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Overexpression of *LeMYB1* enhances shikonin formation by up-regulating key shikonin biosynthesis-related genes in *Lithospermum erythrorhizon*

H. ZHAO[1,2], Q.S. CHANG[1], D.X. ZHANG[1], R.J. FANG[1], H. ZHAO[1], F.Y. WU[1], X.M. WANG[1], G.H. LU[1], J.L. QI[1,3]*, and Y.H. YANG[1]*

State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing 210093, P.R. China[1]
Engineering Technology Research Center of Anti-aging Chinese Herb, School of Life Sciences at Fuyang Normal College, Fuyang 236032, P.R. China[2]
Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720, USA[3]

Abstract

We previously reported that *LeMYB1* might be a crucial transcription factor in regulating shikonin formation in *Lithospermum erythrorhizon*. In this study, by overexpressing *LeMYB1* under the control of CaMV35S promoter in *L. erythrorhizon* hairy roots, we further clarified the role of *LeMYB1* in the shikonin formation and its regulation. The *LeMYB1*-overexpressing transgenic hairy roots were successfully induced by infecting seedling nodes with *Agrobacterium rhizogenes* strain ATCC15834 that carried the pBI121-*LeMYB1* vector. The *LeMYB1* transcripts were significantly up-regulated in the transgenic hairy root lines compared with the wild type lines, and the total content of shikonin and its derivatives was dramatically enhanced by the *LeMYB1* overexpression. Real-time PCR results reveal that the enhanced shikonin biosynthesis in the overexpressing lines were mainly caused by a highly up-regulated expression of genes coding key enzymes (*PAL*, *HMGR*, and *PGT*) and key regulators (*LeDI-2* and *LePS-2*) involved in the shikonin biosynthesis. Overall, our results suggest that *LeMYB1* plays a positive role in regulating the shikonin biosynthesis in *L. erythrorhizon*.

Additional key words: CaMV35S, pBI121, R2R3 repeat, *RolC*, MeJA, transcription factor, transgenic plants.

Introduction

Lithospermum erythrorhizon roots containing shikonin and its derivatives have been widely used for the treatment of wounds, pathogen infections, and tumors (Jang *et al.* 2014, Wang *et al.* 2014) and as natural colorants in food and cosmetic products (Yazaki *et al.* 1999, Yoo *et al.* 2014). The constant increase in a commercial demand for *L. erythrorhizon* roots and their limited supply from natural resources have promoted the use of biotechnology-based approaches, such as cell or hairy root cultures, for plant conservation and for an enhanced secondary metabolite production (Fujita *et al.* 1981, Shimomura *et al.* 1991, Sykłowska-Baranek *et al.* 2012, Hao *et al.* 2014). However, the large-scale production of shikonin and its derivatives by cell or hairy root cultures is limited. The biggest hurdle is the complexity of the shikonin biosynthetic pathway and the regulatory pathways governing shikonin production. For example, merely overexpressing the genes coding biosynthetic enzymes could not significantly increase the shikonin content in *L. erythrorhizon* hairy roots (Boehm *et al.* 2000). Despite the complexity of these pathways, a significant progress has been made in our understanding

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Abbreviations: *GAPDH* - glyceraldehyde-3-phosphate dehydrogenase encoding gene; *HMGR* - 3-hydroxy-3-methylglutaryl-coenzyme A reductase encoding gene; *LeDI-2* - *Lithospermum erythrorhizon* dark-inducible gene; *LePS-2* - *Lithospermum erythrorhizon* pigment callus-specific gene; MeJA - methyl jasmonate; *PAL* - phenylalanine ammonia lyase encoding gene; *PGT* - *p*-hydroxybenzoate:geranyltransferase encoding gene.
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* Corresponding authors; fax: (+86) 25 89682705, e-mails: qijlberkeley@berkeley.edu, yangyh@nju.edu.cn

of the biochemistry and regulation of the shikonin pathway in *L. erythrorhizon* in recent years. In particular, many genes coding the shikonin biosynthetic enzymes and the transcription factors, which are possibly involved in the regulation of shikonin formation, have been cloned (Yazaki *et al.* 1995a,b, Zhang *et al.* 2010, Zou *et al.* 2011, Zhao *et al.* 2014).

In a previous report, we isolated a full-length cDNA (*LeMYB1*) from *L. erythrorhizon* and found that it is highly expressed in response to methyl jasmonate (MeJA), an effective hormone enhancing shikonin formation. The alignment of LeMYB1 with other MYB proteins revealed that it contains an N-terminal R2R3 repeat, which is involved in jasmonic acid signalling and phenylpropanoid biosynthetic pathway regulation (Gális *et al.* 2006, Zhao *et al.* 2014), and a high degree of amino acid identity to NtMYBJS1 from *Nicotiana tabacum*. This protein might act as an important positive regulator in enhancing shikonin formation (Zhao *et al.* 2014).

In this study, we further clarified the role of *LeMYB1* in shikonin formation by overexpressing *LeMYB1* in *L. erythrorhizon*. The content of shikonin and the expression of genes encoding critical enzymes in the shikonin pathway, including phenylalanine ammonia lyase (PAL), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), *p*-hydroxybenzoate:geranyltrans ferase (PGT), and genes encoding two regulating factors, *L. erythrorhizon* dark-inducible gene (*LeDI-2*) and *L. erythrorhizon* pigment callus-specific gene (*LePS-2*), were evaluated. We attempted to introduce new insights into the regulation of *LeMYB1* in the shikonin biosynthesis pathway and test the possibility of controlling shikonin biosynthesis *via* the engineering of *LeMYB1* gene in *L. erythrorhizon.*

Materials and methods

Plants and growth conditions: Mature seeds of *Lithospermum erythrorhizon* Sieb. *et* Zucc were collected from the Northeast region of China. After stratification in a humid sand at 4 °C for about 30 d, the germinated seeds were sterilized and grown in culture vessels containing 5 cm^3 of a Murashige and Skoog (1962; MS) medium. Seedlings were kept under a 16-h photoperiod, an irradiance of 80 μmol m^{-2} s^{-1} (*TLD36 W/54*, *Philips*, Einhoven, The Netherlands), and day/night temperatures of 25/19 °C; one seedling per culture vessel. When all seedlings developed two to three leaves, the stem node sites of the robust seedlings were used as explants for transformation experiments.

Plasmid construction, hairy root generation, and culture conditions: With cDNA from *L. erythrorhizon* cells (Zhao *et al.* 2014) as template, the open reading frame (ORF) of *LeMYB1* gene (GenBank accession number KC818628) was amplified by PCR using primers listed in Table 1 Suppl. The resultant PCR products were cloned into pBI121 vector (Sugikawa *et al.* 2005) to obtain a pBI121-*LeMYB1* overexpression construct. Subsequently, the genetic transformation of *Agrobacterium rhizogenes* strain ATCC15834 was conducted. Hairy roots were generated by infecting the stem nodes of *L. erythrorhizon* seedlings with ATCC15834 (either carrying the construct or non-transformed as controls). The induced hairy roots were excised from the seedlings and screened on a B5 (Gamborg *et al.* 1968) solid medium containing 0.25 g dm^{-3} cefotaxime and were maintained separately as independent clones. After the elimination of bacteria, the hairy roots were transferred into a B5 liquid medium for propagation at a temperature of 25 ± 1 °C and a constant irradiance of 80 μmol m^{-2} s^{-1}, and were subcultured routinely every two weeks. For the production of shikonin and its derivatives, the hairy roots were transferred into an M9 (Fujita *et al*. 1981) liquid medium maintained on a rotary shaker at 100 rpm and grown at 25 °C in the dark. The transgenic roots were confirmed by PCR with a combination of 35S forward and *GFP*-specific reverse primers. The *LeMYB1* over-expression was determined by real-time PCR using the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene as internal standard. The roots were collected separately from two independent *LeMYB1*-over-expressing lines (OM3 and OM9) and control lines (WT1 and WT2), and these roots were used for real-time PCR and shikonin content analyses.

Measurement of content of shikonin and its derivatives: The production of shikonin and its derivatives was determined as previously described by Heide and Tabata (1987). Metabolites were extracted from the hairy roots and culture medium in each flask using petrol ether. The absorption of the extracts was measured at 520 nm using a double beam UV/Vis spectrophotometer (*UV-2800*, *Zhejiang Scientific Instruments*, Zhejiang, China), and the shikonin content was calculated according to a standard curve. The total shikonin production was the sum of shikonin contained in the hairy roots and secreted into the culture medium. The total shikonin production was expressed as the ratio of total shikonin mass to the fresh mass of hairy roots.

Gene expression analysis by real-time PCR: For cDNA preparation, total RNA was isolated from the hairy roots (WT or OM lines) cultured in a M9 liquid medium for 6 d in the dark using *Trizol* (*Invitrogen Life Technologies Co*., Carlsbad, CA, USA) reagent according to the manufacturer's instructions and quantified by a double beam UV/Vis spectrophotometer (*UV-2800*) at 260 nm. Reverse transcription was performed with 1 μg of total

RNA using *SunShineBio*™ reverse transcription system first strand cDNA synthesis kit (*Sunshine Biotech Co.*, Nanjing, China) following the manufacturer's protocol. Resultant cDNAs were subsequently diluted and used as templates for investigating the transcriptional regulation of *PAL*, *HMGR*, *PGT*, *LeDI-2*, *LePS-2*, and *LeMYB1* in the WT and OM lines of *L. erythrorhizon* by real-time PCR as previously described (Portereiko *et al.* 2006, Wu *et al.* 2009, Zhao *et al.* 2014). Primers of these genes are listed in Table 1 Suppl., and the *GAPDH* gene was used as reference. A representative sample from two biological experiments is shown. In each independent experiment, the relative expression at a maximal level was set to 100, and other data were normalized accordingly (Zhao *et al.* 2014).

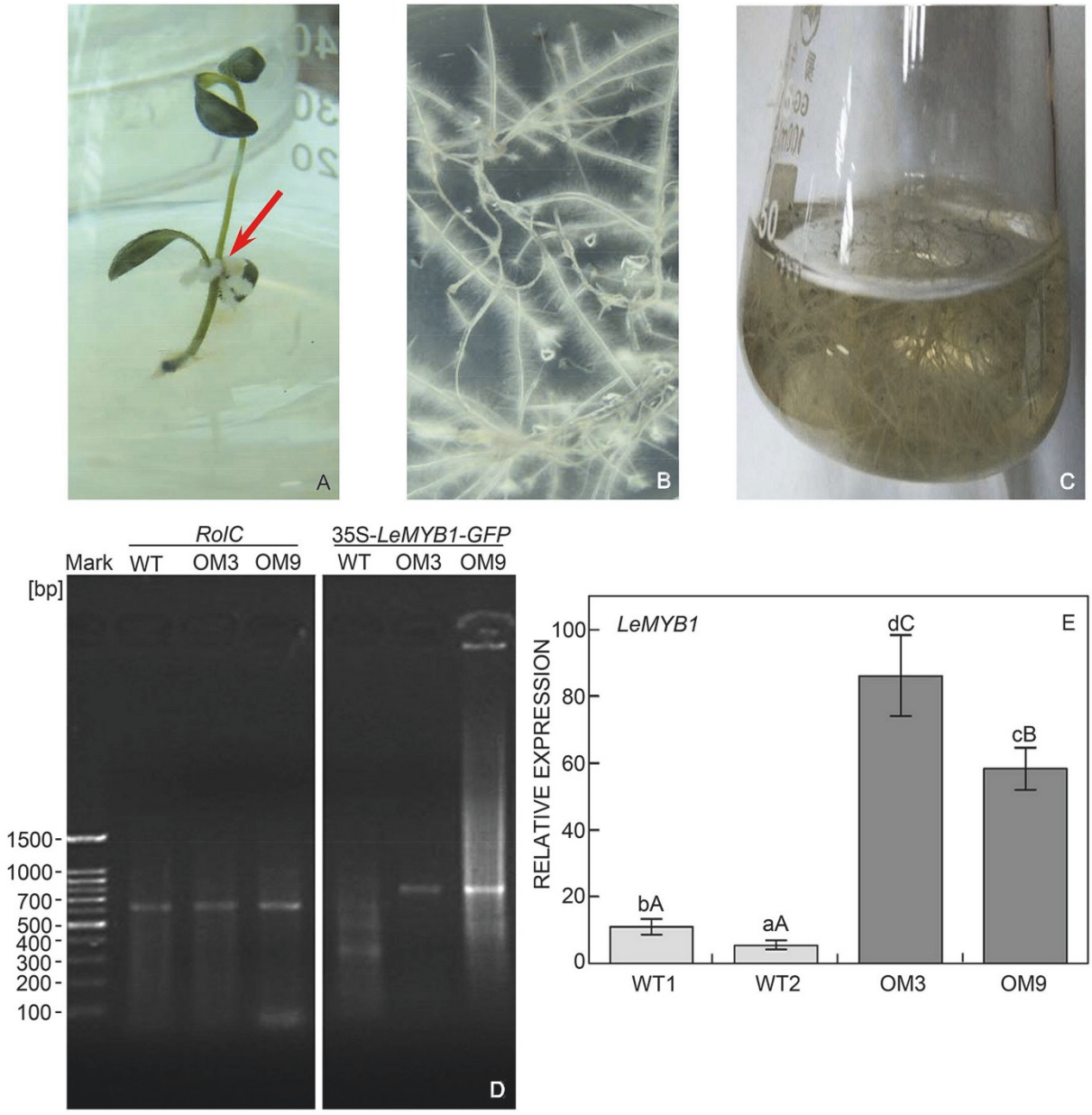

Fig. 1. Generation of *LeMYB1*-overexpressing transgenic hairy roots. *A* - Hairy roots emerging from infection sites. *B* - Hairy roots cultured in a B5 hormone-free solid medium. *C* - Hairy roots cultured in a B5 hormone-free liquid medium. *D* - PCR analysis of *RolC* and 35S-*LeMYB1-GFP* in WT and OM hairy root lines of *L. erythrorhizon*. *E* - Real-time PCR detection of *LeMYB1* mRNA level in WT and OM hairy root lines. All values represent an average ± SD. The *bars* with different letters are significantly different at $P < 0.05$ (*lower case letters*) or $P < 0.01$ (*capital letters*), respectively.

Results and discussion

After infection with *A. rhizogenes* (the wild type strain and a strain harboring pBI121-*LeMYB1* binary construct), stem nodes of *in vitro*-grown *L. erythrorhizon* showed root formation at the infection sites with a high transformation frequency [about one to six hairy root(s) on average for each needle infection site] within two to four weeks of incubation (Fig. 1*A*). Emerging roots exhibited a fast growth and a high degree of lateral branching (Fig. 1*B*). The high efficiency of hairy root induction in *L. erythrorhizon* was in agreement with earlier studies of genetic transformation in this plant using ATCC15834 (Yazaki *et al.* 1998). Individual root lines emerging from stem nodes were excised, transferred, sterilized, and maintained in a B5 liquid medium (Fig. 1*C*). Two putative overexpression lines (OM3 and OM9) were randomly selected for a further identification *via RolC* amplification. The presence of a 676 bp amplification product confirmed the transformation of the selected hairy root lines (Fig. 1*D*). Because the plasmid pBI121-*LeMYB1* contained the coding region of the *L. erythrorhizon LeMYB1* gene under the control of the CaMV 35S promoter, the presence of the transgene in these root lines was further confirmed by 35S-*LeMYB1*-*GFP* fusion segment amplification (807 bp) from their genomic DNA (Fig. 1*D*). Subsequently, the positive transformants were further analyzed by real-time PCR for transgene expression. Real-time PCR analyses show that *LeMYB1* was strongly overexpressed in the OM lines with a relative expression ranging from 4.4- to 16.3-fold of that in the WT lines. Both the OM lines significantly accumulated a higher amount of *LeMYB1* mRNA than the WT hairy root lines ($P < 0.01$) (Fig. 1*E*).

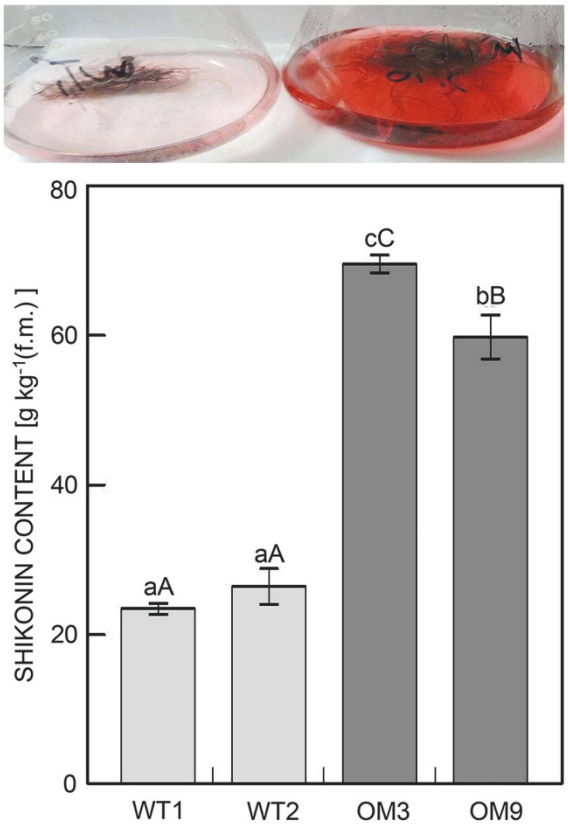

Fig. 2. Production of shikonin and its derivatives in WT and OM hairy root lines. The means ± SD, $n = 3$. The *bars* with different letters are significantly different at $P < 0.05$ (*lower case letters*) or $P < 0.01$ (*capital letters*), respectively.

To investigate the function of *LeMYB1* in regulating the biosynthesis of shikonin and its derivatives, the OM and WT hairy root lines were harvested after 6 d of culture in an M9 shikonin production medium. A higher content of shikonin and its derivatives was apparently detected in two independent OM lines overexpressing *LeMYB1* than in the WT lines ($P < 0.01$). The *LeMYB1*-overexpressing OM lines produced 2.8 times more shikonin and its derivatives than the WT lines (Fig. 2).

Members of the MYB transcription factor family have been widely studied in diverse physiological and biochemical processes, including the control of cell morphogenesis and the response to environmental stresses and phytochromes, as well as the regulation of secondary metabolism (Baldoni *et al.* 2013, Meng *et al.* 2014, Muthamilarasan *et al.* 2014). The overexpression of *VvMYB5a* from *Vitis vinifera* induces a strong accumulation of anthocyanin and flavonol compounds in tobacco (Deluc *et al.* 2006). Ectopically expressed *NtMYBJS1* (MeJA responsive), which shows a high degree of homology with *LeMYB1*, increases the accumulation of caffeoylputrescine and feruloyl-putrescine in unelicited BY-2 tobacco cell cultures (Gális *et al.* 2006). Consistent with these reports, our findings imply that *LeMYB1* also functioned as transcriptional activator in the shikonin biosynthesis.

To clarify the molecular regulation of *LeMYB1* in enhancing the biosynthesis of shikonin and its derivatives, we analyzed the expression patterns of the key genes involved in shikonin biosynthesis (Fig. 3*A*). The results of real-time PCR after *L. erythrorhizon* hairy roots had been transferred to an M9 medium for 6 d indicated differential transcript profiles for shikonin pathway genes and regulators in the OM hairy root lines in comparison with the WT hairy root lines. In response to the *LeMYB1* overexpression, the mRNA transcripts of the key shikonin pathway genes, namely *PAL* (Fig. 3*B*), *HMGR* (Fig. 3*C*), and *PGT* (Fig. 3*D*), were significantly up-regulated in the two OM lines compared with the WT lines; such genes showed 38- to 113-fold, 60- to 90-fold, and 5- to 30-fold increase, respectively ($P < 0.01$). We also analyzed the effect of the *LeMYB1* overexpression on the transcription of two key regulatory genes, namely

LeDI-2 (Fig. 3*E*) and *LePS-2* (Fig. 3*F*), presumably playing important roles in the stability or transport of the intracellular vesicles and in the intra-cell wall trapping of shikonin pigments, respectively (Yazaki *et al.* 2001, Yamamura *et al.* 2003). The expressions of *LeDI-2* and *LePS-2* were also significantly enhanced in the *LeMYB1*-overexpressing hairy root lines ($P < 0.01$).

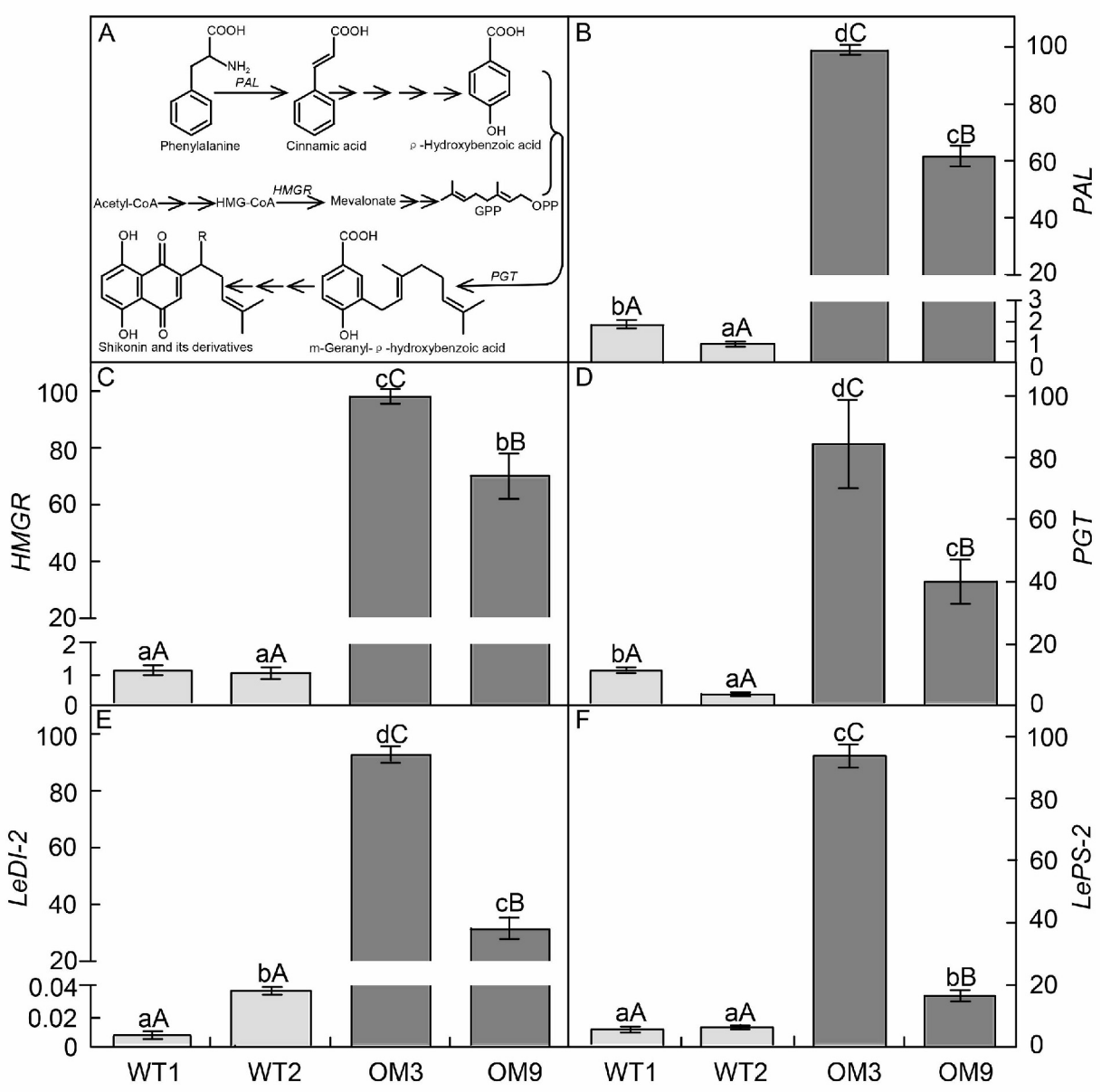

Fig. 3. Expression patterns of key shikonin biosynthesis-related genes in WT and OM hairy root lines. *A* - a brief metabolic pathway for the biosynthesis of shikonin and its derivatives. Relative mRNA amounts of *PAL* (*B*), *HMGR* (*C*), *PGT* (*D*), *LeDI-2* (*E*), and *LePS-2* (*F*) were determined by real-time PCR. All values represent an average ± SD. The *bars* with different letters are statistically significantly different at $P < 0.05$ (*lower case letters*) or $P < 0.01$ (*capital letters*), respectively.

Previous studies have indicated that most plant MYBs are generally transcriptional active regulators. For example, AtMYB12 was found to activate the phenylpropanoid biosynthetic pathway by up-regulating the expression of *CHS* (chalcone isomerase gene), *F3H* (flavanone 3-hydroxylase gene), *FLS* (flavonol synthase gene), and *CHI* (chalcone isomerase gene) (Misra *et al.* 2010). The overexpression of *Osmyb4* in tobacco activates the transcription of several phenylpropanoid pathway genes, such as *PAL*, *C4H*, *4CL1*, and *4CL2*, which specifically triggers the accumulation of chlorogenic and rosmarinic acids (Docimo *et al.* 2013). The expression of a single transcription factor can possibly regulate multiple genes in the phenylpropanoid

pathway (Goff *et al.* 1992, Jin and Martin 1999, Stracke *et al.* 2007). Our results indeed show that the overexpression of *LeMYB1* increased the transcription of *PAL*, *HMGR*, and *PGT* which encode the key enzymes involved in the shikonin biosynthesis pathway of *L. erythrorhizon*.

Quantification of the transcripts of *LeDI-2* and *LePS-2*, two other major factors relevant to shikonin formation in *L. erythrorhizon*, in the transgenic roots revealed a strong correlation between their transcription and the *LeMYB1* overexpression. Ultimately, these changes led to a significant increase in shikonin content in the *LeMYB1*-overexpressing transgenic *L. erythrorhizon* hairy roots. Further study is needed to determine which genes are regulated directly by LeMYB1 using a yeast one-hybrid system or an electrophoresis mobility shift assay.

Gene expression is generally regulated by a multi-protein complex that involves the combined actions of several transcription factors in eukaryotes. For example, C1/P1 (MYB family) and R/B (b-HLH family) are required for the activation of anthocyanin pigmentation in maize (Ludwig *et al.* 1989, Piazza *et al.* 2002). Therefore, other relevant transcription factors or proteins in the shikonin biosynthetic pathway may work together with LeMYB1 to regulate the shikonin biosynthesis.

In summary, our findings show that the over-expression of *LeMYB1* in hairy roots resulted in a significant increase in the transcription of *PAL*, *HMGR*, *PGT*, *LeDI-2*, and *LePS-2*. Such the increase coincided with an ultimate enhancement in shikonin content. Overall, the results confirm the positive function of *LeMYB1* in shikonin biosynthesis. This information could elucidate mechanisms underlying the regulation of shikonin biosynthesis by MYB. Further studies are required to determine various factors influencing shikonin biosynthesis and to provide a conclusive picture of regulatory systems that would facilitate the targeted manipulation of the shikonin pathway, thereby enhancing the shikonin content in *L. erythrorhizon*.

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