

Enzyme Inhibitors Cause Multiple Effects on Accumulation of Monoterpene Indole Alkaloids in *Catharanthus Roseus* Cambial Meristematic Cell Cultures

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ABSTRACT

Background: Enzyme inhibitors have been used for the clarification of biosynthesis of natural products. *Catharanthus roseus* cambial meristematic cell (CMC) culture has been established and proved to be a better monoterpeneindole alkaloid (MIA) producer than *C. roseus* dedifferentiated cell (DDC) culture. However, little is known about the inter-relationship of the MIA-biosynthetic genes with respect to their transcription.

Objective: To clarify effects of alteration of one gene transcription on transcript levels of another genes in MIA-biosynthetic pathway, and how the accumulation of MIAs in CMCs are influenced by the alteration of their biosynthetic gene transcript levels. **Materials and Methods:** 3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR) inhibitor lovastatin and 1-deoxy-D-xylulose 5-phosphate synthase (DXS) inhibitor clomazone were fed to *C. roseus* CMC cultures. The contents of MIAs were qualified by High Performance Liquid Chromatography and the transcript levels of the relevant genes were measured by qRT-PCR. **Results:** Lovastatin improved the accumulation of MIAs via increasing the transcription of their biosynthetic genes encoding DXS1, tryptophan decarboxylase (TDC), loganic acid methyltransferase (LAMT), strictosidine synthase (STR), desacetoxvindoline-4-hydroxylase (D4H) and ORCA3 (a jasmonate-

responsive transcriptional regulator), whereas clomazone reduced the contents of MIAs and the mRNA levels of the corresponding genes.

Conclusion: The biosynthesis of MIAs in *C. roseus* is manipulated via a complex mechanism, the knowledge of which paves the way for rationally tuning metabolic flux to improve MIA production in *C. roseus* CMCs.

Key words: Cambial meristematic cell, *Catharanthus roseus*, enzyme inhibitor, monoterpeneindole alkaloids,

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INTRODUCTION

More than 3000 different monoterpeneindole alkaloids (MIAs) are found in eight plant families (e.g., Apocynaceae, Loganiaceae and Rubiaceae), some of which have been reported to possess powerful biological and pharmacological activities.^[1,2] In *Catharanthus roseus*, over 100 different MIAs have been characterized,^[3] including ajmalicine with anti-arrhythmic and antihypertensive activities,^[4,5] and vinblastine and vincristine used as anticancer medicines.^[6] Due to their high-value pharmacological activities, many efforts have been made to study the biosynthesis of MIAs.^[1,7]

MIA biosynthetic pathway in *C. roseus* is complex and usually illustrated in four stages: (I) monoterpene biosynthesis, including the production of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), and the formation of monoterpene geraniol derived from IPP and DMAPP; (II) iridoid biosynthesis, i.e., the conversion of geraniol to iridoid glycoside secologanin; (III) early MIA biosynthesis, i.e., the production of strictosidine aglycone via the coupling of secologanin and tryptamine derived from tryptophan, and consequent deglycosylation; (IV) late MIA biosynthesis, including synthesis of all the monoindole alkaloids (e.g., vindoline, catharantine and ajmalicine) derived from strictosidine aglycone, and bisindole alkaloids (e.g., vinblastine and vincristine) produced from coupling between vindoline and catharantine.^[7-12]

In plants, the biosynthesis of IPP occurs via two metabolic pathways: the mevalonic acid (MVA) pathway and the methylerythritol 4-phosphate (MEP) pathway.^[7] Clarification of which pathway provides IPP for biosynthesis of MIAs would pave the way for refining metabolic flux to enhance yields of MIAs in plants and in culturable plant cells/tissues.

Different strategies, including inhibitor experiments, incorporation of labeled precursors and analyses of transgenic lines and mutants were employed to elucidate the metabolic source of isoprenoid units, and some progresses were made.^[13] However, all those efforts only focused on early MIA-biosynthesis steps, such as relationships between MVA pathway and MEP pathway or between isoprenoid (IPP and DMAPP) flux and production of iridoid intermediates.^[7,13,14] Inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) and 1-deoxy-xylulose-5-phosphate synthase (DXS) involved in MVA and MEP pathway, respectively, have been used as additional tools to study regulation of isoprenoid production in plants.^[15] Herein, we used HMGR inhibitor lovastatin and DXS inhibitor clomazone to alter the production of IPP and DMAPP derived from either MVA or MEP,^[16-18] and also investigated their effects on downstream MIA-biosynthetic steps. Our previous work has established a *C. roseus* cambial meristematic cell (CMC) culture system, which is a better MIA producer than both *C. roseus* dedifferentiated cell (DDC) cultures and hairy root

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cultures. In this article, we investigated growth characteristics, yields of MIAs (ajmalicine, vindoline and catharanthine) and transcription of key MIA-biosynthetic genes in *C. roseus* CMCs treated with lovastatin and clomazone, respectively. These findings may provide basis for rationally tuning metabolic flux to enhance production of MIAs in *C. roseus* CMCs.

MATERIALS AND METHODS

Chemicals

Vindoline, catharanthine, ajmalicine, lovastatin, clomazone (2-[2-chlorophenyl]-4, 4-dimethyl-3-isoxazolidinone) and ammonium acetate were obtained from Aladdin (Aladdin Reagents Co., Shanghai, China). Trizol, PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time), and SYBR® Premix Ex Taq™ (TliRNaseH Plus) were purchased from Takara (Takara Bio., Kyoto, Japan). HPLC grade methanol and acetonitrile were obtained from Merck (Merck KGaA, Darmstadt, Germany). All other chemicals were of analytical grade.

Plant Materials and Cell Culture Conditions

C. roseus CMCs used in this research have been established and maintained in our research group as described previously.^[19] CMC cultures were maintained at 25°C under continuous dark in MS solid media supplemented with 2% sucrose, 2.0 mg/L α-naphthylacetic acid (NAA) and 4g/L gelrite. Eight weeks prior to the experiments, 12-day-old CMC cultures were transferred to 250-mL Erlenmeyer flasks containing 100 mL MS solid media. The resulting cultures were added 2.0 mg/L NAA and cultivated at 25 °C with a 12/12-h light/dark photo period. Suspension cultures of CMCs were established by inoculating 12-day-old CMCs (5.0 g fresh weight) into 100 mL of fresh MS liquid media supplemented with 2% sucrose and 2.0 mg/L NAA, and were sub-cultured at 12-day intervals. Also, the suspension cultures were carried out on a HZT-2 gyrotory shaker (Donglian Electronic & Technol. Dev. Co., Beijing, China) with an agitation speed of 120 rpm at 25°C under continuous light. CMC growth was determined by grams of dry weight (DW) per liter.

Growth rate = (dry cell weight/initial dry cell weight) × 100%

Inhibitor Treatment

Lovastatin (200 mg) was dissolved in 7.5 mL of ethanol. After adding 11.25 mL of 0.1 M NaOH and incubating at 50 °C for 2h, the pH was adjusted to pH 7.2 with HCl, and distilled water was added to 50 mL to obtain a 10 mM stock solution of active lovastatin.^[20] In the same as lovastatin solution was prepared, control solution was prepared just without adding lovastatin. Clomazone solution was prepared by dissolving 120 mg of it in 50 mL of 50% (v/v) ethanol to give a 20 mM stock solution, while control solution was 50% (v/v) ethanol.

Twelve-day-old suspensions of *C. roseus* CMCs were centrifuged at 300 × g for 10 min, and the media was discarded. CMCs (5.0g fresh weight) were inoculated into 100 mL of fresh MS liquid media in 250-mL Erlenmeyer flasks at 25°C and 120 rpm under continuous light. After being filter-sterilized, lovastatin and clomazone solutions were added individually to 3-day-old suspension CMC cultures to give final concentrations of 10, 50, 100 and 150 μM, respectively. Control experiments were treated with corresponding blank solutions. Cells were harvested for 4, 6 and 8 days after treatment. The harvested cells were separated from liquid media by vacuum filtration, washed with distilled water, and freeze-dried. Experiments were performed in triplicate.

Alkaloid Extraction and Determination

The extraction of alkaloids from cells and liquid media was conducted according to a reported method.^[21] The extracts were dissolved in 1.0

mL of methanol, filtered through 0.22-μm nylon membrane, and analyzed by HPLC. HPLC analysis was performed using an Agilent 1260 series system (Agilent Technologies, Santa Clara, CA, USA) equipped with a UV detector, an infinity quaternary pump and an autosampler. Chromatographic separations were performed by a Phenomenex Gemini C18 column (250 mm × 4.6 mm, 5 μm) (Phenomenex, Inc., Torrance, CA, USA) at 25°C. The mobile phase consisted of methanol/acetonitrile/10 mM ammonium acetate (15:40:45, v/v/v). The flow rate was set to 1.0 mL/min and the injection volume was 10 μL. The detection wavelength was 280 nm. MIAs were identified and quantified by comparing retention time and UV absorbance spectra with the commercial standards. Each sample solution was analyzed in triplicate.

Monitoring Gene Expression by qPCR

CMC cultures were frozen in liquid nitrogen and ground into the powder using a mortar and a pestle. Total RNA was extracted from CMC cultures according to the reported method.^[19] RNA was quantified using a Nano Drop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Total RNA (1 μg) was treated with DNase to remove genomic DNA using a PrimeScript™ reagent kit with gDNA Eraser (Takara Bio., Kyoto, Japan), and then cDNA was synthesized according to manufacturer's instructions (Takara Bio.).

Table 1: The primer sequences for qRT-PCR

<i>C. roseus</i> gene	Primer Sequence
<i>RPS9</i> (AJ749993)	
Forward primer	TGAAGCCCTTTTGAGGAGGATG
Reverse primer	TGCCATCCCAGACTTGAAAACA
Product size (bp)	122
<i>DXS1</i> (KC625536)	
Forward primer	CGTGGGATGATTAGTGGTTC
Reverse primer	ATACTTGTCCGCTGCTCTCTC
Product size (bp)	207
<i>DXS2A</i> (AJ011840)	
Forward primer	AGGTGAGATCCCTTTTCTCTCC
Reverse primer	TTCTTGTGGCTTGCACATTTAG
Product size (bp)	292
<i>DXS2B</i> (DQ8486762)	
Forward primer	GGCTGGCCTAACTCCAAAG
Reverse primer	TGATATTTTCCCTAATTCCACA
Product size (bp)	230
<i>DXR</i> (AF250235)	
Forward primer	TCAAGCAGAAGCTGTAAGTCA
Reverse primer	ACCAATACAAAGAAAACCCAACT
Product size (bp)	159
<i>LAMT</i> (EU057974)	
Forward primer	GAGTAATTGATGCAGCCAAG
Reverse primer	TTGATTGGATCAAAGATTGG
Product size (bp)	88
<i>TDC</i> (M25151)	
Forward primer	TCCGAAAACAAGCCCATCGT
Reverse primer	AAGGAGCGGTTTCGGGGATA
Product size (bp)	126
<i>STR</i> (X61932)	
Forward primer	TGACAGTCCCAGAGGTGTGG
Reverse primer	CGCCGGGAACATGTAGCTCT
Product size (bp)	122
<i>DAH</i> (U71605)	
Forward primer	TACCCTGCATGCCCTCAACC
Reverse primer	TTGAAGGCCCAATTTGAT
Product size (bp)	121
<i>ORCA3</i> (AJ251250)	
Forward primer	CGAATTCATGCGGAAAGC
Reverse primer	CCTTATCTCCGCCGCAACT
Product size (bp)	146

The transcript levels of 40S Ribosomal Protein S9 (RPS9, the housekeeping gene) and the concerned genes (*DXS*, *DXS2A*, *DXS2B*, *DXR*, *TDC*, *LAMT*, *STR*, *D4H* and *ORCA3*) were monitored. The primer sequences for *RPS9*, *LAMT*, *TDC*, *STR*, *D4H*, *GES*, *ORCA3*, *SGD*, *DXS1*, *DXS2A*, *DXS2B* and *DXR*^[10,11,15,22] were shown in Table 1.

The qRT-PCR experiments were performed according to the SYBR® Premix Ex Taq™ (TliRNaseH Plus) kit protocol (Takara Bio.). Using the 96-wells thermal cycler (Bio-Rad, Hercules, CA, USA), all the qRT-PCR reactions were performed under the following conditions: 30 s at 95°C, and 40 cycles of 5 s at 95°C and 20 s at 60°C. Melt curve stage analysis (60°C–95°C) was used to verify the specificity of amplicons. The results of qRT-PCR analyses were subject to expression stability assay using Bio-Rad CFX Manager Software (Bio-Rad). All samples were measured in triplicate.

Statistical Analysis

All the values were reported as mean ± SD. Statistical analyses were performed using independent two-tailed Student's *t* test. All comparisons were made relative to untreated controls. Differences were considered significant at $p < 0.05$ (indicated by *; $p < 0.01$ indicated by **).

RESULTS AND DISCUSSION

Effects of Lovastatin and Clomazone on the Growth of *C. roseus* Cambial Meristematic Cells

Firstly, the growth curves of the untreated, lovastatin-treated and clomazone-treated *C. roseus* CMCs were made. Lovastatin and clomazone were added to 3-day-old suspension CMCs of *C. roseus*. After 4, 6 and 8 days, the CMCs were harvested and the dry cell weight of each group was recorded [Figure 1]. (Note: cell growth rate and the concerned MIA contents dramatically declined after 8-day incubation with enzyme inhibitors, so the longest incubation time was set to 8 days.) For all the groups, the cell weight reached to the maximum on the 8th day. In the presence of low-concentrated lovastatin (10 and 50 µM), the cells grew as well as the control groups did, but high-concentrated lovastatin (100 and 150 µM) dramatically inhibited cell growth as compared to the control groups, especially after day 6. Clomazone did not influence cell growth as much as lovastatin did. Forty six per cent and 67% reductions of cell growth were observed only in the presence of 150 µM clomazone on day 6 and 8, respectively. In order to exclude the possibility of MIA-production

decrease caused by cell-growth inhibition, we focused our efforts on the effects of low-concentrated (10 and 50 µM) lovastatin and clomazone on accumulation of MIAs and transcription of their biosynthetic genes, whereas the effects of high-concentrated (100 and 150 µM) lovastatin and clomazone just served as the references.

Effects of Lovastatin and Clomazone on Vindoline, Catharanthine and Ajmalicine Accumulation in *C. roseus* Cambial Meristematic Cell Cultures

C. roseus CMCs were treated with lovastatin and clomazone as mentioned above and MIA accumulation was monitored. The dose-response and time course of the effect of lovastatin and clomazone on the accumulation of vindoline, catharanthine and ajmalicine were showed in Figure 2.

Except for the content of ajmalicine in the clomazone-treated groups, the accumulation of the concerned compounds increased with extension of culturing time, and the maximal contents occurred on the 8th day [Figure 2]. Low-concentrated (10 and 50 µM) lovastatin improved the accumulation of ajmalicine, vindoline and catharanthine, albeit not much. The contents of ajmalicine, vindoline and catharanthine decreased in the groups treated with 100 and 150 µM lovastatin, which might be caused by the toxic activity of high-concentrated lovastatin against cell growth and/or metabolism. Clomazone evidently reduced the accumulation of vindoline and catharanthine, especially on day 6 and 8, and the data showed a dose-response relationship to some degree. Unexpectedly, clomazone extremely inhibited the content of ajmalicine, and even made the production of ajmalicine slower than its consumption [Figure 2 C2], which implied that clomazone might influence the accumulation of ajmalicine not only by inhibiting MEP pathway but also by impacting other steps involved in synthesis/metabolism of ajmalicine.

Effects of Lovastatin and Clomazone on MIA Gene Transcription in *C. roseus* Cambial Meristematic Cell Cultures

Besides detection of MIA contents in *C. roseus* CMC cultures as mentioned above, the transcript levels of the MIA-biosynthetic genes encoding *DXS*, tryptophan decarboxylase (*TDC*), loganic acid methyltransferase (*LAMT*), strictosidine synthase (*STR*), desacetoxyvindoline-4-hydroxylase (*D4H*) and *ORCA3* (a jasmonate-

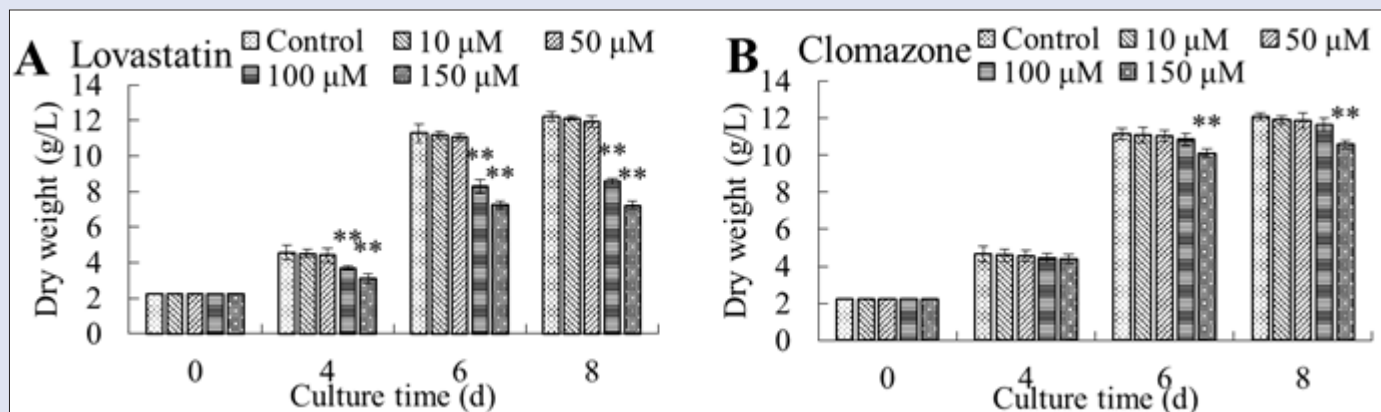


Figure 1: Effects of lovastatin (A) and clomazone (B) on growth of *C. roseus* CMCs in 250-mL Erlenmeyer flasks. Data were analyzed by ANOVA followed by Student's *t* test. Significant differences between treatments and the control are shown as $p < 0.05$ (*) and $p < 0.01$ (**).

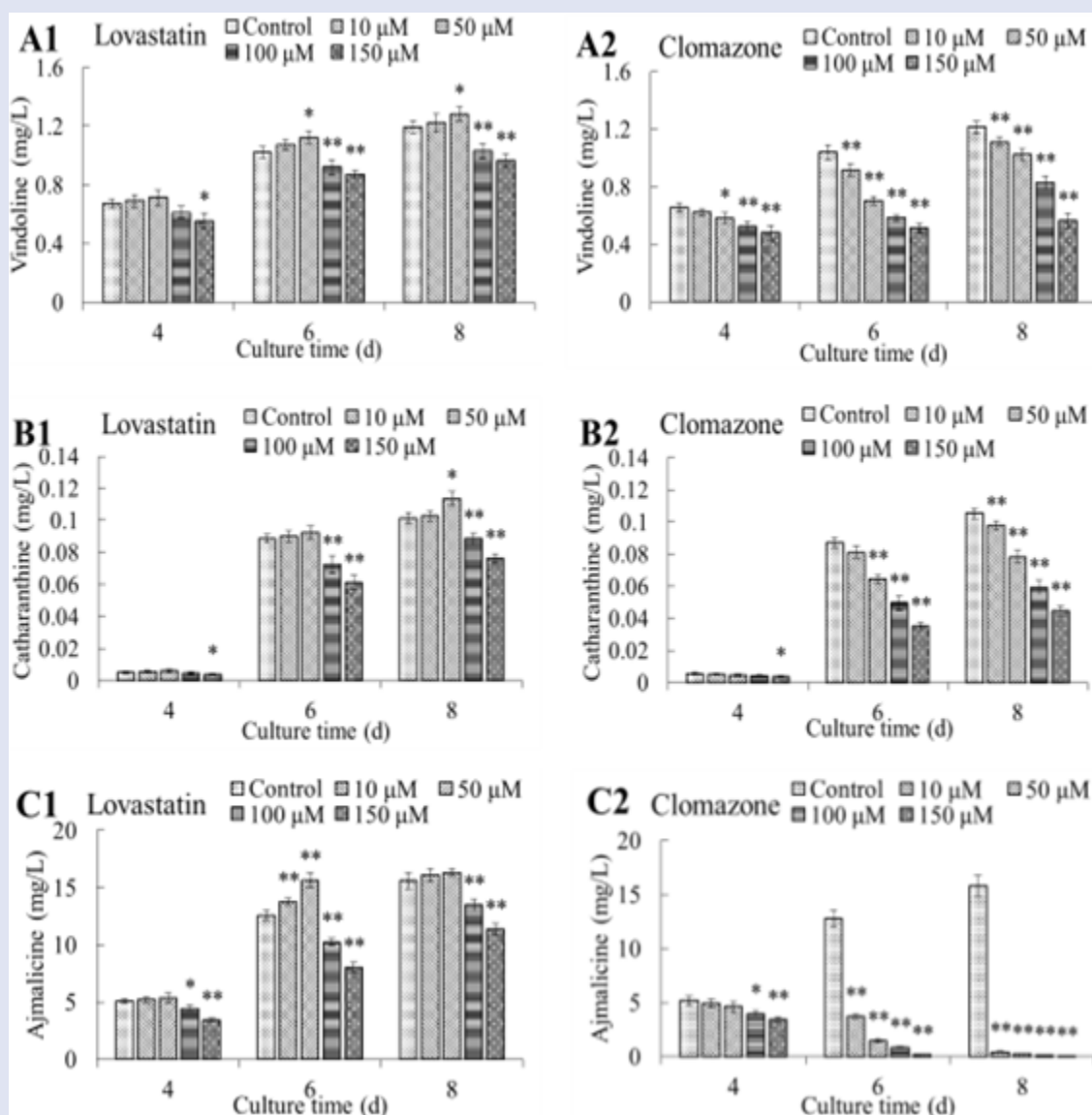


Figure 2: Effects of lovastatin and clomazone on production vindoline (A1 and A2), catharanthine (B1 and B2) and ajmalicine (C1 and C2) in *C. roseus* CMCs. Values are means \pm SD of triplicate experiments. Data were analyzed by ANOVA followed by Student's *t* test. Significant differences between treatments and the control are shown as $p < 0.05$ (*) and $p < 0.01$ (**).

responsive transcriptional regulator) in the untreated and inhibitor-treated *C. roseus* CMCs were monitored in parallel by quantitative reverse transcription (RT)-PCR [Figures 3 and 4]. Among these enzymes, DXS may be derived from three genes, i.e. *DXS1*, *DXS2A* and *DXS2B*. Low-concentrated lovastatin (10 and 50 μ M) slightly increased the transcript amounts of *DXS1* and *DXR* [Figure 3A and 3B] but didn't show effect on *DXS2A* & *2B* transcription (data not shown). However, lovastatin caused dramatic enhancement of the transcript levels of *LAMT*, *TDC*, *STR*, *D4H* and *ORCA3* compared with those of the control. Especially, in the presence of 50 μ M lovastatin, the maximal relative transcript levels of *TDC*, *LAMT*, *STR*, *D4H* and *ORCA3* were 3.1, 2.3, 2.8, 3.4 and 4.0 times higher than those of the control, respectively [Figure 3C–G]. Although it was unclear that how much the transcription of *HMGR* in the CMCs was reduced by lovastatin due to the lack of the knowledge of *HMGR* in *C. roseus*, it is apparent that the inhibition of *HMGR* doesn't decrease accumulation

of MIAs, confirming that the MEP pathway is the major source of IPP used for biosynthesis of MIAs. Inhibition of *HMGR* might cause a global deficiency of IPP and DMAPP in cells, which, together with the crosstalk between MVA and MEP pathways, could lead to the slight increase of the transcription of *DXS1* and *DXR* to overcome the IPP deficiency when the CMCs were treated with 10 and 50 μ M lovastatin [Figure 3A and 3B]. The enzyme DXS is mainly derived for *DXS2A* & *2B* according to the previous report,^[15] the treatment of lovastatin however had no impact on the transcription of *DXS2A* & *2B* (data not shown). Therefore, enhancement of MIA accumulation in lovastatin-treated groups is not due to the increase of *DXS1* mRNA level. Even if the higher *DXS1* mRNA level caused by lovastatin brought into a bit of excess accumulation of IPP, it is unreasonable that the transcription of *TDC*, *LAMT*, *STR* and *D4H* was simultaneously up-regulated because these genes located at the downstream steps of IPP which could inhibit their transcription. The transcription of *ORCA3* and the concerned MIA-

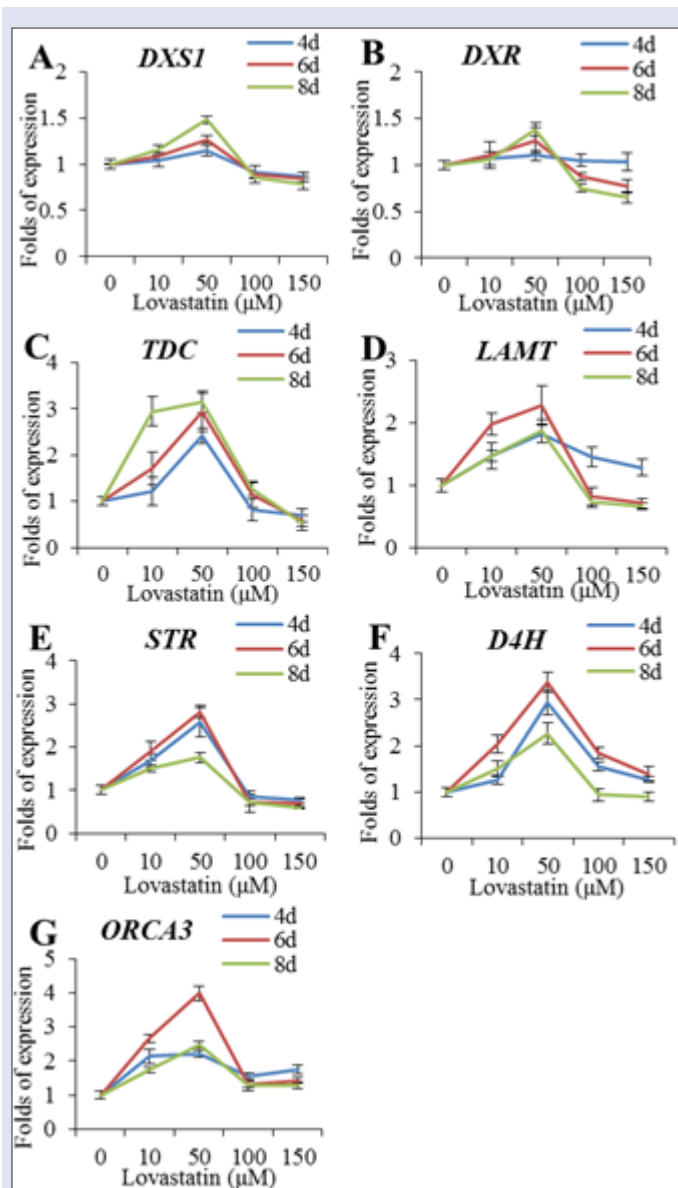


Figure 3: Effects of lovastatin on expression of MIA genes in *C. roseus* CMCs. Values are mean ± SD of triplicate experiments.

biosynthetic genes was almost synchronously induced by lovastatin except that only the maximal induction to *TDC* shifted slightly in time and occurred on day 8 in the presence of 50 M lovastatin, but the magnitude on day 6 was very close to that on day 8 [Figure 3C–G]. Therefore, we reasoned that the transcription of *TDC*, *LAMT*, *STR* and *D4H* was activated by the increase of *ORCA3* transcript level which is induced by lovastatin via an unknown mechanism. This hypothesis is also consistent with the fact that *ORCA3* manipulates the transcription of *TDC*, *STR*, *SGD* and *D4H*.^[23-26]

Four days after the treatment of clomazone, the transcript level of *DXS1* declined and *DXS 2A & 2B* mRNA levels dramatically increased [Figure 4A–C], which was consistent with the reported results.^[15] And the transcript levels of *TDC*, *LAMT*, *STR*, *D4H* and *ORCA3* decreased [Figure 4E–I], which could be the reason that led to decline of MIA accumulation. [Figure 2A2, B2 and C2]

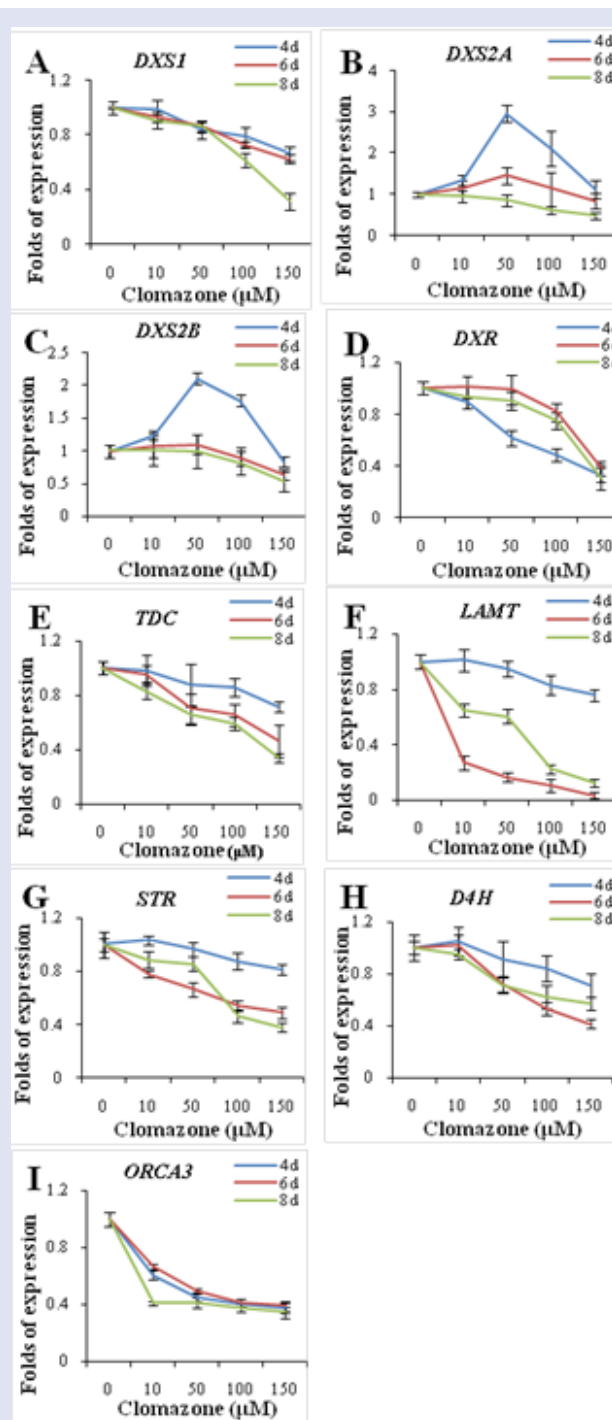


Figure 4: Effects of clomazone on expression of MIA genes in *C. roseus* CMCs. Values are mean ± SD of triplicate experiments.

CONCLUSIONS

In summary, the present study confirmed that *DXS 2A & 2B* mainly contributed to the production of isoprenoid IPP which were used for biosynthesis of MIAs. HMGR inhibitor lovastatin and *DXS1* inhibitor clomazone not only influence the production of IPP and DMAPP, but also cause evident effects on transcription of downstream genes. This indicates that biosynthesis of MIAs in *C. roseus* is manipulated

via a complex mechanism, thus MIA accumulation depends on the comprehensive effects caused by the alteration of the transcription of their biosynthetic genes. These findings pave the way for rationally tuning metabolic flux to improve MIA production in *C. roseus* CMCs.

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