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Molecular cloning, characterization, and expression analysis of *LeMYB1* from *Lithospermum erythrorhizon*

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Abstract

MYB transcription factors (TFs) are known to have important functions in regulating the biosynthesis of secondary metabolites in plants. In this study, *LeMYB1*, a member of the *MYB* gene family of *Lithospermum erythrorhizon*, was cloned *via* the rapid amplification of cDNA ends. The alignment of the predicted translations of *LeMYB1* with other MYB proteins revealed that LeMYB1 contained an N-terminal R2R3 repeat and a high degree of amino acid identity to NtMYBJS1 which is involved in jasmonic acid signalling and phenylpropanoid biosynthetic pathway regulation. To determine the expression pattern of *LeMYB1*, its promoter was cloned and the sequence analysis was performed. The results revealed a number of potential regulatory motifs related to tissue-specific gene expression and abiotic and biotic stress responses. Real-time PCR results suggest that *LeMYB1* was induced transiently during the early stage when *L. erythrorhizon* cells were transferred from a B5 growth medium to a M9 production medium for shikonin formation. Exogenous methyl jasmonate (MeJA), an effective inducer of shikonin biosynthesis, induced the rapid *LeMYB1* expression. In contrast, a treatment with ibuprofen (IBU), an inhibitor of jasmonate biosynthesis, significantly inhibited the *LeMYB1* expression. Another inhibitor of shikonin formation, 2,4-dichlorophenoxyacetic acid (2,4-D), also markedly repressed the expression of *LeMYB1*. Tissue-specific expression analysis showed that *LeMYB1* mRNA was predominantly accumulated in roots where shikonin was synthesized. Thus, the *LeMYB1* gene may be a valuable member of the *R2R3-MYB* family in *L. erythrorhizon* and is possibly involved in the regulation of shikonin biosynthesis.

Additional key words: 2,4-dichlorophenoxyacetic acid, ibuprofen, methyl jasmonate, R2R3 repeat, RACE, real-time PCR, shikonin.

Introduction

Shikonin and its derivatives are a class of naphthoquinone-containing pigments that are only synthesized in plants of family *Boraginaceae*, such as *Lithospermum erythrorhizon* (Han *et al.* 2008), *Onosma paniculatum*

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; 4CL - 4-coumarate:CoA-ligase; GAPDH - glyceraldehydephosphate dehydrogenase; GPP - geranylpyrophosphate; HMGR - 3-hydroxy-3-methylglutaryl-CoA reductase; HTH - helix-turn-helix; IBU - ibuprofen; MeJA - methyl jasmonate; NJ - neighbor-joining; NCBI - National Center for Biotechnology Information; ORF - open reading frame; PAL - phenylalanine ammonia-lyase; PHB - *p*-hydroxybenzoic acid; PGT - *p*-hydroxybenzoate-3-geranyltransferase; PLACE - plant cis-acting regulatory DNA elements; RACE - rapid amplification of cDNA ends; RT-PCR - reverse transcription polymerase chain reaction; TD-PCR - touch-down PCR; TFs - transcription factors.
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(Rinner *et al.* 2010), and *Arnebia euchroma* (Damianakos *et al.* 2012). Shikonin and its derivatives can increase pathogen resistance and improve viral defense (Chen *et al.* 2003, Papageorgiou *et al.* 2008), or can be used as natural colorants in food and cosmetic products (Yazaki *et al.* 1999, Papageorgiou *et al.* 2006). A two-stage cell culture system had been successfully established by Mitsui Petrochemical Industries for the production of shikonin and its derivatives at the industrial level. In the first stage, a growth medium, such as B5 growth medium, is used for the proliferation of callus cells. In the second stage, a production medium, such as M9 medium, is used for the production of shikonin and its derivatives (Yazaki *et al.* 1999, Zhang *et al.* 2010a, Zou *et al.* 2011). Shikonin and its derivatives are biosynthetically derived from two key precursors from cultured *L. erythrorhizon* cells, *p*-hydroxybenzoic acid (PHB), which is synthesized from phenylpropanoid metabolites, and geranyl pyrophosphate (GPP), which is derived from the isoprenoid pathway (Heide and Berger 1989a, Yamaga *et al.* 1993, Gaisser and Heide 1996). In the B5 medium, the cultured cells accumulate a large amount of *p*-hydroxybenzoic acid-*O*-glucoside in their vacuoles whose aglycone form is one of the precursors of shikonin biosynthesis (Yazaki *et al.* 1986a, 1995). Upon the transfer of cells from the B5 to M9 medium, glucoside is enzymatically hydrolyzed to produce free hydroxybenzoic acid that is consequently prenylated to form *m*-geranyl-*p*-hydroxybenzoic acid (Yazaki *et al.* 1986a, Heide *et al.* 1989b), a key intermediate in shikonin formation. Although the biochemistry and enzymology of the shikonin biosynthetic pathway are well understood, the key transcription factor (TF) that may control this pathway is largely unknown.

R2R3-MYB TFs regulate the activity of several branches of phenylpropanoid metabolism. This plant-specific R2R3-MYB TF family is defined by a common DNA-binding domain of two repeats of about 50 amino acids (Ogata *et al.* 1996). The examination of R2R3-MYB TFs *via* phylogenetic analysis revealed functionally distinct subgroups (Kranz *et al.* 1998, Stracke *et al.* 2001, Dubos *et al.* 2010), several of which are involved in the regulation of particular branches of phenylpropanoid metabolism (Paz-Ares *et al.* 1987, Quattrocchio *et al.* 1998, Schwinn *et al.* 2006), such as anthocyanin production (Ahmed *et al.* 2009), phlobaphene biosynthesis (Grotewold *et al.* 1994), flavonol biosynthesis (Mehrtens *et al.* 2005), hydroxycinnamic acid biosynthesis (Docimo *et al.* 2013), and monolignol biosynthesis (Zhou *et al.* 2009, Zhong *et al.* 2010). For the common phenylpropanoid metabolism pathway of shikonin biosynthesis, we speculate that R2R3-MYB TFs may have important functions in regulating the formation of shikonin. In this report, we isolated a *R2R3-MYB* gene, named *LeMYB1*, and characterized the promoter of *LeMYB1* from cultured cells of *L. erythrorhizon*. The expression patterns of *LeMYB1* were also analyzed.

Materials and methods

Plants and treatments: The callus cells (line Y8) used in this study were derived from young shoots of *Lithospermum erythrorhizon* Sieb. et Zucc (Zhang *et al.* 2010b) and were maintained in a B5 (Gamborg *et al.* 1968) growth medium for proliferation under an 8-h photoperiod (an irradiance of 80 μmol m^{-2} s^{-1}, *TLD36 W/54*, *Philips*, Einhoven, The Netherlands) and a temperature of 25 °C. For the formation of shikonin and its derivatives, and for *LeMYB1* expression analysis, the cells were transferred into an M9 (Fujita *et al.* 1981) production medium and were kept in darkness at 25 °C on a shaker at 120 rpm. Approximately 1 g of cells from the B5 solid medium were transferred to 100 cm^3 conical flasks containing 20 cm^3 of the M9 liquid medium (Yang *et al.* 1999). Methyl jasmonate (MeJA) at a concentration of 10 μM, 50 μM ibuprofen (IBU), or 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D) (*Sigma*, St. Louis, MO, USA) were used to treat the cultured cells to analyze the gene expression patterns.

Full-length cDNA cloning of *LeMYB1*: To clone the *LeMYB1* gene, the total RNA of cells cultured in the M9 production medium was isolated as described by Zhang *et al.* (2010a). The first-strand cDNA was synthesized using *M-MLV* reverse transcriptase (*Promega*, Madison, WI, USA) with an adaptor oligo (dT_{15}) primer. A combination of touch-down PCR (TD-PCR) and the rapid amplification of cDNA ends (RACE) were applied for cloning the full-length cDNA of *LeMYB1* as described by Zou *et al.* (2011). Initially, PCR was performed using the cDNA prepared above as the template, and the degenerated primers (Yang *et al.* 2008) were designed according to the conserved regions of *Glycine max* R2R3-type MYB sequences (Table 1 Suppl.), and gene-specific 5' and 3' RACE primers were designed based on the cloned sequence. The PCR products were cloned into a pMD18-T vector (*TaKaRa*, Dalian, China) for sequencing. The resulting sequences were aligned together to obtain a full-length cDNA designated as *LeMYB1*. To confirm the full-length cDNA sequence of *LeMYB1*, we redesigned the 5′ and 3′ primers based on the integrated full-length cDNA to clone the open reading frame (ORF) of *LeMYB1*.

Characterization and bioinformatic analysis of LeMYB1: The full-length cDNA of *LeMYB1* was

subjected to online *BLASTn* and *BLASTx* analyses and the ORF was identified using the online *ORF Finder* program (http://ncbi.nlm.nih.gov). For the multiple sequence alignment analysis, the amino acid sequences of LeMYB1 and MYB homologs of different plant species that were retrieved from NCBI were aligned, and the phylogenetic tree was constructed based on the full-length amino acid sequences by using the bootstrap neighbor-joining (NJ) method in the *DNAMAN 5.2.2*. The following proteins were included for the construction of the evolutionary tree: *Arabidopsis thaliana* AtMYB4 (BAA21619), AtMYB12 (AF062864), AtMYB29 (NP_196386), AtMYB75 (NP_17605), AtMYB90 (NP_176813), and AtMYB122 (NP_177548), *Antirrhinum majus* AmMixta (CAA55725), *Lotus japonicus* LjMYB12 (BAF74782), *Fragaria* × *ananassa* FaMYB1 (AAK84064), *Nicotiana tabacum* NtMYBJS1 (AB236951), *Nicotiana attenuata* NaMYB8 (ADD59978), *Vitis vinifera* VvMYBF1 (FJ948477), VvMYBPA1 (AM259482), VvMYB5a (AY555190), and VvMYB5b (AY899404), *Diospyros kaki* DkMYB4 (BAI49721), *Picea mariana* PmMBF1 (AAA82943), *Malus domestica* MdMYB1 (ABK58136), *Zea mays* ZmC1 (AAA33482), and *Gynura bicolor* GbMYB1 (AB550244).

Cloning *LeMYB1* promoter and sequence analysis: The promoter of *LeMYB1* was isolated from the genomic DNA of *L. erythrorhizon via* PCR walking as described by Siebert *et al.* (1995). For the PCR reaction, *LeMYB1* gene-specific primers GSP1, GSP2, and GSP3 (Table 1) and adaptor primers AP1 and AP2 (Siebert *et al.* 1995) were used. Putative functional *cis*-acting elements of the *LeMYB1* promoter were identified using the *Plant cis-acting regulatory DNA elements* (*PLACE*) database (Higo *et al.* 1999).

Expression analyses of *LeMYB1* in cell cultures of *L. erythrorhizon*: The callus cells were subcultured in the B5 growth medium for 16 - 18 d, then were transferred to the M9 production medium. MeJA, IBU, or 2,4-D was added to the M9 medium to investigate the expression patterns of *LeMYB1*. Samples for the *LeMYB1* expression analysis were collected at 0, 3, 6, and 12 h, and 1, 2, 3, and 6 d after the cells were transferred to the M9 production medium (Zhang *et al.* 2010a). Mature seeds of *L. erythrorhizon* were germinated and grown in a greenhouse, and the roots, stems, and leaves of the intact seedlings with 16 true leaves were chosen for the tissue-specific expression analysis of *LeMYB1*. Real-time PCR was performed as described by Portereiko *et al*. (2006) and Wu *et al.* (2009). In each independent experiment, the relative expression at a maximal level was set to 100, other data were normalized accordingly. The primer pair of LeMYB1-Q-F and LeMYB1-Q-R was used to determine a *LeMYB1* gene expression, and the glyceraldehydephosphate dehydrogenase (*GAPDH*) gene was used as reference.

Results

A PCR-based method was performed to isolate *R2R3-MYB* TF genes from the cultured cells of *L. erythrorhizon*. We amplified a 168 bp fragment sequence based on the design of the degenerated primers of *G. max*. By performing *BLAST* analysis on the cDNA fragment, the sequence was shown to have high homology with *R2R3-MYB* genes. By RACE, the corresponding full-length cDNA sequence was subsequently obtained (Fig. 1) and designated as *LeMYB1* (GenBank accession number KC818628). The deduced LeMYB1 contained an N-terminal R2R3 repeat that corresponds to the DNA binding domain of plant MYB-

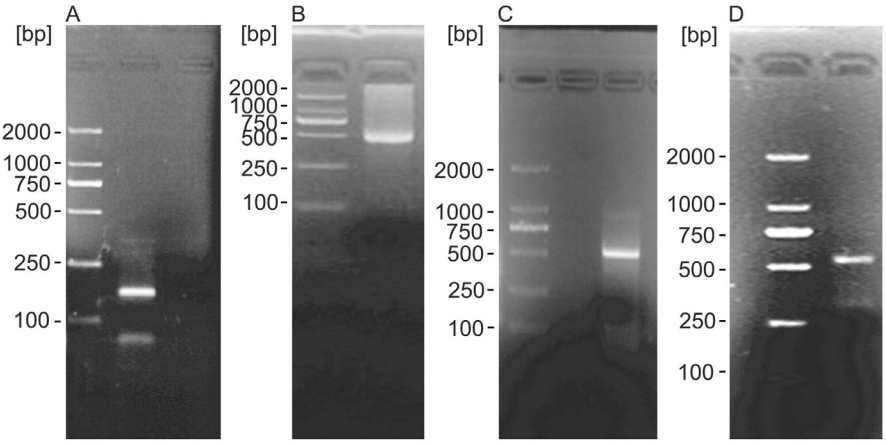

Fig. 1. Cloning *LeMYB1* cDNA from *L. erythrorhizon* cell cultures. *A* - The PCR product of the specific cDNA fragment of *LeMYB1* cloned *via* the TD-PCR method; *B* - the PCR product of the 5' cDNA end of *LeMYB1* cloned *via* the 5' RACE method; *C* - the PCR product of the 3' cDNA end of *LeMYB1*cloned *via* the 3' RACE method; *D* - the PCR product of the *LeMYB1* ORF.

Table 1. Putative *cis*-acting elements in the promoter sequence of *LeMYB1*. The positions of the *cis*-elements are with respect to the upstream position of the transcription start site.

Elements	*Cis*-element	Position	Sequence 5' to 3'
Myb, Myc binding	MYB2AT	42	TAACTG
	MYBCOREATCYCB1	1707	AACGG
	MYBST1	1419	GGATA
	TATCCAOSAMY	1447	TATCCA
	MYCATERD1	1786	CATGTG
Light regulation	RBCSCONSENSUS	165	AATCCAA
	REALPHALGLHCB21	536	AACCAA
	SORLIP1AT	550	GCCAC
	SORLIP2AT	52, 72, 78	GGGCC
Tissue-specific gene expression	ACGTOSGLUB1	698	GTACGTG
	CEREGLUBOX2PSLEGA	1282	TGAAAACT
	CACTFTPPCA1	742, 1389, 1422, 1460, 1614	TACT
	DOFCORE	338, 453, 622, 814, 931, 1187, 1537, 1582, 1725, 1736, 1792	AAAG
	TAAAGSTKST1	452, 1186, 1735	TAAAG
	POLLEN1LELAT52	1021, 1580, 1648, 1659, 1675	AGAAA
	ROOTMOTIFTAPOX1	303, 431, 487, 566, 991, 1338, 1822, 1891	ATATT
Pathogen/elicitor response	WBOXNTERF3	49, 599	TGACT
	WBOXATNPR1	727, 1510	TTGAC
	WRKY71OS	46, 599, 728, 1372, 1511, 1867	TGAC
	BIHD10S	691, 1115	TGTCA
	GT1GMSCAM4	619, 1022	GAAAAA
Abiotic stress response	ABRELATERD1	700, 883, 940	ACGTG
	ARR1AT	221, 635, 914, 1131	TGATT
	CCAATBOX1	160, 168, 538	CCAAT
	SURECOREATSULTR11	1308	GAGAC
	ANAERO2CONSENSUS	1572	AGCAGC
	RAV1AAT	419, 923, 1139, 1805	CAACA
Me-JA response	ASF1MOTIFCAMV	728, 1372	TGACG
Cu^{2+} response	CURECORECR	239, 698, 1205	GTAC

type proteins. The alignment of the predicted translations of the *LeMYB1* gene with other MYB TFs at the R2R3 domain indicated a high degree of homology (Fig. 1 Suppl.), whereas the C-terminal region showed little homology. LeMYB1 showed 75, 74, and 72 % identity to NtMYBJS1, VvMYBF1, and AtMYB12, respectively.

A phylogenetic tree was constructed with a selected set of full-length amino acid sequences of R2R3-type MYB factors from various plant species. They act as regulators of glucosinolate clade and cell shape (Glover *et al.* 1998, Dubos *et al.* 2010), C2 repressors of the flavonoid pathway (Jin *et al.* 2000, Aharoni *et al.* 2001), regulators of the phenylpropaniod pathway (Galis *et al.* 2006, Kaur *et al.* 2010), regulators of proanthocyanidins (Bogs *et al.* 2007, Akagi *et al.* 2009), general flavonoid pathway regulators (Deluc *et al.* 2006), and anthocyanin regulators (Borevitz *et al.* 2000, Takos *et al.* 2006, Shimizu *et al.* 2011, Gatica-Arias *et al.* 2012), respectively. The *LeMYB1* gene was located in subfamily 2 of *A. thaliana* which comprises *N. tabacum* NtMYBJS1 and *N. attenuata* NaMYB8. These genes are involved in jasmonic acid signaling and regulating the phenylpropanoid pathway (Galis *et al.* 2006, Kaur *et al.* 2010) (Fig. 2).

A 1 907 bp promoter fragment of *LeMYB1* was isolated from the genomic DNA of *L. erythrorhizon via* PCR walking (GenBank accession number KC818629). The transcription starting site was presumed to be at the 60^{th} nucleotide upstream of the translation initiation codon (ATG) of the *LeMYB1* cDNA. The *LeMYB1* promoter was then analyzed for putative *cis*-acting elements by using the *PLACE* database (Higo *et al.* 1999) which revealed a number of potential regulatory motifs corresponding to several known *cis*-acting elements related to tissue-specific gene expression and abiotic and biotic stress responses. Several consensus *cis*-acting elements, such as MYB, MYC, WBOX, and WRKY, were also found (Table 1). Several important *cis*-acting elements related to shikonin formation, such as MeJA response and Cu^{2+} response elements, were involved in the promoter region. The *LeMYB1* promoter also contained four light regulation elements, including RBCSCONSENSUS, REALPHALGLHCB21, SORLIP1AT, and SORLIP2AT.

Changes in the *LeMYB1* transcript levels after the medium transition were confirmed *via* real-time PCR (Fig. 3). The transcripts for *LeMYB1* showed a significant increase in the expression within 12 h, after which the

LeMYB1 mRNA expression showed a more than 2-fold decrease to a steady-state level. This gene expression pattern is consistent with those of the shikonin-biosynthetic genes phenylalanine ammonia-lyase (*PAL*), 4-coumarate:CoA-ligase (*4CL*), 3-hydroxy-3-methyl-glutaryl-CoA reductase (*HMGR*), and *p*-hydroxybenzoate-3-geranyl transferase (*PGT*) (Zhang *et al.* 2010a), or that of the other transcription factor gene *LeEIL-1* (Zou *et al.* 2011) which indicates that a stimulating effect occurred on the transcription of *LeMYB1*.

We characterized the expression of the *LeMYB1* gene of *L. erythrorhizon* cells in response to MeJA, an effective inducer of shikonin accumulation (Yazaki *et al.* 1997), and IBU, a specific inhibitor of JA biosynthesis.

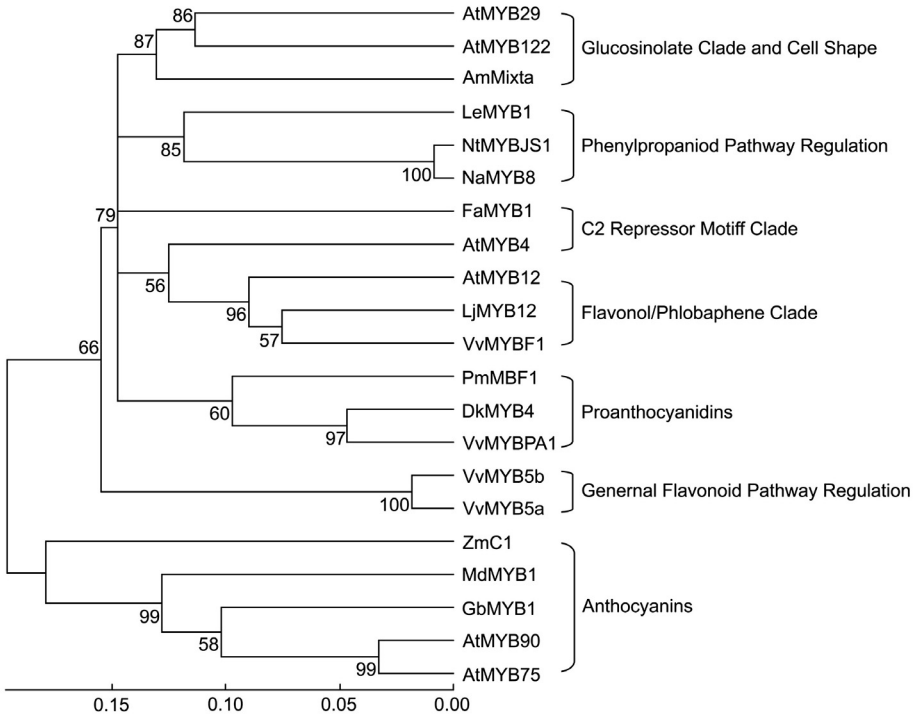

Fig. 2. A phylogenetic tree showing selected plant MYB transcription factors from the GenBank database (accession numbers are listed in the Materials and methods. The *scale bar* represents 0.1 substitutions per site, and the numbers next to the nodes are bootstrap values from 1 000 replicates.

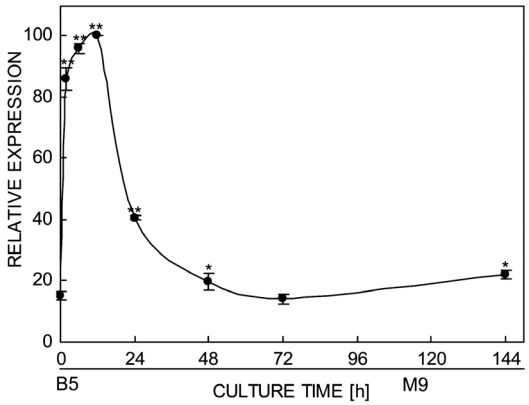

Fig. 3. The expression patterns of *LeMYB1* when cell cultures of *L. erythrorhizon* were transferred from a B5 growth medium to an M9 production medium. The *GAPDH* gene was used as reference. A representative sample from two biological replicates is shown. Means ± SD for three technical replicates (* and ** - significant differences between the B5 medium at 0 h and the time points in the M9 medium at $P < 0.05$ and $P < 0.01$, respectively).

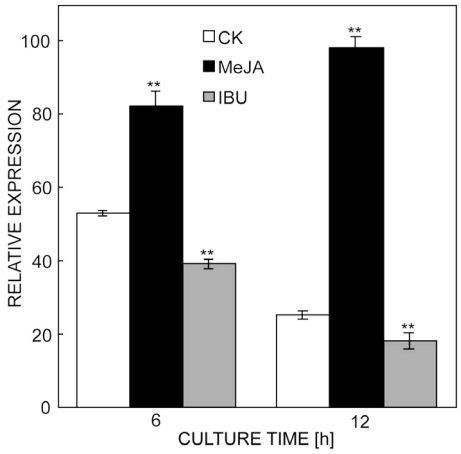

Fig. 4. The effects of MeJA (an effective inducer of shikonin biosynthesis) and IBU (an inhibitor of jasmonate biosynthesis) on the expression of *LeMYB1* in cell cultures of *L. erythrorhizon*. Untreated cells used as the control sample (CK) (** - significant difference between CK and the treatment at the same time point at $P < 0.01$).

The *LeMYB1* expression of *L. erythrorhizon* cells treated with 10 μM MeJA was significantly higher compared with that of the control sample, whereas the *LeMYB1* expression was lower than that of the control sample after the *L. erythrorhizon* cells were treated with 50 μM IBU for 6 h. The inducing effect of MeJA on *LeMYB1* expression was more significant at 12 h than at 6 h (Fig. 4). These results indicate that MeJA was involved in regulating the expression of *LeMYB1* in *L. erythrorhizon*.

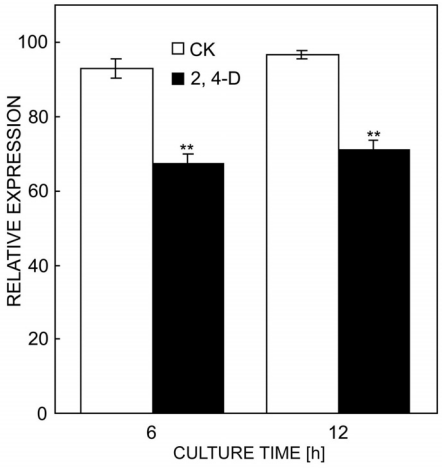

Fig. 5. The effect of 2,4-D (an inhibitor of shikonin formation) on the expression of *LeMYB1* in cell cultures of *L. erythrorhizon*. Untreated cells used as the control sample (CK) (** - significant difference between CK and the treatment at the same time point at $P < 0.01$).

2,4-D, a strong inhibitor of shikonin biosynthesis, has an irreversible inhibitory effect on shikonin formation (Tabata *et al.* 1974, Yazaki *et al.* 1986b). To evaluate if the inhibitory effect of 2,4-D on shikonin biosynthesis was possibly mediated by the suppression of the *LeMYB1* gene expression, *LeMYB1* mRNA transcripts of *L. erythrorhizon* cells treated with 2,4-D (1 μM) were analyzed *via* real-time PCR, and the result shows that 2,4-D significantly repressed the expression of *LeMYB1* (Fig. 5). The level of the *LeMYB1* expression was 72.4 and 73.3 % of the control sample at 6 h and 12 h, respectively, which shows that the suppression of the *LeMYB1* gene expression by 2,4-D might contribute to the inhibition of shikonin biosynthesis.

Real-time PCR was performed to clarify the spatial expression pattern of the *LeMYB1* gene in various organs of the *L. erythrorhizon* seedling. Roots, stems, and leaves were collected from intact seedlings with 16 true leaves. *LeMYB1* transcripts were detected in all organs, but the relative *LeMYB1* mRNA level in the roots, where shikonin was biosynthesized, was significantly higher than that in the stems ($P < 0.01$) or leaves ($P < 0.01$) (data not shown). We previously cloned two crucial regulator genes that are possibly related to shikonin formation, *LeERF-1* and *LeEIL-1*, and these two genes were also dominantly expressed in roots of intact *L. erythrorhizon* seedlings (Zhang *et al.* 2010b, Zou *et al.* 2011). In the present study, the results indicate that LeMYB1 may be a pivotal factor in regulating the formation of shikonin.

Discussion

The MYB family is one of the most abundant classes of transcription factors in plants, and its subfamily of a R2R3 type is the largest in higher plants. A common feature of R2R3-type MYB proteins is the presence of a functional DNA binding domain that typically consists of two imperfect repeats (R2 and R3). Each repeat is of about 50 to 53 amino acids in length and encodes two α-helices that form a helix-turn-helix (HTH) structure which intercalates in the major groove of DNA when bound to it (Ogata *et al.* 1996). In higher plants, the R2R3-type *MYB*-related genes constitute a rather large family of genes and have important functions in the regulation of gene expression, including the control of cell morphogenesis, responses to stresses and phytohormones, and a regulation of the phenylpropanoid biosynthetic pathway (Ballesteros *et al.* 2001, Nesi *et al.* 2001, Schmitz *et al.* 2002, Galis *et al.* 2006, Pasquali *et al.* 2008, Liu *et al.* 2011, Petroni and Tonelli 2011). Systematic analyses of MYB TFs were previously performed in *Arabidopsis* and rice (Chen *et al.* 2006). However, relatively few *MYB* genes have been studied in medicinal plants, especially of *Boraginaceae* family. In the present study, we isolated an R2R3-type *MYB* gene from *L. erythrorhizon*. The structural analysis of the LeMYB1 protein indicates that the protein possess a highly conserved N-terminal DNA-binding domain containing two typical motifs, whereas the C-terminal region is highly diverse compared with other members of the R2R3-MYB protein family in *Arabidopsis* or other species (Stracke *et al.* 2001). Despite the C-terminal diversity, the MYB factor families in *Arabidopsis* have been categorized into 22 subgroups based on the conserved amino acid sequence motifs detected in the C terminus of the MYB proteins. By analyzing the deduced amino acid sequence and phylogeny, LeMYB1 exhibits a remarkable similarity to *N. tabacum* NtMYBJS1 and *N. attenuata* NaMYB8 which are involved in phenylpropanoid metabolism and are classified as subfamily 2 motif of the *A. thaliana* R2R3-MYB TFs. Previous studies have shown that the overexpression of *NtMYBJS1* in tobacco BY-2 cells specifically induces the expression of core phenyl-propanoid pathway genes, such as *PAL* and *4CL,* and causes the accumulation of specific phenylpropanoid conjugates in the cells (Galis *et al.* 2006). Given the high homology of LeMYB1 with NtMYBJS1 and the common

phenylpropanoid metabolism pathway, LeMYB1 possibly regulates *PAL* and *4CL* expressions in the phenylpropanoid metabolism of *L. erythrorhizon* which controls shikonin biosynthesis.

To understand its induction expression pattern, the promoter sequence of *LeMYB1* was amplified from the *L. erythrorhizon* genome. Sequence analysis by using *PLACE* revealed the presence of a number of putative tissue-specific or shikonin formation-related regulatory motifs corresponding to known *cis*-elements of plant genes, such as the MYB recognition site (Zahur *et al.* 2012), light stress, MeJA, and Cu^{2+} response elements which implies that the *LeMYB1* promoter may be involved in a complex regulation mechanism.

MeJA is a well-established signal molecule in plant defense responses and is an effective inducer of secondary metabolite accumulation in plant cell cultures, such as taxol (paclitaxel) in *Taxus* (Laskaris *et al.* 1999). Previous studies have shown that MeJA as well as jasmonic acid are capable of inducing the biosynthesis of shikonin in *L. erythrorhizon* cells, however, its molecular mechanism remains to be determined. In the present study, *LeMYB1* was up-regulated by MeJA which indicates that MeJA is a signal for the activation of the *MYB* gene expression and shikonin biosynthesis activities of *L. erythrorhizon* cells. 2,4-D, a strong inhibitor of shikonin biosynthesis, has an irreversible inhibitory effect on shikonin formation. 2,4-D reportedly stimulates the growth of plant cells (Jacobs *et al.* 1966). A competition between plant secondary metabolite production and growth was also previously hypothesized (Van der Plas *et al.* 1995). Thus, we speculate that the inhibitory effect of 2,4-D on shikonin formation may be involved in the promotion of growth of *L. erythrorhizon* cells. As previously mentioned, *MYB* genes have important functions in cell proliferation, cell determination, cell differentiation, and secondary metabolite production in plants. Our results indicate that the addition of 2,4-D in the M9 medium remarkably altered the expression of the *LeMYB1* gene which suggests that the inhibitory effect of 2,4-D on shikonin formation was possibly mediated by the suppression of the *LeMYB1* expression. The tissue-specific expression analysis showed that the amount of *LeMYB1* transcripts was higher in the roots of the *L. erythrorhizon* seedlings where shikonin accumulated compared with the aboveground parts. These results collectively demonstrate that *LeMYB1* might have an important function in regulating shikonin formation.

References

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