Pak. J. Bot., 50(4): 1451-1458, 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)

YONGHUI LIAO[1,2†], CHENGYI TANG[1,2†], RONGJUN FANG[1,2], YU ZHU[1,2], FENGYAO WU[1,2], PINGZHI ZHAO[1,2], SHOUCHENG HUANG[1,3], XIAOMING WANG[1,2], YANJUN PANG[1,2], RONGWU YANG[1,2], GUIHUA LU[1,2*], JINLIANG QI[1,2*] AND YONGHUA YANG[1,2*]

[1]Institute of Plant Molecular Biology, State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing 210093, P.R. China
[2]Co-Innovation Center for Sustainable Forestry in Southern China, Nanjing Forestry University, Nanjing 210037, P. R. China
[3]College of Life Science, Anhui Science and Technology University, Fengyang 233100, P.R. China
[*]Corresponding author's email: yangyh@nju.edu.cn; qijl@nju.edu.cn; guihua.lu@nju.edu.cn
[†]These authors contributed equally to this work

Abstract

The synthetic growth hormone2,4-dichlorophenoxyacetic acid (2,4-D) significantly inhibits shikonin and its derivatives biosynthesis in the medicinal plant *Lithospermum erythrorhizon*. However, the molecular mechanism of this regulation remains unclear.In this study, we attempt to address this issue by comparing the transcriptome of 2,4-D treated cell cultureswith that of the control (CK). A total of 216 up-regulated and 269 down-regulated genes by 2,4-D were discovered. Gene ontology (GO) enrichment analysis revealed that differentially expressed genes (DEGs) were statistically significantly related to the metabolic process. Pathway classification enrichment analysis further confirmed that the DEGs were mainly related to the secondary metabolism. More importantly, 2,4-D has no effect on the two key enzyme genes *ACS* and *ACO* for ethylene biosynthesis. However,2,4-D significantly down-regulated the genes directly involved in ethylene signal transduction pathway (ESTP), including *HMA5*, *EIN3*, and *ORCA3*. 2,4-D also down-regulated two indirect genes, *LOX* and *GA2ox*, thereby conferring the jasmonate (JA) and GA biosynthesis, which regulates ESTP by targeting EIN3. By blocking ESTP, 2,4-D finally down-regulated the phenylpropanoid formation-related genes, thereby conferring shikonin and its derivatives biosynthesis. Our findingsprovide new hints for the deep understanding of the molecular mechanism of shikonin formation.

Key words: 2, 4-D, Ethylene signal transduction pathway, *Lithospermum erythrorhizon*, Shikonin, Transcriptome.

Introduction

Secondary metabolites play multiple pivotal roles in plant growth, development, stress, and resistance. Many kinds of these metabolites, especially compounds formed in medicinal plants, are also widely used in clinical application. Shikonin and its derivatives are a kind of naphthoquinone metabolites only synthesized in the root organs of the medicinal Boraginaceae plants, such as *Lithospermum erythrorhizon*, *Onosma paniculatum*, *Arnebia euchroma* and *Arnebia guttata*. These compounds have significant antimicrobial, anti-inflammatory, antitumor, and anti-HIV activities (Chen *et al.,* 2003; Papageorgiou *et al.,* 2006; Chen *et al.,* 2011; Gong & Li 2011; Lu *et al.,* 2011; Chen *et al.,* 2012; Tian *et al.,* 2015; Yang *et al.,* 2015). Given that these compounds are red pigments, they are also widely used as natural colorant either for food or pharmacy and cosmetic industry.

The metabolic pathway of shikonin and its derivatives formation has been well clarified. Many regulators, such as methyl jasmonate (MeJA) (Yazaki*et al*., 1997a), mineral elements (Fujita *et al*., 1981), light signal (Yazaki *et al*., 1999), nitric oxide (Wu *et al.,* 2009), andethylene (ET) (Touno *et al.,* 2005; Fang *et al.,* 2016a,b), have also been involved in their formation.

The synthetic growth hormone 2,4-dichlorophenoxyacetic acid (2,4-D) is widely used in tissue culture system. However, unlike the positive phytohormones, (such as ET and MeJA) used for regulating shikonin biosynthesis, 2,4-D can dramatically inhibit shikonin and its derivatives biosynthesis (Yazaki *et al.,* 1999), which provides an excellent reverse experimental system for analyzing the molecular mechanism of shikonin formation. However, the molecular regulatory mechanism remains unknown. In this study, we constructed and compared the expression profiling of 2,4-D treated cell cultures with that of the control (CK) by using the advanced high-throughput transcriptome sequencing strategy to address this question. Our findings can provide new hints for the deep understanding of the molecular mechanism of shikonin biosynthesis.

Materials and Methods

Plant material and 2,4-D treatment: The Y8 cell line used in the present research was induced from the *Lithospermum erythrorhizon*young shoots (Zhao *et al.,* 2014) and maintained in B5 growth medium for cell proliferation (Gamborg *et al.,* 1968)under 16 h dark/8 h light (80 μM M^{-2} S^{-1}, TLD36W/54, Philips, Einhoven, The Netherlands) at 25°C (Zhao *et al.,* 2015). For the formation of shikonin and its derivatives, the cells should be transferred into M9 production medium from B5 growth medium (Fujita *et al.*,1981) and cultured in darkness in a shaker at 120 rpm at 25°C. About 1 g of callus cells were cultured in 20 mL of M9 liquid medium in the 100 mL flasks. For treatment, callus cells were transferred into M9 medium containing 1 μM 2,4-D and collected on day three (this period is the early evoke stage for shikonin biosynthesis) for transcriptome analysis the callus cells treated with ddH_2O were used as control (Zhao *et al.,* 2014).

RNA isolation, library construction, and sequencing: Total RNA was prepared with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). After mRNA was purified with oligo-d(T) beads, the libraries were constructed and then sequenced by using IlluminaMiSeq. Raw reads with adaptors, without uncertainty nucleotides, length shorter than 50 base pairs, and of low quality (<Q20) were discarded (Shanghai Personal Biotechnology Co., Ltd. Shanghai, China).

Sequence bioinformatics analyses and functional annotation: The sequences were spliced by using the Velvet/ Oases software (http://www.ebi.ac.uk/~zerbino/oases; http://www.ebi.ac.uk/~zerbino/velvet) after trimming and quality evaluation. Unigenes were obtained by BLASTanalysis with the spliced transcripts. The cluster and annotation of unigenes were analyzed by using the non-redundantdatabase in NCBI (http://www.ncbi.nlm.nih.gov/). Evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were analyzed at http://eggnog.embl.de/andhttp://www.genome.jp/kegg/, respectively. The normalized basemean of the gene with two-fold change and $p<0.05$ was designated as differentially expressed gene (DEG). Gene Ontology (GO) classification of all DEGs was conducted as previously reported (Ashburner *et al.*, 2010).

Results

Characterization of the transcriptome sequences: The RNA-seq strategy was applied to analyze the genome-wide transcriptional response of the callus cells to 2, 4-D regulator. The callus cells treated with or without 2, 4-D were sequenced by using IlluminaMiSeq. We obtained approximately 7,779,424 and 6,464,274 high-quality reads of 214 and 191 bp from 2, 4-D and CK sample, respectively. The percentage of the useful reads of 2,4-D and CK sample is 85.90% and 85.44%, respectively. The average length of the contig, transcript, and unigene is 172, 587, and 1,162, respectively (Table 1, Fig. 1).

Identification of DEGs in response to 2,4-D: According to the criteria (two-fold change and $p<0.05$) for screening the preceding DEGs mentioned, we completely identified 485 DEGs, in which 269 and 216 genes were down-regulated and up-regulated genes by 2,4-D, respectively (Fig. 2). A total of 23814 genes did not show significant expression difference. The percentage of the down-regulated and up-regulated genes accounted for 0.89% and 1.11% of total genes, respectively.

Functional analyses and annotations of the DEGs: GO enrichment analysis reveals that the DEGs were preferentially statistically related to the metabolic process, the response to endogenous stimulus, transport, cellular process, and binding, with the –log10 (*p*-value) of 66.69, 24.46, 16.14, 15.75, and 13.16, respectively (Fig. 3). The functions of the DEGs were dramatically involved in the biological process, which suggests that 2,4-D treatment affected shikonin and its derivatives biosynthesis mainly by regulating multiple biological processes of the callus cells. Pathway classification enrichment analysis further confirmed the GO enrichment analysis result. As shown in Fig. 4, the DEGs were mainly statistically related to the metabolism process, in which the DEGs were further preferentially statistically related to the biosynthesis of secondary metabolites (the –log10 (*p*-value) is 8.77), followed by amino acid metabolism and energy metabolism..

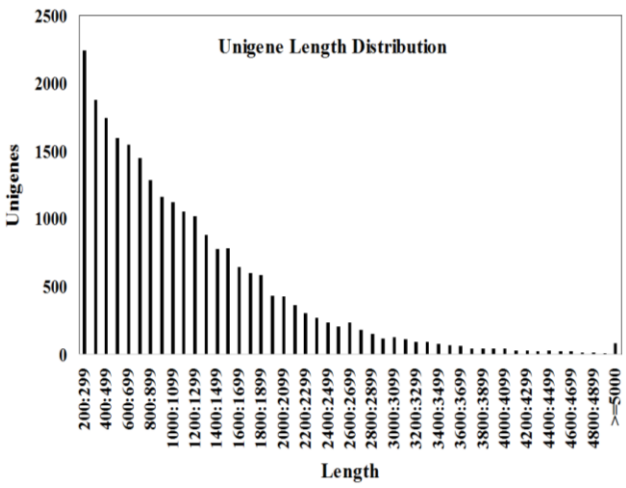

Fig. 1. Length distribution of the unigenes.

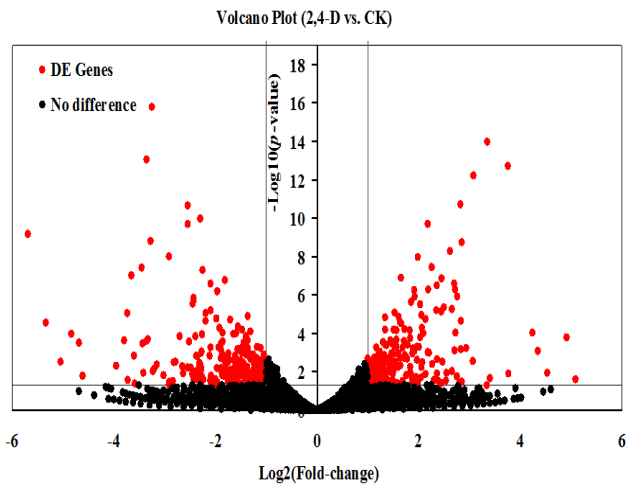

Fig. 2. Volcano plot of the EDGs of 2,4-D versus CK. The genes with two-fold change and $p<0.05$ were designated as DEGs. Red dots represent DEGs, and the black dots indicate the genes with no difference. The two vertical lines and one horizontal line within the graph indicated the threshold value of two-fold change and $p<0.05$, respectively.

Table 1.The characterization of contig, transcript, and unigene.

Type	Total length (bp)	Sequence/transcript/ unigene No.	Max length (bp)	Ave length (bp)	N50	>N50 reads No.	GC %
Contig	44,743,510	259,717	1,400	172	177	90,495	41.8%
Transcript	70,693,280	120,363	9,054	587	1,359	16,116	42.3%
Unigene	28,235,987	24,299	9,054	1,162	1,582	5,908	42.16%

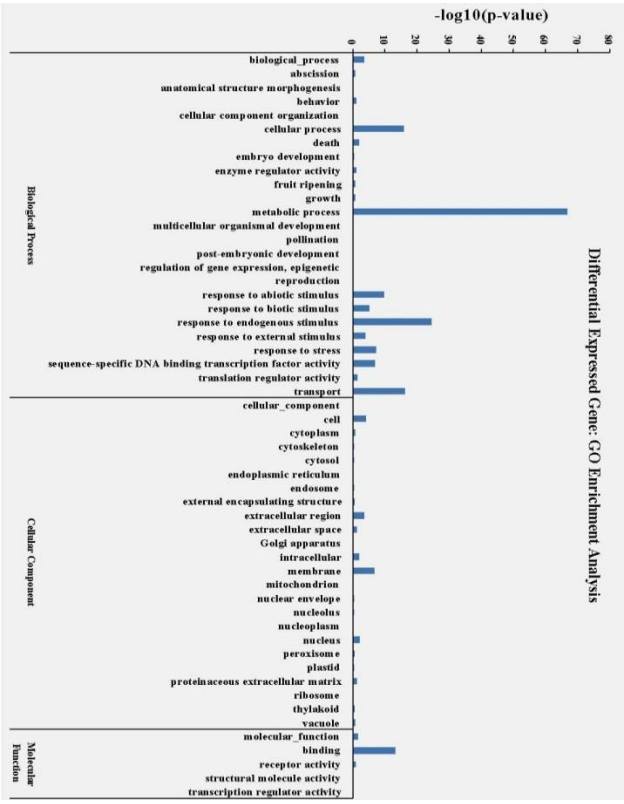

Fig. 3. GO enrichment analysis of the DEGs. The GO functions were mainly classified into three categories, namely, biological process, cellular component, and molecular function. The bar with high –log10 (*p*-value) indicated that the DEGs were preferentially statistically related to this function category.

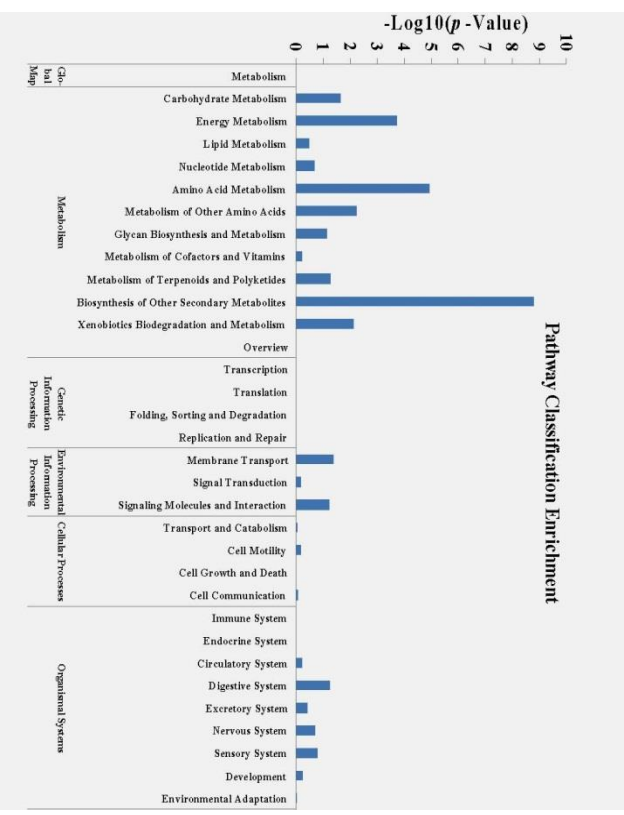

Fig. 4. Pathway classification enrichment of the DEGs.The bar with high –log10 (*p*-value) indicated thatthe DEGs were preferentially statistically related to this function pathway.

DEGs involved in secondary metabolite biosynthesis in response to 2,4-D: The DEGs in the biosynthesis of secondary metabolites in response to 2,4-D are listed in Table 2. All the DEGs involved in the biosynthesis of phenolic (phenylpropanoid, stilbenoid, diarylheptanoid and gingerol, flavonoid, and anthocyanin) and alkaloid compounds (isoquinoline alkaloid, tropane, piperidine, and pyridine alkaloid) were down-regulated by 2,4-D. As for terpenoid compounds, the gene related to terpenoid backbone biosynthesis was down-regulated, whereas other genes related to diterpenoid, brassinosteroid, and zeatin biosynthesis were up-regulated.

We also detected a few reported genes involved in the pathway of shikonin biosynthesis. Although the expression of these genes was not designated as DEGs based on our strict screening criteria, these genes were down-regulated by 2,4-D to some extent, except the two gene members *PGT-2* and *C4H-1*, which were up-regulated by 2,4-D to some extent.The two genes were possibly not main participants in shikonin and its derivatives formation as *PGT-1* and *C4H-2* (Table 3).

Influence of 2,4-D on the genes related to ethylene formation and signal transduction pathway: Two genes involved in ethylene biosynthesis, ACC synthase gene (*ACS*) and ACC oxidase gene (*ACO*), were detected in the transcriptome data, but their expression was not regulated by 2,4-D. However, the genes in ethylene signal transduction pathway (ESTP) were all significantly down-regulated by 2,4-D, including the copper-transporting ATPase gene (*HMA5*), which transports copper ion to the ethylene receptor to activate its activity for highaffinity with ethylene signal; the EIN3-like gene (*EIL*), which acts as the pivotal positive transcription factor for ESTP; the JA-responsive APETALA2 (AP2)-domain transcription factor gene from *Catharanthusroseus* (*ORCA3*); the key enzyme lipoxygenase gene (*LOX*) conferring the biosynthesis of JA, which regulates the ESTP by targeting EIN3; and the key enzyme gibberellin 2-oxidase gene (*GA2ox*) conferring the degradation of GA, which also regulates the ESTP by targeting EIN3 (Table 4, Fig. 5).

Discussion

As one kind of artificial auxin, 2,4-D is crucial in plant growth, development, and somatic embryogenesis, similar to other phytohormones belonging to the auxin category, such as indole-3-acetic acid (IAA),3-indolebutyric acid, and 1-naphthaleneacetic acid (Cruz *et al.,* 1990; Cleland, 1995; Ljung*et al.,* 2002; Bakhtiar *et al.,*2014). A few studies showed that 2,4-D can also positively or negatively regulate the formation of secondary metabolites in higher plants. The 2,4-D treatment suppressed the biosynthesis of anthocyanin (Ozeki *et al.,* 1993; Takeda, 1990). The cell lines of red beet showing different colors were induced from secondary callus with specific strategies. The colors, including white, yellow, orange, red, deep violet,and green, represent all kinds of pigments in red beet plant. The ratio of 2,4-D to 6-BAP was acritical factor in the regulation of these processes (Girod & Zryd, 1991). Many other secondary compounds, such as lycopene (Robertson *et al.,* 1995), indole alkaloids (Carpin*et al.,* 1997), nicotine (Takahashi & Yamada, 1973), saponin(Lu *et al.,* 2001), and coumarin (Bais *et al.,* 2001; Lu *et al.,* 2001) can be negatively regulated by 2,4-D but activated by the supply

of IAA or zeatin. The 2,4-D also regulated the production of low-molecular-weight secondary metabolite phytoalexin. In *Arabidopsis* seedlings, the treatment of 2,4-D yielded a decrease in camalexin production (Glawischnig*et al.*, 2004). However, the 6-methoxymellein production in carrot cells was promoted by 2,4-D but not by IAA in the medium (Kurosaki *et al.,* 1985). The regulator 2,4-D significantly irreversibly inhibited shikonin and its derivatives biosynthesis even at a very low concentration (Yazaki*et al.,* 1999). However, the molecular regulatory mechanism of this process remains unknown.

In recent years, the advanced high-throughput transcriptome sequencing strategy has been applied to uncover the molecular mechanisms of diverse plant physiological processes (Morozova*et al.*, 2008; Shendure & Ji, 2008; Wang *et al.*, 2009; Calzadilla *et al.*, 2016; Cao *et al.*, 2016; Chen *et al.*, 2016; Islam *et al.*, 2016; Muñoz-Espinoza *et al.*, 2016; Vojta*et al.*, 2016; Xu*et al.*, 2016; Cheng *et al.*, 2018). We used this approach in the present study to compare the transcriptome differences between 2,4-D treated callus cells and the CK. The results showed that 485 genes were significantly regulated by 2,4-D. GO enrichment analysis revealed that the DEGs were preferentially statistically related to the metabolic process, response to endogenous stimulus, transport, cellular process, and binding, with the higher –log10(*p*-value) of 66.69, 24.46, 16.14, 15.75, and 13.16, respectively. These results revealed that 2,4-D mainly affected the metabolic processes of the callus cells, and this regulation was possibly controlled by the endogenous stimulus induced by 2,4-D. In addition, due to the absence of shikonin formation under 2,4-D treatment, the genes related to the transport of shikonin and its derivatives and other substrates for shikonin biosynthesis were significantly influenced by 2,4-D. Moreover, the functions of the DEGs were dramatically involved in the biological process, suggesting that 2,4-D treatment affected shikonin and its derivatives biosynthesis mainly by regulating multiple biological processes of the callus cells. Pathway classification enrichment analysis showed that the DEGs were mainly statistically related to the metabolism process, in which the DEGs were preferentially statistically related to the biosynthesis of secondary metabolites with the highest –log10 (*p*-value) of 8.77, thereby further confirming the GO enrichment analysis result.

Shikonin and its derivatives are formed from the following two crucial precursors: geranyl pyrophosphate derived from the isoprenoid pathway and *p*-hydroxybenzoic acid formed from the phenylpropanoid pathway. Therefore, the factors affecting the two pathways can influence shikonin and its derivatives biosynthesis. The functional annotations proved that the DEGs related to the biosynthesis of secondary metabolites were dramatically regulated by the compound 2,4-D. Among these genes, all the DEGs involved in the phenylpropanoid biosynthesis pathway were down-regulated by the compound 2,4-D. Although the genes related to diterpenoid, brassinosteroid, and zeatin biosynthesis were up-regulated, the rate-limiting enzyme gene of the isoprenoid pathway (Campbell *et al.*, 1998; Berthelot*et al.*, 2012), isopentenyldiphosphate isomerase gene, was dramatically down-regulated by 2,4-D. Therefore, the two pathways for shikonin biosynthesis were all blocked by 2,4-D to a considerable extent. In addition, a few reported genes related to the biosynthesis pathway of shikonin and its derivatives (i.e., *PAL-1*, *PAL-2* (Yazaki *et al.*, 1997b), *C4H-2* (Yamamura *et al.*, 2001), *4CL-1*, *4CL-2* (Yazaki *et al.*, 1995), and *PGT-1* (Yazaki *et al.*,2002)), and the important gene possibly involve in the trapping and/or intra-cell wall excretion of shikonin and its derivatives, *LePS-2*, were all down-regulated by 2,4-D to some extent although the expression of these genes was not designated as DEGs based on our strict screening criteria.

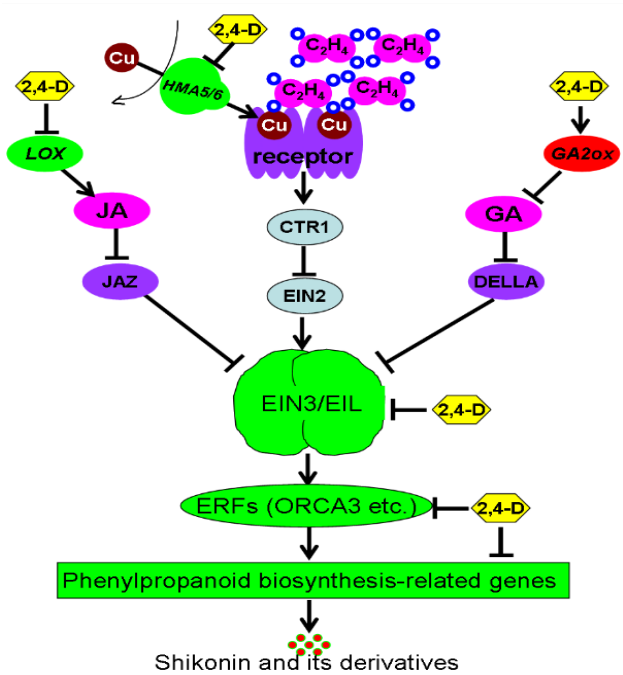

Fig. 5. Proposed model illustrating the molecular mechanism of 2,4-D in regulating the biosynthesis of shikonin and its derivatives by blocking the ESTP. The arrow and bars represented positive and negative regulation, respectively. The green and red background indicated the gene down-regulated and up-regulated by 2,4-D, respectively.

Since the previous studies proved that ethylene signal plays a pivotal role in regulating shikonin and its derivatives biosynthesis (Touno *et al.*, 2005; Fang *et al.*, 2016a,b), the factors affecting the ethylene biosynthesis and ESTP would finally influence the formation of shikonin pigments. In this study, two genes involved in ethylene biosynthesis, *ACS* and *ACO*, were not regulated by the compound 2,4-D. However, the genes involved in ESTP were all significantly down-regulated by the compound 2,4-D, in which the P-type copper-transporting ATPase gene *HMA5* transported copper ion to the ethylene receptor to activate its activity for highaffinity with ethylene signal. The *EIL* acted as the pivotal positive transcription factor gene for ESTP; the JA-responsive APETALA2 (AP2)-domain transcription factor gene from *C. roseus ORCA3* activated the transcription of the shikonin biosynthesis-related genes. Although the *LOX* and *GA2ox*genes were not directly involved in the ESTP, the two genes were key enzyme genes conferring the biosynthesis of JA and GA, which indirectly regulated the ESTP by targeting EIN3 through JAZ and DELLA protein, respectively (Fig. 5). These results suggested that blocking the ESTP is possibly the key point for 2,4-D-inhibited shikonin biosynthesis in *L. erythrorhizon*. This speculation provided new directions for the comprehensive investigation of the molecular regulation of shikonin biosynthesis by 2,4-D in future studies.

Table 2. The regulation of the enzyme transcripts involved in the biosynthesis of secondary metabolites by 2,4-D.

KEEG pathway	Secondary pathway	KO catalogy	Transcript locus	Function annotation	2,4-D vs. CK (fold change≥2)
	Phenolic compounds				
ko00940	Phenylpropanoid biosynthesis	ko:K00083	Locus_1596_Transcript_1/1	Cinnamyl-alcohol dehydrogenase [EC:1.1.1.195]	0.20
		ko:K00083	Locus_2354_Transcript_28/37	Cinnamyl-alcohol dehydrogenase [EC:1.1.1.195]	0.49
		ko:K00430	Locus_2_Transcript_51161/65694	Peroxidase [EC:1.11.1.7]	0.09
		ko:K00430	Locus_2_Transcript_51158/65694	Peroxidase [EC:1.11.1.7]	0.14
		ko:K00430	Locus_6462_Transcript_1/1	Peroxidase [EC:1.11.1.7]	0.30
		ko:K00430	Locus_2_Transcript_29767/65694	Peroxidase [EC:1.11.1.7]	0.31
		ko:K00430	Locus_2_Transcript_29825/65694	Peroxidase [EC:1.11.1.7]	0.34
		ko:K00430	Locus_2_Transcript_37809/65694	Peroxidase [EC:1.11.1.7]	0.38
ko00945	Stilbenoid, diarylheptanoid and gingerol biosynthesis	ko:K00588	Locus_12545_Transcript_1/4	Caffeoyl-CoA O-methyltransferase [EC:2.1.1.104]	0.46
		ko:K00588	Locus_13326_Transcript_2/2	Caffeoyl-CoA O-methyltransferase [EC:2.1.1.104]	0.47
ko00941	Flavonoid biosynthesis	ko:K00588	Locus_12545_Transcript_1/4	Caffeoyl-CoA O-methyltransferase [EC:2.1.1.104]	0.46
		ko:K00588	Locus_13326_Transcript_2/2	Caffeoyl-CoA O-methyltransferase [EC:2.1.1.104]	0.47
ko00942	Anthocyanin biosynthesis	ko:K00588	Locus_12545_Transcript_1/4	Caffeoyl-CoA O-methyltransferase [EC:2.1.1.104]	0.46
		ko:K00588	Locus_13326_Transcript_2/2	Caffeoyl-CoA O-methyltransferase [EC:2.1.1.104]	0.47
		ko:K12930	Locus_2_Transcript_50243/65694	BZ1; anthocyanidin 3-O-glucosyltransferase [EC:2.4.1.115]	0.33
	Alkaloid compounds				
ko00950	Isoquinoline alkaloid biosynthesis	ko:K00815	Locus_2_Transcript_58734/65694	TAT; tyrosine aminotransferase [EC:2.6.1.5]	0.41
ko00960	Tropane, piperidine and pyridine alkaloid biosynthesis	ko:K00815	Locus_2_Transcript_58734/65694	TAT; tyrosine aminotransferase [EC:2.6.1.5]	0.41
	Terpenoid compounds				
ko00900	Terpenoid backbone biosynthesis	ko:K01823	Locus_2_Transcript_30811/65694	IDI; isopentenyl-diphosphate delta-isomerase, IDI [EC:5.3.3.2]	0.44
ko00904	Diterpenoid biosynthesis	ko:K04125	Locus_14694_Transcript_1/1	Gibberellin 2-oxidase [EC:1.14.11.13]	5.83
ko00905	Brassinosteroid biosynthesis	ko:K09588	Locus_17776_Transcript_2/2	CYP90A1, CPD; cytochrome P450 [EC:1.14.-.-]	3.31
ko00908	Zeatin biosynthesis	ko:K00279	Locus_17614_Transcript_1/2	Cytokinin dehydrogenase [EC:1.5.99.12]	6.15
		ko:K00279	Locus_28580_Transcript_1/1	Cytokinin dehydrogenase [EC:1.5.99.12]	83.29

Table 3.The effect of 2,4-D on the expression pattern of some reported genes involved in the pathway of shikonin biosynthesis.

Transcript locus	CK base mean	2,4-D base mean	2,4-D vs CK (fold change)	Function annotation	Gene accession number
Locus_16289_Transcript_10/10	11723	8725	0.74 [a]	*PAL-1* [*Lithospermumerythrorhizon*]	gi|3914261|sp|O49835.1
Locus_2_Transcript_39794/65694	74	73	0.98 [a]	*PAL-2* [*Lithospermumerythrorhizon*]	gi|3914262|sp|O49836.1
Locus_2_Transcript_45675/65694	117	51	0.44 [b]	*C4H-2* [*Lithospermumerythrorhizon*]	gi|16555879|dbj|BAB71717.1
Locus_207_Transcript_20/21	2054	1518	0.74 [a]	*4CL-1* [*Lithospermumerythrorhizon*]	gi|1117778|dbj|BAA08365.1
Locus_2_Transcript_42332/65694	535	473	0.88 [a]	*4CL-2* [*Lithospermumerythrorhizon*]	gi|9988455|dbj|BAA08366.2
Locus_577_Transcript_4/5	554	504	0.91 [a]	*PGT-1* [*Lithospermumerythrorhizon*]	gi|75162373|sp|Q8W405.1
Locus_2_Transcript_57569/65694	119	54	0.46 [b]	*LePS-2* [*Lithospermumerythrorhizon*]	gi|14575593|dbj|BAB61106.1
Locus_15525_Transcript_1/1	214	271	1.27 [a]	*PGT-2* [*Lithospermumerythrorhizon*]	gi|75162372|sp|Q8W404.1
Locus_2_Transcript_49062/65694	12	23	1.85 [a]	*C4H-1* [*Lithospermumerythrorhizon*]	gi|16555877|dbj|BAB71716.1

Note: The superscript letter ([a]) indicates that the gene is not up to 2-fold change. The superscript letter ([b]) indicates that although the fold change of these two genes is more than 2, the *p*-value is more than 0.05. Therefore, these genes were all not designated as differentially expressed genes

Table 4.The effect of 2,4-D on the expression pattern of the genes involved in ethylene biosynthesis or ethylene signal transduction pathway acting as enzyme genes or regulator genes.

Transcript locus	CK base mean	2,4-D base mean	2,4-D vs CK (fold change)	Function annotation	Gene accession number
Genes involved in ethylene biosynthesis					
Locus_2_Transcript_28823/65694	365	372	1.02[a]	ACC synthase 1 [*Lithospermumerythrorhizon*]	gi|261363609|gb|ACX71871.1
Locus_3325_Transcript_7/7	1431	1581	1.10 [a]	ACC oxidase 1 [*Lithospermumerythrorhizon*]	gi|261363611|gb|ACX71872.1
Genes involved in ethylene signal transduction pathway acting as transcription factor					
Locus_1999_ Transcript_1/1	568	150	0.26	Copper-transporting ATPase P-type, putative (HAM5) [*Ricinuscommunis*]	gi|255537433|ref|XP_002509783.1
Locus_14933_Transcript_1/1	3058	2180	0.71[a]	EIN3-like protein [*Lithospermumerythrorhizon*]	gi|228065860|gb|ACP56697.1
Locus_12414_ Transcript_1/2	387	178	0.46	AP2-domain DNA-binding protein ORCA3 [*Catharanthusroseus*]	gi|158702391|gb|ABW77571.1
Genes involved in JA biosynthesis; JA is a positive regulator of the ethylene signal transduction pathway					
Locus_1955_ Transcript_1/2	211	68	0.32	PREDICTED: lipoxygenase 6, choloroplastic-like (LOX6) [*Glycine max*]	gi|356525142|ref|XP_003531186.1
Genes involved in GA degradation; GA is a positive regulator of the ethylene signal transduction pathway					
Locus_14694_Transcript_1/1	13	78	5.83	GA 2-oxidase [*Populustrichocarpa*]	gi|224064641|ref|XP_002301530.1

Note: The superscript letter ([a]) indicates that the gene is not up to 2-fold change

Conclusions

In conclusion, unlike ET and MeJA signals that act as positive regulators for shikonin biosynthesis, 2,4-D negatively regulates the accumulation of shikonin and its derivatives, thereby providingan excellent experimental system for dissecting the molecular mechanism of these valuable secondary metabolites. However, the molecular mechanism remains unknown. By using the advanced high-throughput transcriptome sequencing strategy, we dissected the expression profiling of the DEGs regulated by 2,4-D in *L. erythrorhizon* callus cells. The results revealed that 2,4-D dramatically affected the metabolism process. In particular, it down-regulated the biosynthesis-related genes for phenylpropanoidbiosynthesis.This regulation might be achieved by directly blocking the ESTP or indirectly regulating the JA and GA to target EIN3 of the ESTP, whereas 2,4-D has no regulation on ET biosynthesis. Our findings provide new hints for the comprehensive understanding of the molecular mechanism of shikonin biosynthesis in future studies.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (NSFC) (31670298, 31470384, 31170275and 31171161), the Program for Changjiang Scholars and Innovative Research Team in University (IRT_14R27),and the Natural Science Foundations of the Jiangsu Bureau of Science and Technology (BK2011414).

References

Ashburner *et al.,* 2010. Missing in ref. list

Ashburner, M., C.A. Ball, J.A. Blake, D. Botstein, H. Butler, J.M. Cherry, A.P. Davis, K. Dolinski, S.S. Dwight, J.T. Eppig and M.A. Harris. 2000. Gene Ontology: tool for the unification of biology. *Nature Genet*., 25: 25-29.

Bais, H.P., G. Sudha, J. George and G.A. Ravishankar. 2001. Influence of exogenous hormones on growth and secondary metabolite production in hairy root cultures of *Cichorium intybus* L. cv. Lucknow Local. *In Vitro Cell. Dev. Biol. Plant*, 37: 293-299.

Bakhtiar, Z., H. Mirjalili, A. Sonboli, M.M. Farimani and M. Ayyari. 2014. *In vitro* propagation, genetic and phytochemical assessment of *Thymus persicus*-a medicinally important source of pentacyclictriterpenoids. *Biologia*, 69: 594-603.

Berthelot, K., Y. Estevez, A. Deffieux and F. Peruch. 2012. Isopentenyldiphosphateisomerase: A checkpoint to isoprenoid biosynthesis. *Biochimie*, 94: 1621-1634.

Calzadilla, P.I., S.J. Maiale, O.A. Ruiz and F.J. Escaray. 2016. Transcriptome response mediated by cold stress in *Lotus japonicus*. *Front. Plant Sci*., 7: 374.

Campbell, M., F.M. Hahn, C.D. Poulter and T. Leustek. 1998. Analysis of the isopentenyldiphosphateisomerase gene family from *Arabidopsis thaliana*. *Plant Mol. Biol*., 36: 323-328.

Cao, Q., A. Li, J. Chen, Y. Sun, J. Tang, A. Zhang, Z. Zhou, D. Zhao, D. Ma and S. Gao. 2016. Transcriptome sequencing of the sweet potato progenitor (*Ipomoea Trifida* (HBK) G. Don.) and discovery of drought tolerance genes. *Trop. Plant Biol*., 9: 63-72.

Carpin, S., L. Ouelhazi, M. Filali, J.C. Chenieux, M. Rideau and S. Hamdi. 1997. The relationship between the accumulation of a 28 kD polypeptide and that of indole alkaloids in *Catharanthus roseus* cell suspension cultures. *J. Plant Physiol*., 150: 452-457.

Chen, H.M., P.H. Wang, S.S. Chen, C.C. Wen, Y.H. Chen, W.C. Yangand N.S. Yang. 2012. Shikonin induces immunogenic cell death in tumor cells and enhances dendritic cell-based cancer vaccine. *Cancer Imm. Immunother*., 61: 1989-2002.

Chen, J., J. Xie, Z. Jiang, B. Wang, Y. Wang and X. Hu. 2011. Shikonin and its analogs inhibit cancer cell glycolysis by targeting tumor pyruvate kinase-M2. *Oncogene*, 30: 4297-4306.

Chen, N., M. Su, X. Chi, Z. Zhang, L. Pan, M. Chen, T. Wang, M. Wang, Z. Yang and S. Yu. 2016. Transcriptome analysis reveals salt-stress-regulated biological processes and key pathways in roots of peanut (*Arachis hypogaea* L.). *Genes Genom*., 38: 493-507.

Chen, X., L.Yang, N. Zhang, J.A. Turpin, R.W. Buckheit, C. Osterling, J.J. Oppenheim and O.M. Howard. 2003. Shikonin, a component of Chinese herbal medicine, inhibits chemokine receptor function and suppresses human immunodeficiency virus type 1. *Antimicrob. Agents Chemother*., 47: 2810-2816.

Cheng, S., Y. Zong, M. Chen, J. Wang, M. Liao and F. Liu. 2018. De novo assembly and characterization of *Rhododendron hybridum* Hort. (Ericaceae) global transcriptome using Illumina sequencing. *Par. J. Bot*., 50: 757-761.

Cleland, R.S. 1995. Auxin and cell elongation. Pages 214-227 *In* P.J. Davies, ed. Plant hormones: physiology, biochemistry and molecular biology, Kluwer Academic Publishers, Dordrecht, Netherlands.

Cruz, G.S., J.M. Canhoto and M.A.V.Abreu. 1990. Somatic embryogenesis and plant regeneration from zygotic embryos of *Feijoa sellowiana* Berg. *Plant Sci*., 66: 263-270.

Fang, R.J., A.L. Zou, H. Zhao, F.Y. Wu, Y. Zhu, H. Zhao, Y.H. Liao, R.J. Tang, Y.J. Pang, R.W. Yang, X.M. Wang, J.L. Qi, G.H. Lu and Y.H. Yang. 2016b. Transgenic studies reveal the positive role of *LeEIL-1* in regulating shikonin biosynthesis in *Lithospermum erythrorhizon* hairy roots. *BMC Plant Biol*., 16: 121.

Fang, R.J., F.Y. Wu, A.L. Zou, Y. Zhu, H. Zhao, H. Zhao, Y.H. Liao, R.J. Tang, T.Y. Yang, Y.J. Pang, X.M. Wang, R.W. Yang, J.L. Qi, G.H. Lu and Y.H. Yang. 2016a. Transgenic analysis reveals *LeACS-1* as a positive regulator of ethylene-induced shikonin biosynthesis in *Lithospermum erythrorhizon* hairy roots. *Plant Mol. Biol*., 90: 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: 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: 151-158.

Girod, P.A. and J.P. Zryd. 1991. Secondary metabolism in cultured red beet (beta-vulgaris l) cells-differential regulation of betaxanthin and betacyanin biosynthesis. *Plant Cell Tiss. Org. Cult*., 25: 1-12.

Glawischnig, E., B.G. Hansen, C.E. Olsen and B.A. Halkier. 2004. Camalexin is synthesized from indole-3-acetaidoxime, a key branching point between primary and secondary metabolism in *Arabidopsis*. *PNAS*, 101: 8245-8250.

Gong, K. and W.Li. 2011. Shikonin, a Chinese plant-derived naphthoquinone, induces apoptosis in hepatocellular carcinoma cells through reactive oxygen species: A potential new treatment for hepatocellular carcinoma. *Free Rad. Biol. Med*., 51: 2259-2271.

Islam, M.S., D.D. Fang, G.N. Thyssen, C.D. Delhom, Y. Liu and H.J. Kim. 2016. Comparative fiber property and transcriptome analyses reveal key genes potentially related to high fiber strength in cotton (*Gossypium hirsutum* L.) line MD52ne. *BMC Plant Biol*., 16: 36.

Kurosaki, F., K. Futamura and A. Nishi. 1985. Factors affecting phytoalexin production in cultured carrot cells. *Plant Cell Physiol*., 26: 693-700.

Ljung, K., A.K. Hull, M. Kowalczyk, A. Marchant, J. Celenza, J.D. Cohen and G. Sandberg. 2002. Biosynthesis, conjugation, catabolism and homeostasis of indole-3-acetic acid in *Arabidopsis thaliana*. *Plant Mol. Biol*., 50: 309-332.

Lu, L., A. Qin, H. Huang, P. Zhou, C. Zhang, N. Liu, S. Li, G. Wen, W. Dong, X. Wang, Q.P. Dou and J. Liu. 2011. Shikonin extracted from medicinal Chinese herbs exerts anti-inflammatory effect via proteasome inhibition. *Europ. J. Pharmacol*., 658: 242-247.

Lu, M.B., H.L.Wong and W.L. Teng. 2001. Effects of elicitation on the production of saponin in cell culture of *Panax ginseng*. *Plant Cell Rep*., 20: 674-677.

Morozova, O. and M.A. Marra. 2008. Applications of next-generation sequencing technologies in functional genomics.*Genomics*, 92: 255-264.

Muñoz-Espinoza, C., A. DiGenova, J. Correa, R. Silva, A. Maass, M. González-Agüero, A. Orellana and P. Hinrichsen. 2016. Transcriptome profiling of grapevine seedless segregants during berry development reveals candidate genes associated with berry weight. *BMC Plant Biol*., 16: 104.

Ozeki, Y., E. Davies and J. Takeda. 1993. Structure and expression of chalcone synthase gene in carrot suspension-cultured cells regulated by 2,4-D. *Plant Cell Physiol*., 34: 1029-1037.

Papageorgiou, V.P., A.N. Assimopoulou, V.F. Samanidou and I.N. Papadoyannis. 2006. Recent advances in chemistry, biology and biotechnology of alkannins and shikonins. *Curr. Org. Chem*., 10: 2123-2142.

Robertson, G.H., N.E. Mahoney, N. Goodman and A.E. Pavlath. 1995. Regulation of lycopene formation in cell-suspension culture of VFNT tomato (*Lycopersicon esculentum*) by CPTA, growth-regulators, sucrose, and temperature.*J. Exp. Bot*., 46: 667-673.

Shendure, J. and H. Ji. 2008. Next-generation DNA sequencing. *Nature Biotechnol*., 26: 1135-1145.

Takahashi, M. and Y. Yamada. 1973. Regulation of nicotine production by auxins in tobacco cultured cells *In vitro*. *Agricul. Biol. Chem*., 37: 1755-1757.

Takeda, J. 1990. Light-induced synthesis of anthocyanin in carrot cells in suspension. II: Effects of light and 2,4-D on induction and reduction of enzyme-activities related to anthocyanin synthesis. *J. Exp. Bot*., 41: 749-755.

Tian, R., Y. Li and M. Gao. 2015. Shikonin causes cell-cycle arrest and induces apoptosis by regulating the EGFR-NF-kBsignalling pathway in human epidermoid carcinoma A431 cells. *Biosci. Rep*., 35: e00189.

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

Vojta, P., F. Kokáš, A. Husičková, J. Grúz, V. Bergougnoux, C.F. Marchetti, E. Jiskrová, E. Ježilová, V. Mik, Y. Ikeda and P. Galuszka. 2016. Whole transcriptome analysis of transgenic barley with altered cytokinin homeostasis and increased tolerance to drought stress. *New Biotechnol*., 33: 676-691.

Wang, Z., M. Gerstein and M. Snyder. 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nature Rev. Genet*., 10: 57-63.

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, Y.J. Pang, H.G. Shen and Y.H. Yang. 2009. Nitric oxide regulates shikonin formation in suspension-cultured *Onosma paniculatum* cells. *Plant Cell Physiol*., 50: 118-128.

Xu, S., Q. Huang, C. Lin, L. Lin, Q. Zhou, F. Lin and E. He. 2016. Transcriptome comparison reveals candidate genes responsible for the betalain-/anthocyanidin-production in bougainvilleas. *Funct. Plant Biol*., 43: 278-286.

Yamamura, Y., Y. Ogihara and H. Mizukami. 2001. Cinnamic acid4-hydroxylase from *Lithospermum erythrorhizon*: cDNA cloning and gene expression. *Plant Cell Rep*., 20: 655-662.

Yang, N.S., S.Y. Lin and T.S. Wu. 2015. Differential effects of phytochemical shikonin on induction of immunogenic cell death versus autophagy: Potential application to DC-based cancer vaccine. *FASEB J*., 29: Supplement LB117.

Yazaki, K., A. Ogawa and M. Tabata. 1995. Isolation and characterization of two cDNAs encoding 4-coumarate: CoA ligase in *Lithospermum* cell cultures. *Plant Cell Physiol*., 36: 1319-1329.

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: 335-342.

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

Yazaki, K., M. Kataoka, G. Honda, K. Severin and L. Heide. 1997b. cDNA cloning and gene expression of phenylalanine ammonia-lyase in *Lithospermum erythrorhizon*. *Biosci. Biotechnol. Biochem*., 61: 1995-2003.

Yazaki, K., M. Kunihisa, T. Fujisaki and F. Sato. 2002. Geranyl Diphosphate: 4-Hydroxybenzoate Geranyltransferase from *Lithospermum erythrorhizon*-Cloning and characterization of a key enzyme in shikonin biosynthesis. *J. Biol. Chem*., 277: 6240-6246.

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: 429-435.

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: 436-444.

(Received for publication 8 October 2017)