Pak. J. Bot., 53(2): 473-482, 2021. DOI: http://dx.doi.org/10.30848/PJB2021-2(26)

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED miRNAs IN RESPONSE TO WHITE LIGHT SIGNAL IN *LITHOSPERMUM ERYTHRORHIZON* CELLS

HUA ZHAO[1,2†], RONGJUN FANG[1†], SHOUCHENG HUANG[1†], ZHONGLING WEN[1,4], MINKAI YANG[1,4], HU ZHAO[1], BAO LIU[3], TONGMING YIN[4], GUIHUA LU[1,4], JINLIANG QI[1,4*] AND YONGHUA YANG[1,4*]

[1]*State Key Laboratory of Pharmaceutical Biotechnology, Institute of Plant Molecular Biology, School of Life Sciences, Nanjing University, Nanjing 210023, China*
[2]*School of Life Sciences, Nantong University, Nantong 226019, China*
[3]*Key Laboratory of Molecular Epigenetics of the Ministry of Education (MOE), Northeast Normal University, Changchun 130024, China*
[4]*Co-Innovation Center for Sustainable Forestry in Southern China, Nanjing Forestry University, Nanjing 210037, China*
**Corresponding author's e-mails: yangyh@nju.edu.cn; qijl@nju.edu.cn*
[†]*Theses authors contributed equally to this work*

Abstract

Light signal plays a key negative regulatory role in the biosynthesis of shikonin and its derivatives which are important pharmaceutical secondary metabolites in *Lithospermum erythrorhizon*. Although microRNAs (miRNAs) could regulate multiple biological and metabolic processes, the miRNAs involved in the light-regulated shikonin formation still remain unclear. In the present study, we constructed two small RNA libraries of *L. erythrorhizon* callus cells cultured under different light conditions (Dark, White light) and carried out high-throughput sequencing using Miseq platform. From a total of 11,909,475 raw reads, we acquired a global miRNA profiling of *L. erythrorhizon* in response to different light conditions. A total of 32 annotated miRNA families and 50 putative miRNA-targeted transcripts were identified. A total of 18 miRNAs were differentially expressed in the Dark vs White light small RNA libraries, and their putative target genes were involved in FERONIA signaling, phytohormone signaling, etc. The expression levels of some important miRNAs under different light conditions and the target TCP transcription factor of miR319 were validated by Q-PCR and RLM-RACE, respectively. We further proposed a possible regulatory pathways of light-responsive miRNA implicated in shikonin biosynthesis. Our results provide new insights into the regulation of shikonin biosynthesis in *L. erythrorhizon*.

Key words: Expression; Light signal; *Lithospermum erythrorhizon*; MicroRNA; Shikonin.

Introduction

The root of *Lithospermum erythrorhizon* has been used as natural red textile dyes or medicine for thousands of years in East Aisa. The application of *L. erythrorhizon* is attributed to the red naphthoquinone shikonin derivatives accumulated in its root with the properties of heat-clearing and detoxicating (Papageorgiou *et al.*, 1999). Modern medical research shows that shikonin derivatives have extensive pharmacological activities including anti-inflammatory, antibacteria, antivirus and anti-tumor (Andujar *et al.*, 2013; Liang *et al.*, 2013; Zhang *et al.*, 2017; Zhao *et al.*, 2017; Boulos *et al.*, 2019; Zhu *et al.*, 2019). With the more discoveries of the diversification of their medicinal value, shikonin derivatives have become more prominent in the pharmaceutical industry and attracted more and more attention.

Efforts were made to obtain abundant shikonin derivatives independent of whole plants and an excellent two-phase culture system of *L. erythrorhizon* was invented in 1981 (Fujita *et al.*, 1981). Shoot culture and hairy root system have also been developed to enhance the yields of shikonin (Shimomura *et al.*, 1991; Touno *et al.*, 2000). Based on these culture methods combined with gene cloning or omics analysis, the biosynthesis pathway of shikonin is basically understood (Yazaki *et al.*, 1999; Wu *et al.*, 2017; Liao *et al.*, 2018; Kojiro *et al.*, 2019). The amounts of shikonin can be promoted by several positive factors including nitric oxide (Wu *et al.*, 2009), copper ion (Fujita *et al.*, 1981), fungal elicitors (Sim *et al.*, 1994), ethylene (Touno *et al.*, 2005) and jasmonates (Yazaki *et al.*, 1997).

Surprisingly, light is a negative regulator of shikonin biosynthesis (Tabata *et al.*, 1974; Yazaki *et al.*, 1999). As for most plants, the synthesis of secondary metabolites is facilitated by light (Matsuura *et al.*, 2016; Namdar *et al.*, 2019; Thakur *et al.*, 2019). Previous studies about light inhibition of shikonin biosynthesis have been focused on the discovery of light suppressive genes involved in the metabolic pathway of shikonin (Yazaki *et al.*, 1999; Liu *et al.*, 2006; Qi *et al.*, 2008; Zhang *et al.*, 2010).

MicroRNAs (miRNAs) are a group of endogenous non-coding small RNAs of approximately 22 nucleotide in length, which have been identified as one of the most distinguishable post-transcriptional regulators throughout the plant and animal kingdom (Wittstock *et al.*, 2004; Wu *et al.*, 2010). Since the first plant miRNA identified from *Arabidopsis thaliana*, miRNAs research has become one of the hotspots (Reinhart *et al.*, 2002). The diversity and wide distribution of miRNAs, as well as miRNA-mediating regulatory mechanisms, have received much attention. The newly released miRbase 22.1 collected 10,414 mature miRNAs from 82 plant species (Kozomara *et al.*, 2019). These miRNAs are found to regulate almost all biological and metabolic processes in plants with diverse functions such as the control of cell fate, stress responses and the involvement in signal transduction (Sun, 2012; Baloch *et al.*, 2019).

Although the light-regulated shikonin biosynthesis mechanism has been researched for about decades, there is little information available for miRNAs from *L. erythrorhizon*. Therefore, to uncover the differentially expressed miRNAs (DEMs) in response to light signal in *L.*

erythrorhizon will give new insights into the biosynthetic mechanism of shikonin and its derivatives. In this study, we constructed two small RNA libraries of *L. erythrorhizon* cell culture under different light conditions (Dark, White light) and carried out high-throughput sequencing using Miseq platform. By comparing the sequencing data of continuous illuminated callus culture with that of the control, the light-responsive miRNA possibly involved in regulating the biosynthesis of shikonin were found. Our results will provide crucial clue to the elucidation of the functions of miRNA in *L. erythrorhizon*.

Materials and Methods

Plant materials and light treatments: First, *L. erythrorhizon* aseptic seedlings were obtained and cultured according to the previous description (Fang *et al.*, 2016). Then the hypocotyls were cut into 5 mm in length for callus induction in B5 growth medium (Gamborg *et al.*, 1968) containing 0.025 mg/L indoleacetic acid (IAA) and 1 mg/L 6-benzyladenine (BA) in darkness at 25℃ for about 12 days. Subsequently, the induced fresh yellow callus cells were subcultured in the same growth medium every 12 days for multiplication under 8 h/day illumination at 25℃. Finally, the well-grown subcultured calli were inoculated into the M9 liquid production medium in a certain proportion placed in a rotary shaker at 120 r/min, and grew either in the dark condition or under continuous white light intensity with 80 $\mu M^{m-2\ s-1}$ (Zhao *et al.*, 2014). Suspended cells were picked at 72 h removing excess moisture, and stored in -80℃ refrigerator following liquid nitrogen flash-freezing until used for transcriptome analysis. The callus cells suspended in M9 liquid medium under darkness were performed as control.

Small RNA library construction and deep sequencing: Total RNA was extracted using RNAiso Plus reagent (TaKaRa Bio Inc., Japan). RNA quality and integrity were checked by ultraviolet spectrophotometer combined with electrophoresis. This RNA was then applied to construct small RNA library according to the manual in the Illumina TruSeq RNA Sample Prep Kit. Two kinds of small RNA libraries were constructed and nominated as DARK and WHITE according to the culture condition. Small RNA sequencing was performed on Illumina MiSeq Personal Sequencer at Shanghai Personalbio, China.

Identification of known and novel miRNAs: For miRNA annotation, raw reads underwent qualification by removing adapters and trimming low quality reads, choice of 18-30 nt long sequences and deduplication. The obtained unique sequence reads were compared to Rfam 11.0. The sequences annotated as miRNAs were analyzed by BLAST against plant microRNA database (PMRD) (Zhang *et al.*, 2010) with miRBase 22.1 to identify the known miRNAs in *L. erythrorhizon*. Subsequently, the representative sequences that existed in at least three species and had an approximate sequence with at least one target species were selected for comparison with the corresponding mature miRNA sequences of ten species to investigate *L. erythrorhizon* miRNAs evolutionary conservation.

For novel miRNA identification, the left sequences were aligned with the transcriptome sequences of *L. erythrorhizon*. The candidate pre-miRNAs were then folded into a secondary structure using online RNA-folding program mfold (Zuker, 2003). The criteria chosen for miRNA precursors were as described elsewhere (Zhang *et al.*, 2006a).

Profiling miRNA expression difference: Differential miRNA expression profile analysis was based on the sequence reads in each library. The qualified miRNA sequence reads were normalized to calculate Fold_change by the log2 ratio. |log2 fold_change (WHITE/DARK)|≥1.0 was used as a threshold for DEMs judgment.

Prediction and validation of miRNA targets: The potential targets of the *L. erythrorhizon*-specific miRNAs were predicted using the psRNATarget program with default parameters based on the transcriptome data of *L. erythrorhizon* and mRNA sequence of *Boraginaceae* submitted to NCBI (National Center of Biotechnology Information) (Dai *et al.*, 2018). Further analysis of the targets was made through Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation. The target validation was conducted by RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) using a GeneRacer Kit (Invitrogen, Carlsbad, CA,USA) as described previously (Huang *et al.*, 2018).

Validation of miRNA by RT-PCR: Candidate miRNAs related to light-regulated shikonin biosynthesis were verified by adding poly (A) tail RT-PCR (Shi & Chiang, 2005). RNA polyadenylation and then reverse transcription were manipulated according to the TAKARA SYBR® PrimeScript™ miRNA RT-PCR Kit (TaKaRa Bio Inc., Japan). The following qPCR reactions were performed in triplicate using SYBR® Premix Ex Taq™ Kit (TaKaRa Bio Inc., Japan) on ABI 7300 (Life Tech, Applied Biosystems, Foster City, USA) real-time PCR system. *L. erythrorhizon* 5.8S rRNA was used for the endogenous reference. The relative expression levels of miRNAs were calculated by $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008).

Results

Sequence analysis of short RNAs: Two independent small RNA libraries from suspended microcalli of *L. erythrorhizon* cultured 72 h in dark (DARK) or white light (WHITE) were sequenced using Illumina Miseq platform. A total of 6,411,595 (DARK) and 5,497,880 (WHITE) raw reads were obtained. After removal of adapter sequences, low quality reads and sequences shorter than 15 nt, 5,330,477 and 3,645,908 total clean reads remained in the two libraries, respectively. The number of clean reads fall in the size range 18-30 nt were 3,021,119 and 3,057,307 for the DARK and WHITE, respectively (Table 1). The size distributions of the clean reads ranged from 15 to 30 nt in the two libraries were quite similar. The majority of small RNAs were distributed between 18 to 24 nt. There were two distinct peaks around 21 and 24 nt, which was consistent with most of the previous results. Sequences with length of 24

nt were shown to be the most abundant, while 21 nt small RNAs, the typical length for mature plant miRNAs, were the second highest abundant in the libraries (Fig. 1). Statistics analysis of the first nucleotides of the small RNA sequences ranged from 18 to 25 nt showed that the group of 20, 21 and 22 nt had a predominant bias of U and the group of 24 nt had a predominant bias of A (Fig. 2). Subsequently, we annotated the unique and total sRNAs from the two libraries by blasting sRNA sequences against Rfam. The annotation and distribution of different noncoding RNAs (snoRNAs, rRNAs, miRNA and tRNAs, etc.) were summarized in Table 2.

Table 1. Raw and clean read statistics of small RNAs.

Sample	Raw reads	Clean reads	18-30nt
Dark	6,411,595	5,330,477	3,021,119
White	5,497,880	3,645,908	3,057,307

Known miRNAs and evolutionary conservation: Sequences annotated as miRNA by Rfam alignment were subjected to the homolog search against PMRD with miRBase 22.1 integrated. We found that a number of 25,229 reads (592 unique sequences) in DARK library matched known miRNAs from PMRD, which accounts for 0.84% of clean reads. In WHITE library, 49,193 reads (732 unique sequences) were found to be orthologs of known miRNAs, which accounts for 1.61% of clean reads (Table 3).

With no more than 3 mismatches between sequences, a total of 1001 unique sequences from the two libraries represented 173 conservative miRNAs belonging to 88 families and 18 miRNA* putatively produced from 14 gene families, including *MIR157*, *MIR160*, *MIR166*, *MIR167*, *MIR168*, *MIR171*, *MIR390*, *MIR393*, *MIR395*, *MIR838*, *MIR853*, *MIR2111*, *MIR5274*, *MIR5648*. Among them, 78 miRNAs were found in each library accounted for 44.57% of 175 known miRNAs; However, 95 miRNAs were detected either in the DARK or in the WHITE library in which 36 miRNAs were expressed in DARK sample and 59 miRNAs were expressed in WHITE sample.

To investigate the conservation of these known miRNAs between species, we selected representative sequences to compare with known miRNAs in 10 plant species, including *Arabidopsis lyrata*, *Arabidopsis thaliana*, *Glycine max*, *Medicago sativa*, *Oryza sativa*, *Populus trichocarpa*, *Triticum aestivum*, *Vigna unguiculata*, *Vitis vinifera*, *Zea mays*. These miRNAs repsented 24 miRNA families. The miRNA families could be classified into highly conserved miRNAs, moderately conserved miRNAs, low conserved and non-conserved miRNAs according to their distribution. Highly conserved miRNAs distributed in more than 10 different plant families. Those found in 5-9 different plant families should be classified as moderately conserved miRNAs. Low conserved miRNAs distributed in 2-4 different plant families. Non-conserved miRNAs meant the miRNAs distributed in 1 plant species (Zhang *et al.*, 2006b). Based on this rule, *MIR156/157*, *MIR159/319*, *MIR165/166*, *MIR167*, *MIR171*, and *MIR172* families are deeply conserved. *MIR160*, *MIR162*, *MIR168*, *MIR390*, *MIR393*, *MIR394*, *MIR396*, *MIR398* and *MIR399* are moderately conserved. *MIR164*, *MIR477*, *MIR482*, *MIR827* and *MIR858* are low conserved. Moreover, *MIR166* is the most abundant miRNA family in the callus cells of *L. erythrorhizon*, which was consistent with the discovery in *Pananx ginseng* (Wu *et al.*, 2012). It has approximately 16142 reads in DARK library. *MIR159* is the second abundant miRNA family, which was sequenced 1208 counts in DARK library. The low conserved miRNAs expressed relatively low level (Fig. 3).

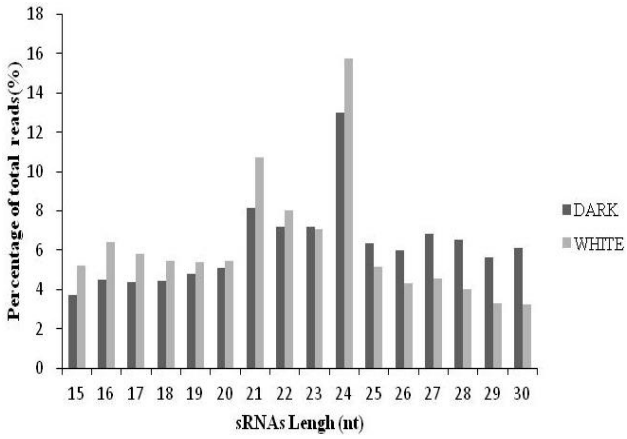

Fig. 1. Length distribution of sRNA in *L. erythrorhizon*. The length distribution of high-quality sequences were obtained from DARK and WHITE libraries. The distribution of the clean reads was shown as percentages. DARK: microcalli cultured in dark; WHITE: microcalli cultured under white light.

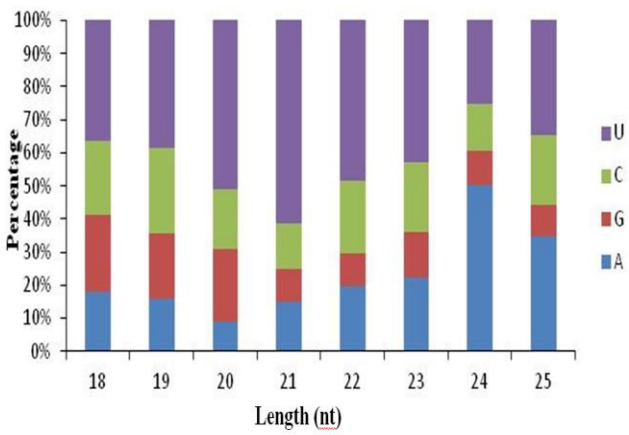

Fig. 2. *L. erythrorhizon* sRNA first site base bias in every length.

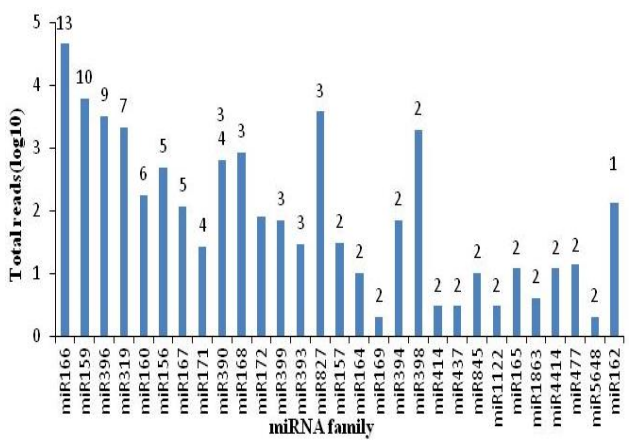

Fig. 3. Number and reads of identified miRNAs in each conserved miRNA family.

Table 2. sRNAs annotation and distribution in the callus cells of *L. erythrorhizon*.

Category	Dark		White	
	Unique sRNAs	Total sRNAs	Unique sRNAs	Total sRNAs
tRNA	9,761	95,540	10,330	110,464
rRNA	37,882	611,394	35,465	586,584
snoRNA	3,838	23,294	4,922	28,451
miRNA	2,511	48,218	3,275	67,072
Intro	3,470	92,967	3,485	68,201
Others	25,171	264,427	65,584	861,055
Total	82,634	1,135,841	123,067	1,721,833

*Note: the miRNA results by comparison with Rfam were not the final miRNA identification results

Table 3. Distribution of miRNAs in the callus cells *L. erythrorhizon*.

	Dark	white
miRNAs	164	208
Unique sequences	592	732
Total sequences	25,229	49,193
Total clean reads	3,021,119	3,057,307

Expression patterns of miRNAs subjected to light resources: The abundance of miRNAs provides clues for light-responsive miRNA analysis. Light quality affected expression levels of most of the miRNAs we detected. Members of the same miRNA families expressed differently in response to the same illumination. A total of 36 miRNAs were only detected in dark condition and their expression were inhibited when the cells exposed to light. While 59 miRNAs were only detected in white light treatment sample. After normalization, scatter plot (Fig. 4.) was mapped about DEMs. It directly displays the distribution of DEMs. We note that the expression of miR164c, miR167b and miR396d was special. The miR164c and miR167b were only found in WHITE while miR396d only found in DARK with the reads more than 5 less than 10; But miR396c was induced by 1.04 fold in WHITE library.

Further differential expression analysis excluded miRNAs with less than 40 total reads in the two libraries, because the low miRNA abundance might lead to the wrong analysis results (Zeng *et al.*, 2012). The abundance of miRNAs were normalized and the fold_change was calculated. The |fold_change|≥1 was considered as the difference in miRNA expression in statistics. We found 18 miRNAs detected between DARK and WHITE were significantly differentially expressed. After white light illumination, a total of 17 miRNAs were up-regulated and only 1 miRNA were down-regulated. The up-regulated miRNAs were miR156a, miR159a, miR159b, miR319b, miR319e, miR166b, miR166*, miR390a, miR394a, miR396c, miR399a, miR827a, miR827b, miR858, miR894, miR5054 and miR5139. The relative abundance of miR156a, miR159b, miR394a and miR858 in the WHITE library was 2 times higher than in the DARK library. The expression of miR168a* was notable down-regulated by 1.29 fold.

Novel miRNA prediction: After the previous conservative miRNA identification, the rest of the miRNA sequences were mapped to our *Lithospermum* transcriptome data using miReap tool with no mismatches allowed as the novel miRNA candidates. About 329 candidate miRNA genes were selected to predict novel miRNA using the Mfold online software. MFEI (minimum free energy indexes) was computed according to the formula MFEI = [100 × MFE/L] / (G + C) %. Most miRNA's MFEI value were above 0.85, which is greater than tRNAs (0.64), rRNAs (0.59) and mRNAs (0.62-0.66) ($p < 0.001$) (Zhang *et al.*, 2006a). As a result, we found 18 new miRNA, from 16 miRNA genes. They are less abundant than the conserved miRNA. The results are shown in Table 4.

Prediction of miRNA targets in *L. erythrorhizon*: Knowledge of miRNA targets is important for understanding miRNA function. The online psRNATarget program was applied to predict *L. erythrorhizon* miRNAs' targets. The transcriptome data of *L. erythrorhizon* and mRNA sequence of *Boraginaceae* submitted to NCBI were used as reference. The candidate target sequence were then annotated by online BLASTX. The results showed that the sequence of no blast or undefined protein were eliminated. The target TEOSINTE RANCHED/ CYCLOIDEA/PCF (TCP) transcription factor of miR319 was validated by RLM-RACE with result showed in Fig. 5.

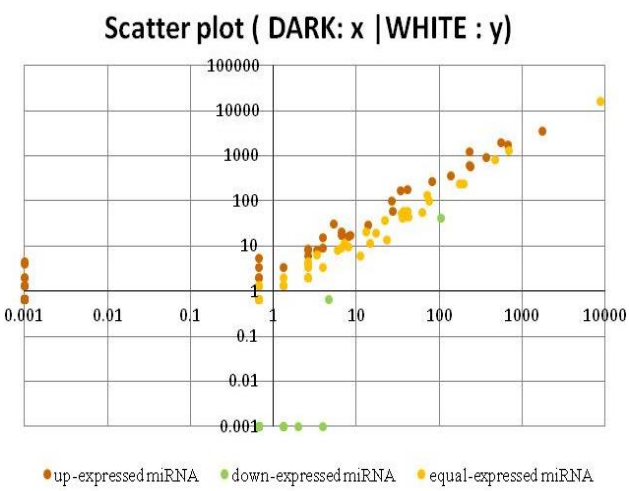

Fig. 4. Comparison of the expression patterns of identified miRNAs between DARK and WHITE.

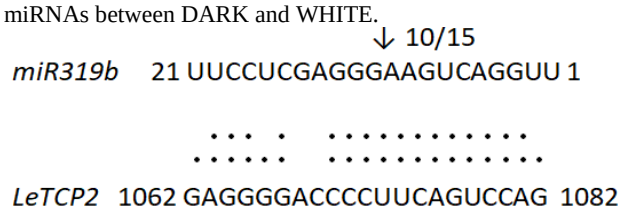

Fig. 5. Cleavage site validation of target gene of miR319 in *L. erythrorhizon*.

Table 4. Predicated novel miRNAs in the callus cells of *L. erythrorhizon*.

miRNA No.	miRNA sequence (5'→3')	LM/LP (nt)	(A+U) %	MFE	MFEI
ler-n01-3p	GATAAAGATCTTGGAAAACC	20/163	55.83	-117.4	1.63
ler-n02-3p	AAGACTGCTAAGGCTCTTGG	20/163	55.83	-117.4	1.63
ler-n03-5p	AATTATAGGAACATGAACACC	21/186	63.98	-114.5	1.71
ler-n04-3p	TATATCTGTATTGTGTACCC	21/122	67.21	-62.3	1.56
ler-n05-3p	TATATCTGTATTGTGTACCC	20/123	69.92	-59.3	1.60
ler-n06-3p	TATATCTGTATTGTGTACCCT	23/125	70.4	-68.2	1.84
ler-n07-3p	ATGAGACATCTGAATGGAGGA	21/141	56.74	-108.9	1.79
ler-n08-3p	GAAGGAGGTAGAGATGGAGA	20/133	52.63	-64.1	1.02
ler-n09-3p	AGGGACGACCAACATATAACT	21/159	62.29	-49.9	0.85
ler-n10-5p	CACCATCCCCACCAAATAGCC	21/128	36.72	-93.1	1.15
ler-n10-3p	TTGGTGAGGGTGGAGGGATG	20/128	36.72	-93.1	1.15
ler-n11-3p	TTGAGAATTGTGTTTTGAATA	21/140	74.29	-52.4	1.46
ler-n12-5p	CCGAAAGTGACAAATGTTGC	20/201	61.69	-67.60	0.88
ler-n13-5p	TTTCATGTAGAAAGTACGCCT	21/256	62.89	-200.8	2.11
ler-n14-5p	CCCCAGAACCACATCAAAACT	21/123	56.10	-102.2	1.89
ler-n15-3p	TCGGTTCGGTTCGGTTTT	18/106	60.38	-35.4	0.84
ler-n16-5p	CAATGAGAGGATCTGATACGT	21/123	55.28	-40.7	0.74
ler-n16-3p	CGTATTCAGACTCTCTTGCCT	21/123	55.28	-40.7	0.74

LP Length of precursor, LM Length of mature miRNA, MFE Minimum free energy, MFEI minimum free energy indexes

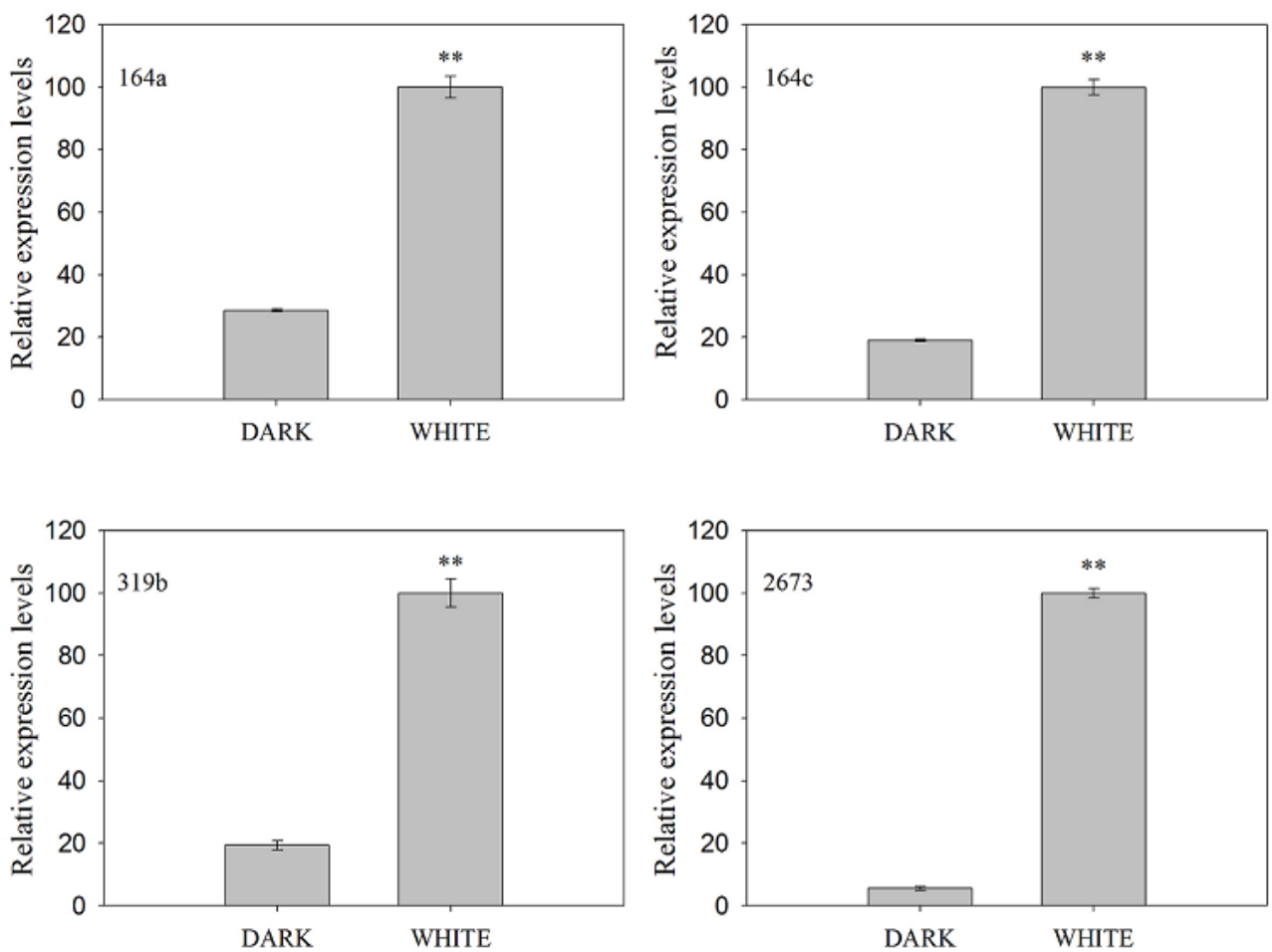

Fig. 6. Expression analysis of miRNAs by RT-qPCR. A representative sample from two biological replicates is shown. Means ± SD for three technical replicates (** - significant differences between DARK and WHITE, $p<0.01$). (Error bars represent SD for three replicates).

Only 28 conserved miRNAs were found their target genes. A total of 9 target genes were detected for only 6 out of the 18 novel miRNAs we acquired. The different miRNA targets can be classified to several groups according to their functions, including transcription factors, enzymes which may play important roles in plant primary metabolisms and secondary metabolism. Among these putative targets, most of them encoded transcription factors which display the conservation of miRNA targets. The corresponding target relations such as miR156/ miR157-SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) miR160-auxin response factor (ARF), miR165/miR166-homeobox-leucine zipper protein (HD-Zip), miR164-NAC domain-containing protein, miR169-nuclear transcription factor Y subunit A-3-like, miR319-TCP and miR858-myeloblastosis (MYB) were also found in *L. erythrorhizon*. Some targets encoded enzymes such as 2-alkenal reductase (target of miR394), arginine decarboxylase (target of miR845), HMGR (target of miR2673) and histone deacetylase (target of miR414). Interestingly, miR845 repressed its target at translation level based on the online prediction (Dai & Zhao, 2011). In addition, other targets included F-box (target of miR393), nucleotide binding site-leucine rich repeat (NBS-LRR) (target of miR1507), mitogen-activated protein kinase kinase phosphatase (MKP) (target of miR159), LRR receptor-like serine/threonine-protein kinase (target of miR2611), kinase-like FERONIA protein (target of miR396), ATP binding cassette transporter (ABC transporter) (targets of miR172 and miR396), etc., which were related to plant hormone signal transduction, plant-pathogen interaction or secondary metabolites transports. For functional annotation, gene ontology (GO) and KEGG analysis were done for the predicted targets, which indicated the diversity of regulatory functions.

Verification of miRNAs: In order to confirm the miRNAs obtained from the high-throughput sequencing, several potential secondary metabolism related miRNAs were measured the expression patterns by RT-qPCR. All the transcripts of the examined miRNAs showed significant increased when the *L. erythrorhizon* cells were exposed to illumination within 72 h (Fig. 6).

Discussion

Light is something that sustains all life on the earth. It not only affords energy for photosynthesis but also acts as the most important environmental signals. Plants have evolved sophisticated light signaling pathway discovered as photoreceptors-E3 ubiquitin ligase complex-transcription factors system regulating plant growth and development as well as plant secondary metabolism (Jiao *et al.*, 2007). There are at least four types of photoreceptors including phytochromes, cryptochromes, phototropins and UV RESISTANCE LOCUS 8 (UVR8) perceiving different light spectrum (Rizzini *et al.*, 2011; Li *et al.*, 2012).

The mechanism of light regulated secondary metabolism is very complex. For most plants, light makes little effect on the production of secondary metabolites or stimulates the production on the contrary. However, light negative controls shikonin biosynthesis by down regulating some key enzymes and transcription factors involved in the formation of shikonin in the important medicinal plant *L. erythrorhizon* (Liu *et al.*, 2006). Shikonin and its derivatives are biosynthesized from two important intermediates metabolites: *p*-hydroxybenzoic acid (PHB) and geranyl pyrophosphate (GPP) which are derived from phenylpropanoid and isoprenoid pathway, respectively. The two intermediates are immediately catalyzed by geranyltransferase and other enzymes through cascade reaction to form shikonin. Shikonin is initially formed in the endoplasmic reticulum and then transported to the cell wall by extracellular secretion (Yazaki *et al.*, 1999).

MiRNAs play a special role in the life cycle, such as plant developmental phase transition, metabolism and stress response (Jung *et al.*, 2009). It is revealed in the model plant *A. thaliana* that light regulate miRNA transcription, miRNA processing and miRNA function (Sanchez-Retuerta *et al.*, 2018). Little is known about the relationship between light and miRNAs of *L. erythrorhizon*. The differential expression and abundance of miRNAs in *L. erythrorhizon* under different light conditions (Dark and White light) provide the clues about different light-responsive miRNAs. Our results show that miR156, miR159, miR319, miR166, miR390, miR394, miR396, miR399, miR827, miR858, miR894, miR2054 and miR5139 are representative light-responsive miRNAs.

The composite and variable structure of light responsive elements (LREs) could explain the specific properties of individual light-responsive promoters (Argüello-Astorga & Herrera-Estrella, 1998). The results from *A. thaliana* and *Populus tremula* showed that the promoter of UV-B responsive miRNA contained LREs. Light-responsive miRNA and their targets shared light regulation pattern. Promoter of UV-B responsive miRNAs was associated with of LREs. The expression level of miR159, miR169 and miR393 are up-regulated in *A. thaliana* but down-regulated in wheat, which suggests the light-responsive miRNAs are species specific (Zhou *et al.*, 2007; Jia *et al.*, 2009). Therefore, we speculate that the promoter of differential expressed *L. erythrorhizon* miRNAs responding to different light irradiation also contains specific LREs.

MiRNAs play their biological functions through their down-regulated targets and the relationship between the miRNA family and their direct target constitute regulation node (for example, miR319-TCP node appears to affect biosynthesis of the hormone jasmonic acid (JA)) (Schommer *et al.*, 2008; Rubio-Somoza & Weigel, 2011). The different miRNA nodes interact with each other and participate in miRNA networks that regulate plant metabolic pathway (Rubio-Somoza & Weigel, 2011).We noted that the targets of some up-regulated miRNAs under illumination are associated with plant secondary metabolism. For example, *Ler*-miR319 targets TCP transcription factors which controlled the biosynthesis of JA by lipoxygenase (LOX) (Schommer *et al.*, 2008). JA can improve the production of secondary metabolites. *Ler*-miR858 targets R2R3-MYB which is a transcrption factor of the phenylpropanoid metabolic pathway. R2R3-MYB can induce the expression of key phenylpropanoid

pathway enzyme transcripts including phenylalanine ammonia-lyase (PAL) and cinnamate-4 hydroxylase (C4H) (Endt *et al.*, 2002; Zhao *et al.*, 2015). The expression levels of miR319 and miR858 were induced significantly in the callus of *L. erythrorhizon* under illumination. This partially explains the reason of the light-down regulated the formation of shikonin in *L. erythrorhizon*. There are also concerns that miR2673 is identified from WHITE library and its potential target is 3-hydroxy-3-methylglutary-coenzyme A reductase (HMGR) gene *HMGR* transcripts. HMGR is a key enzyme of shikonin biosynthesis. White light strongly represses HMGR mRNA levels in *L. erythrorhizon* (Lange *et al.*, 1998). The relationship between miR2673 and HMGR needs to be further validation.

In the analysis of the predicted targets, *Ler*-miR159 targets MKP related to MAPK signal pathway, *Ler*-miR394 targets 2-alkenal reductase response to cadmium ion and response to oxidative stress, *Ler*-miR396 targets receptor protein kinase-like FERONIA protein, mildew resistance locus o (MLO) protein and multidrug-resistance-associated protein (MRP). MAPK cascade is an important signal pathway and play crucial roles in response to biotic or abiotic stress as well as plant growth and development. MKP is one of the important negative-regulated factors. The JA-activated MKK3–MPK6 signal negatively regulates the JA pathway through the inhibition of MYC2 (Takahashi *et al.*, 2007). Whether MKP6 dephosphorylation by MKP could promote JA pathway remain unknown. 2-alkenal reductase can catalyze hydrogenation and reduction of unsaturated double bonds in 2-alkenal or 4-hydroxy-2-alkenals to form saturated aldehydes for detoxification of reactive oxygen species (ROS)-induced peroxides. Dbr1, a 2-alkenal reductase from *Artemisia annua* was found to catalyze artemisinic aldehyde reduction to form dihydroartemisinic aldehyde *in viro* (Zhang *et al.*, 2009). The involvement of 2-alkenal reductase in *L. erythrorhizon* is unknown. FERONIA is a receptor- like kinase (RLK) subfamily involved in many signaling pathway regulating plant growth, reproduction, fruit ripening and pathogen responses. FERONIA perceives environment stimulus signals through extracellular domain and transmits the signal to the downstream components, eg. MLO (Li *et al.*, 2016). MLO is a conserved seven transmembrane calmodulin (CaM) protein as tethering factors for Ca^{2+} channels in cell responses to environmental stimuli and calcium absorption and efflux are affected in MLO-mutant (Meng *et al.*, 2020). A certain concentration of calcium and CaM-mediated Ca^{2+} signaling are important for shikonin accumulation (Liu *et al.*, 2011). 1-aminocyclopropane-1-carboxylic acid synthases (ACS), rate-limiting enzyme in the ethylene synthesis, needs phosphorylation activation by calcium-dependent protein kinase (CPK) pathway or MAPK pathway (Li *et al.*, 2018). Transgenic *LeACS L. erythrorhizon* hairy roots accumulated more shikonin (Fang *et al.*, 2016). But FERONIA may inhibit ethylene production by interaction with S-adenosylmethionine synthetases (SAMS) (Mao *et al.*, 2015). *Le*MRP is possibly directly or indirectly involved in shikonin transport and biosynthesis. *LeMRP* overexpressing *L. erythrorhizon* hairy roots produced more shikonin than control, while *LeMRP* RNAi decreased the accumulation of shikonin (Zhu *et al.*, 2018).

In *Arabidopsis*, miR156 is newly described as relative to secondary metabolism except for the regulation of phase transition and flowering. The node of miR156-SPL9 negatively regulate the anthocyanin pathway by destabilizing MYB-bHLH-WD40 (Gou *et al.*, 2011). SPL9 is also found to regulate the biosynthesis of sesquiterpenes by binding to the sesquiterpene synthase gene promoter (Yu *et al.*, 2015). The miR156-SPL15 module regulates the carotenoigenesis (Wei *et al.*, 2010). Moreover, miR156 targets *SPL15*, and SPL15 has negative feedback on the miR156 expression (Wei *et al.*, 2012). In our study, *Ler*-miR156a is up-regulated when the calli of *L. erythrorhizon* were exposed to continuous white light of 72 h. Whether the node of *Ler*-miR156-SPL are involved in the accumulation of shikonin needs to be identified later.

The miRNA is explored as potential biomarkers in plant stress responses in recent years. Some miRNAs respond to biotic stress (virus, bacteria, fungi, nematode) or abiotic stress (drought, salt, heat, cold, nutrient, mechanical injury, UV, etc.) are summarized (Bej & Basak, 2014). According to this idea, based on the obtained information about light-regulated miRNAs and their targets in *L. erythrorhizon* cells, we proposed a regulatory pathways of light-responsive miRNA mediated shikonin biosynthesis, as shown in Fig. 7. When *L. erythrorhizon* cells in M9 production medium were subjected to white light illumination, miR319, miR858 and miR396 were up-regulated while their target down-regulated. TCP is a positive regulator of JA synthesis. JA and MYB strongly induce the key enzymes of phenylpropanoid pathway such as PAL and C4H, therefore shikonin production is increased. FERONIA and MLO possibly regulated the synthesis of endogenous ethylene which is positive regulator of shinkon production. MRP transports shikonin out of the cytoplasm, which inturn promotes the accumulation of shikonin.

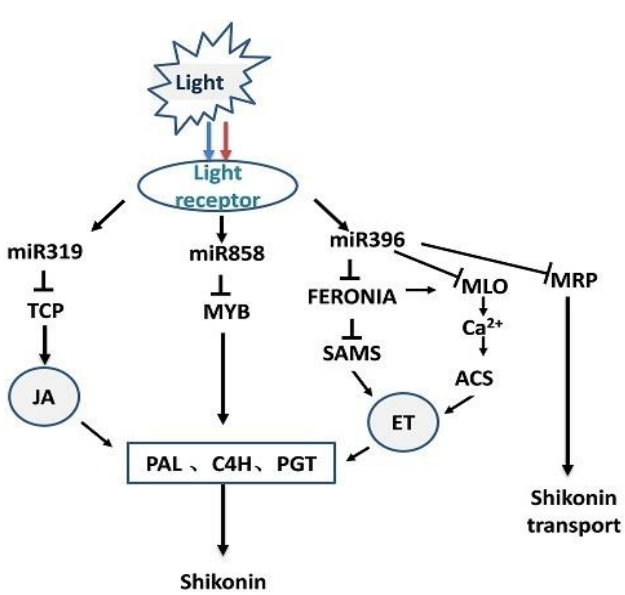

Fig. 7. A proposed regulatory pathways of light-responsive miRNAs mediated shikonin biosynthesis. The arrow and bars represented positive and negative regulation, respectively.

Conclusion

White light could completely inhibit the shikonin derivatives biosynthesis. However, there is little information about the light-responsive miRNAs possibly involved in this process. In the present study, we uncovered the miRNAs expression profile in response to light signal by high-throughput sequencing of two sRNA libraries of *L. erythrorhizon* callus cells cultured under darkness and white light. A total of 18 conservative miRNA family members are notable up-regulated under white light. The possible regulatory pathways of light-responsive miRNAs involved in shikonin biosynthesis were also proposed based on the obtained information about light-regulated miRNAs and their targets. Our results provide new insights into the regulation of shikonin biosynthesis in *L. erythrorhizon*.

Acknowledgments

This research was supported by the grants from the Natural Science Foundation of China (NSFC) (U1903201, 31670298, 31771413, 31970321), the Open Project Program from MOE Key Laboratory of Molecular Epigenetics of China, the Program for Changjiang Scholars and Innovative Research Team in University (IRT_14R27), and the Natural Science Foundation of Jiangsu Bureau of Science and Technology (BK20191254).

References

Andujar, I., M.C. Recio, R.M. Giner and J.L. Rios. 2013. Traditional Chinese medicine remedy to jury: The pharmacological basis for the use of shikonin as an anticancer therapy. *Curr. Med. Chem.*, 20(23): 2892-2898.

Argüello-Astorga, G. and L. Herrera-Estrella. 1998. Evolution of light-regulated plant promoters. *Annu. Rev. Plant Biol.*, 49(1): 525-555.

Baloch, I.A., M.Y.K. Barozai, A.H. Baloch and M. Din. 2019. Bioinformatics prediction and annotation of apple microRNAs and their targets. *Pak. J. Bot.*, 51(3): 909-922.

Bej, S. and J. Basak. 2014. MicroRNAs: The potential biomarkers in plant stress response. *Amer. J. Plant Sci.*, 5(5): 748-759.

Boulos, J.C., M. Rahama, M.E.F. Hegazy and T. Efferth. 2019. Shikonin derivatives for cancer prevention and therapy. *Cancer Lett.*, 459: 248-267.

Dai, X. and P.X. Zhao. 2011. PsRNATarget: A plant small RNA target analysis server. *Nucl. Acids Res.*, 39: W155-W159.

Dai, X., Z. Zhuang and P.X. Zhao. 2018. PsRNATarget: A plant small RNA target analysis server (2017 release). *Nucl. Acids Res.*, 46(W1): W49-W54.

Endt, D.V., J.W. Kijne and J. Memelink. 2002. Transcription factors controlling plant secondary metabolism: What regulates the regulators? *Phytochem.*, 61(2): 107-114.

Fang, R., F. Wu, A. Zou, Y. Zhu, H. Zhao, H. Zhao, Y. Liao, R.J. Tang, T. Yang and Y. Pang. 2016. Transgenic analysis reveals *LeACS-1* as a positive regulator of ethylene-induced shikonin biosynthesis in *Lithospermum erythrorhizon* hairy roots. *Plant Mol. Biol.*, 90(4-5): 345-358.

Fujita, Y., Y. Hara, C. Suga and T. Morimoto. 1981. Production of shikonin derivatives by cell suspension cultures of *Lithospermum erythrorhizon*. *Plant Cell Rep.*, 1(2): 61-63.

Gamborg, O.L., R.A. Miller and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.*, 50(1): 151-158.

Gou, J.Y., F.F. Felippes, C.J. Liu, D. Weigel and J.W. Wang. 2011. Negative regulation of anthocyanin biosynthesis in *Arabidopsis* by a miR156-targeted SPL transcription factor. *Plant Cell*, 23(4): 1512-1522.

Huang, S.C., G.H. Lu, C.Y. Tang, Y.J. Ji, G.S. Tan, D.Q. Hu, J. Cheng, G.H. Wang, J.L. Qi and Y.H. Yang. 2018. Identification and comparative analysis of aluminum-induced micrornas conferring plant tolerance to aluminum stress in soybean. *Biol. Plant.*, 62(1): 97-108.

Jia, X., L. Ren, Q.J. Chen, R. Li and G. Tang. 2009. UV-B-responsive microRNAs in *Populus tremula*. *J. Plant Physiol.*, 166(18): 2046-2057.

Jiao, Y.L., O.S. Lau and X.W. Deng. 2007. Light-regulated transcriptional networks in higher plants. *Nat. Rev. Genet.*, 8(3): 217-230.

Jung, J.-H., P.J. Seo and C.-M. Park. 2009. MicroRNA biogenesis and function in higher plants. *Plant Biotechnol. Rep.*, 3(2): 111-126.

Kojiro, T., N. Yukimi, A. Shunsuke, K. Kenta, A. Wataru, S.-M. Yuka, S. Akifumi, U. Mitsuyoshi and Y. Kazufumi. 2019. Comparative proteomic analysis of *Lithospermum erythrorhizon* reveals regulation of a variety of metabolic enzymes leading to comprehensive understanding of the shikonin biosynthetic pathway. *Plant Cell Physiol.*, 60(1): 19-28.

Kozomara, A., M. Birgaoanu and S. Griffiths-Jones. 2019. miRBase: from microRNA sequences to function. *Nucl. Acids Res.*, 47(D1): D155-D162.

Lange, B.M., K. Severin, A. Bechthold and L. Heide. 1998. Regulatory role of microsomal 3-hydroxy-3-methylglutaryl-coenzyme a reductase for shikonin biosynthesis in *Lithospermum erythrorhizo*n cell suspension cultures. *Planta*, 204(2): 234-241.

Li, C., H.M. Wu and A.Y. Cheung. 2016. Feronia and her pals: Functions and mechanisms. *Plant Physiol.*, 171(4): 2379-2392.

Li, J., W. Terzaghi and X.W. Deng. 2012. Genomic basis for light control of plant development. *Protein Cell*, 3(2): 106-116.

Li, S., X. Han, L. Yang, X. Deng, H. Wu, M. Zhang, Y. Liu, S. Zhang and J. Xu. 2018. Mitogen-activated protein kinases and calcium-dependent protein kinases are involved in wounding-induced ethylene biosynthesis in *Arabidopsis thaliana*. *Plant Cell Environ.*, 41(1): 134-147.

Liang, D., Y. Sun, Y. Shen, F. Li, X. Song, E. Zhou, F. Zhao, Z. Liu, Y. Fu, M. Guo, N. Zhang, Z. Yang and Y. Cao. 2013. Shikonin exerts anti-inflammatory effects in a murine model of lipopolysaccharide-induced acute lung injury by inhibiting the nuclear factor-kappaB signaling pathway. *Int. Immunopharmacol.*, 16(4): 475-480.

Liao, Y.H., C.Y. Tang, R.J. Fang, Y. Zhu, F.Y. Wu, P.Z. Zhao, S.C. Huang, X.M. Wang, Y.J. Pang, R.W. Yang, G.H. Lu, J.L. Qi and Y.H. Yang. 2018. Transcriptome analysis reveals that blocking the ethylene signal transduction pathway is a key point for 2,4-D-inhibited shikonin biosynthesis in *Lithospermum erythrorhizon* (Boraginaceae). *Pak. J. Bot.*, 50(4): 1451-1458.

Liu, Z., Y. Li, T. Yang, J. Su, M. Zhang, R. Tian, X. Li, Y. Pang, J. Qi and Y. Yang. 2011. Shikonin accumulation is related to calcium homeostasis in *Onosma paniculata* cell cultures. *Phyton Ann. Rei Bot.*, 51(1): 103-113.

Liu, Z., J.L. Qi, L. Chen, M.S. Zhang, X.Q. Wang, Y.J. Pang and Y.H. Yang. 2006. Effect of light on gene expression and shikonin formation in cultured O*nosma paniculatum* cells. *Plant Cell Tiss. Org. Cult.*, 84(1): 38-48.

Mao, D., F. Yu, J. Li, B. Van de Poel, D. Tan, J. Li, Y. Liu, X. Li, M. Dong, L. Chen, D. Li and S. Luan. 2015. Feronia receptor kinase interacts with S-adenosylmethionine synthetase and suppresses S-adenosylmethionine production and ethylene biosynthesis in *Arabidopsis*. *Plant Cell Environ.*, 38(12): 2566-2574.

Matsuura, H.N., V. Fragoso, J.T. Paranhos, M.R. Rau and A.G. Fett-Neto. 2016. The bioactive monoterpene indole alkaloid n,beta-d-glucopyranosyl vincosamide is regulated by irradiance quality and development in *Pychotria leiocarpa*. *Ind. Crops & Prod.*, 86: 210-218.

Meng, J.G., L. Liang, P.F. Jia, Y.C. Wang, H.J. Li and W.C. Yang. 2020. Integration of ovular signals and exocytosis of a Ca^{2+} channel by mlos in pollen tube guidance. *Nat. Plants*, 6(2): 143-153.

Namdar, D., D. Charuvi, V. Ajjampura, M. Mazuz, A. Ion, I. Kamara and H. Koltai. 2019. Led lighting affects the composition and biological activity of *Cannabis sativa* secondary metabolites. *Ind. Crops & Prod.*, 132: 177-185.

Papageorgiou, V.P., A.N. Assimopoulou, E.A. Couladouros, D. Hepworth and K.C. Nicolaou. 1999. The chemistry and biology of alkannin, shikonin, and related naphthazarin natural products. *Angew. Chem.Int. Ed.*, 38(3): 270-301.

Qi, J.L., W.J. Zhang, S.H. Liu, H. Wang, D.Y. Sun, G.H. Xu, M.W. Shi, Z. Liu, M.S. Zhang, H.M. Zhang and Y.H. Yang. 2008. Expression analysis of light-regulated genes isolated from a full-length-enriched cdna library of *Onosma paniculatum* cell cultures. *J. Plant Physiol.*, 165(14): 1474-1482.

Reinhart, B.J., E.G. Weinstein, M.W. Rhoades, B. Bartel and D.P. Bartel. 2002. MicroRNAs in plants. *Genes Dev.*, 16(13): 1616-1626.

Rizzini, L., J.J. Favory, C. Cloix, D. Faggionato, A. O'Hara, E. Kaiserli, R. Baumeister, E. Schafer, F. Nagy, G.I. Jenkins and R. Ulm. 2011. Perception of UV-B by the *Arabidopsis* UVR8 protein. *Science*, 332(6025): 103-106.

Rubio-Somoza, I. and D. Weigel. 2011. MicroRNA networks and developmental plasticity in plants. *Trends Plant Sci.*, 16(5): 258-264.

Sanchez-Retuerta, C., P. Suarez-Lopez and R. Henriques. 2018. Under a new light: Regulation of light-dependent pathways by non-coding RNAs. *Front. Plant Sci.*, 9: 962-978.

Schmittgen, T.D. and K.J. Livak. 2008. Analyzing real-time PCR data by the comparative C-T method. *Nat. Protocols*, 3(6): 1101-1108.

Schommer, C., J.F. Palatnik, P. Aggarwal, A. Chételat, P. Cubas, E.E. Farmer, U. Nath, D. Weigel and J.C.J.P.B. Carrington. 2008. Control of jasmonate biosynthesis and senescence by miR319 targets. *PLoS Biol.*, 6(9): e230.

Shi, R. and V.L. Chiang. 2005. Facile means for quantifying microRNA expression by real-time PCR. *BioTechniques*, 39(4): 519-525.

Shimomura, K., H. Sudo, H. Saga and H. Kamada. 1991. Shikonin production and secretion by hairy root cultures of *Lithospermum erythrorhizon*. *Plant Cell Rep.*, 10(6-7): 282-285.

Sim, S.J., D.J. Kim and H.N. Chang. 1994. Shikonin production by extractive cultivation in transformed-suspension and hairy root cultures of *Lithospermum erythrorhizon*. *Ann. N. Y. Acad. Sci.*, 745: 442-454.

Sun, G. 2012. MicroRNAs and their diverse functions in plants. *Plant Mol. Biol.*, 80(1): 17-36.

Tabata, M., H. Mizukami, N. Hiraoka and M. Konoshim. 1974. Pigment formation in callus cultures of *Lithospermum erythrorhizon*. *Phytochem.*, 13(6): 927-932.

Takahashi, F., R. Yoshida, K. Ichimura, T. Mizoguchi, S. Seo, M. Yonezawa, K. Maruyama, K. Yamaguchi-Shinozaki and K. Shinozaki. 2007. The mitogen-activated potein kinase cascade MKK3-MPK6 is an important part of the jasmonate signal transduction pathway in *Arabidopsis*. *Plant Cell*, 19(3): 805-818.

Thakur, M., S. Bhattacharya, P.K. Khosla and S. Puri. 2019. Improving production of plant secondary metabolites through biotic and abiotic elicitation. *J. Appl. Res. Med. Arom.*, 12: 1-12.

Touno, K., K. Harada, K. Yoshimatsu, K. Yazaki and K. Shimomura. 2000. Shikonin derivative formation on the stem of cultured shoots in *Lithospermum erythrorhizon*. *Plant Cell Rep.*, 19(11): 1121-1126.

Touno, K., J. Tamaoka, Y. Ohashi and K. Shimomura. 2005. Ethylene induced shikonin biosynthesis in shoot culture of *Lithospermum erythrorhizon*. *Plant Physiol. Biochem.*, 43(2): 101-105.

Wei, S., M.Y. Gruber, B. Yu, M.J. Gao, G.G. Khachatourians, D.D. Hegedus, I.A. Parkin and A. Hannoufa. 2012. *Arabidopsis* mutant *sk156* reveals complex regulation of SPL15 in a miR156-controlled gene network. *BMC Plant Biol.*, 12: 169.

Wei, S., B. Yu, M.Y. Gruber, G.G. Khachatourians, D.D. Hegedus and A. Hannoufa. 2010. Enhanced seed carotenoid levels and branching in transgenic *Brassica napus* expressing the *Arabidopsis miR156b* gene. *J. Agri. Food Chem.*, 58(17): 9572-9578.

Wittstock, U., N. Agerbirk, E.J. Stauber, C.E. Olsen, M. Hippler, T. Mitchell-Olds, J. Gershenzon and H. Vogel. 2004. Successful herbivore attack due to metabolic diversion of a plant chemical defense. *Proc. Natl. Acad. Sci. USA*, 101(14): 4859-4864.

Wu, B., M. Wang, Y. Ma, L. Yuan and S. Lu. 2012. High-throughput sequencing and characterization of the small RNA transcriptome reveal features of novel and conserved microRNAs in *Panax ginseng*. *PLoS One*, 7(9): e44385.

Wu, F.Y., C.Y. Tang, Y.M. Guo, Z.W. Bian, J.Y. Fu, G.H. Lu, J.L. Qi, Y.J. Pang and Y.H. Yang. 2017. Transcriptome analysis explores genes related to shikonin biosynthesis in *Lithospermeae* plants and provides insights into *Boraginales*' evolutionary history. *Sci. Rep.*, 7(1): 4477.

Wu, L., H. Zhou, Q. Zhang, J. Zhang, F. Ni, C. Liu and Y. Qi. 2010. DNA methylation mediated by a microRNA pathway. *Mol. Cell*, 38(3): 465-475.

Wu, S.J., J.L. Qi, W.J. Zhang, S.H. Liu, F.H. Xiao, M.S. Zhang, G.H. Xu, W.G. Zhao, M.W. Shi and Y.J. Pang. 2009. Nitric oxide regulates shikonin formation in suspension-cultured *Onosma paniculatum* cells. *Plant Cell Physiol.*, 50(1): 118-128.

Yazaki, K., H. Matsuoka, T. Ujihara and F. Sato. 1999. Shikonin biosynthesis in *Lithospermum erythrorhizon*: Light-induced negative regulation of secondary metabolism. *Plant Biotechnol.*, 16(5): 335-342.

Yazaki, K., K. Takeda and M. Tabata. 1997. Effects of methyl jasmonate on shikonin and dihydroechinofuran production in *Lithospermum* cell cultures. *Plant Cell Physiol.*, 38(7): 776-782.

Yu, Z.X., L.J. Wang, B. Zhao, C.M. Shan, Y.H. Zhang, D.F. Chen and X.Y. Chen. 2015. Progressive regulation of sesquiterpene biosynthesis in *Arabidopsis* and *Patchouli (Pogostemon cablin*) by the miR156-targeted spl transcription factors. *Mol. Plant*, 8(1): 98-110.

Zeng, Q.Y., C.Y. Yang, Q.B. Ma, X.P. Li, W.W. Dong and H. Nian. 2012. Identification of wild soybean mirnas and their target genes responsive to aluminum stress. *BMC Plant Biol.*, 12: e182.

Zhang, B.H., X.P. Pan, C.H. Cannon, G.P. Cobb and T.A. Anderson. 2006b. Conservation and divergence of plant microRNA genes. *Plant J.*, 46(2): 243-259.

Zhang, B.H., X.P. Pan, S.B. Cox, G.P. Cobb and T.A. Anderson. 2006a. Evidence that miRNAs are different from other RNAs. *Cell. Mol. Life Sci.*, 63(2): 246-254.

Zhang, W.J., J. Su, M.Y. Tan, G.L. Liu, Y.J. Pang, H.G. Shen, J.L. Qi and Y. Yang. 2010. Expression analysis of shikonin-biosynthetic genes in response to M9 medium and light in *Lithospermum erythrorhizon* cell cultures. *Plant Cell Tiss. Organ Cult.*, 101(2): 135-142.

Zhang, Y., H. Han, H. Qiu, H. Lin, L. Yu, W. Zhu, J. Qia, R. Yang, Y. Pang, X. Wang, G. Lu and Y. Yang. 2017. Antiviral activity of a synthesized shikonin ester against influenza A (H1N1) virus and insights into its mechanism. *Biomed. & Pharmacoth.*, 93: 636-645.

Zhang, Y., K.H. Teoh, D.W. Reed and P.S. Covello. 2009. Molecular cloning and characterization of Dbr1, a 2-alkenal reductase from *Artemisia*. *Ann. Bot.*, 87(6): 643-649.

Zhang, Z., J. Yu, D. Li, Z. Zhang, F. Liu, X. Zhou, T. Wang, Y. Ling and Z. Su. 2010. PMRD: Plant microRNA database. *Nucleic Acids Res.*, 38: D806-D813.

Zhao, H., S.K. Baloch, L.R. Kong, W.J. Zhang, A.L. Zou, X.M. Wang, J.L. Qi and Y.H. Yang. 2014. Molecular cloning, characterization, and expression analysis of *LeMYB1* from *Lithospermum erythrorhizon*. *Biol. Plant.*, 58(3): 436-444.

Zhao, H., Q.S. Chang, D.X. Zhang, R.J. Fang, H. Zhao, F.Y. Wu, X.M. Wang, G.H. Lu, J.L. Qi and Y.H. Yang. 2015. Overexpression of *LeMYB1* enhances shikonin formation by up-regulating key shikonin biosynthesis-related genes in *Lithospermum erythrorhizon*. *Biol. Plant.*, 59(3): 429-435.

Zhao, X.R., Y.L. Zhou, L.Y. Wang, M. Li, D.X. Shi, D. Li and J.F. Wang. 2017. Shikonin alleviates the biotoxicity produced by pneumococcal pneumolysin. *Life Sci.*, 177: 1-7.

Zhou, X.F., G.D. Wang and W.X. Zhang. 2007. UV-B responsive microRNA genes in *Arabidopsis thaliana*. *Mol. Syst. Biol.*, 3(1): 103.

Zhu, J., L. Zhao, B. Luo and W. Sheng. 2019. Shikonin regulates invasion and autophagy of cultured colon cancer cells by inhibiting yes-associated protein. *Oncol. Lett.*, 18(6): 6117-6125.

Zhu, Y., S.J. Chu, Y.L. Luo, J.Y. Fu and Y.H. Yang. 2018. Involvement of *LeMRP*, an ATP-binding cassette transporter, in shikonin transport and biosynthesis in *Lithospermum erythrorhizon*. *Plant Biol.*, 20(2): 365-373.

Zuker, M. 2003. MFold web server for nucleic acid folding and hybridization prediction. *Nucl. Acids Res.*, 31(13): 3406-3415.

(Received for publication 20 May 2019)