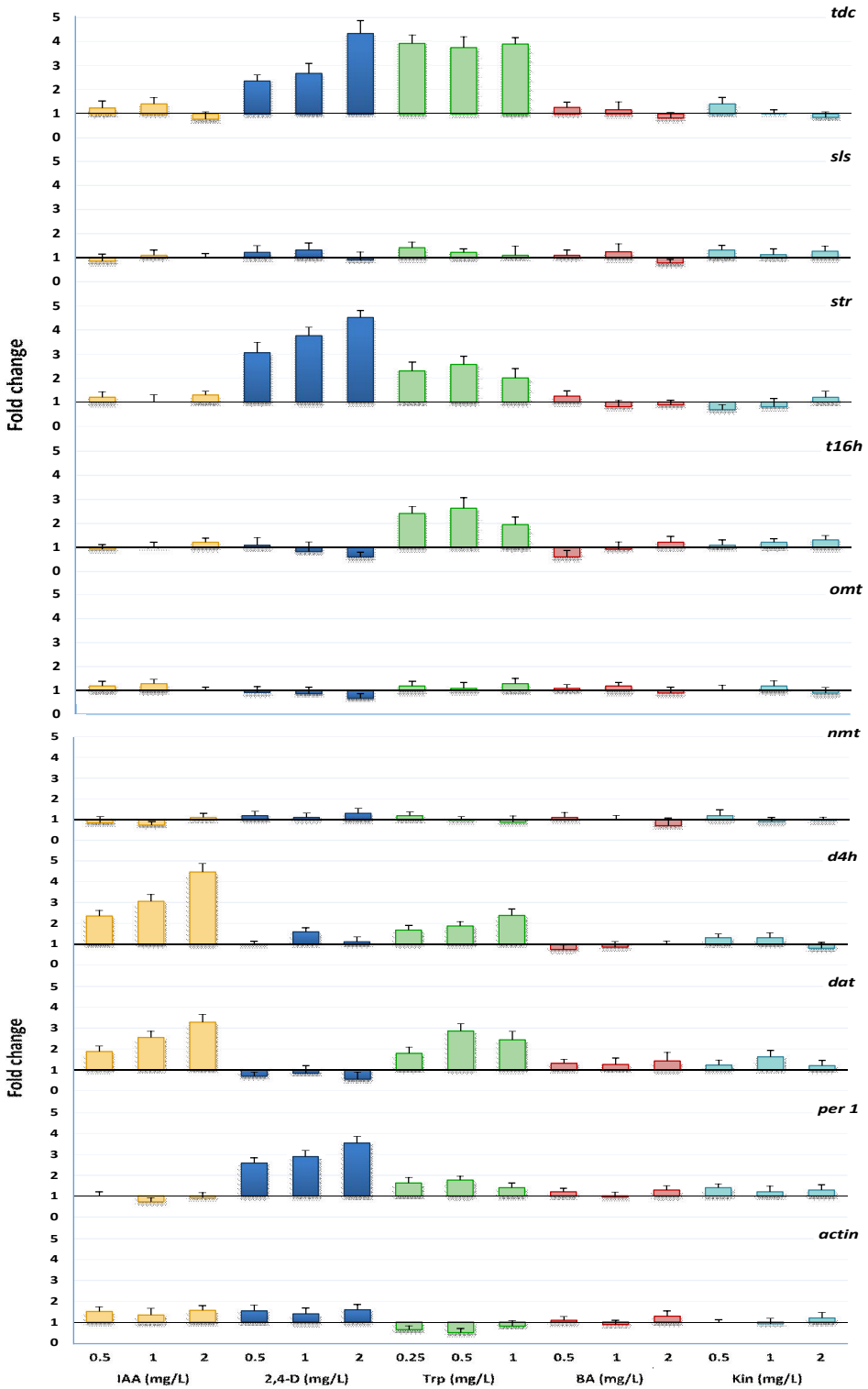

Figure 4. Expression levels (fold change) of nine genes related to the TIA biosynthetic pathway in *C. roseus* leaves treated with different chemical elicitors at different concentrations as compared to the control (1) untreated leaves. The house-keeping gene actin

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