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Transcriptome changes in *Polygonum multiflorum* Thunb. roots induced by methyl jasmonate*#

Hong-chang LIU[1], Wei WU†‡[1], Kai HOU[1], Jun-wen CHEN[1], Zhi ZHAO[2]

([1]*College of Agronomy, Sichuan Agricultural University, Chengdu 611130, China*)

([2]*Guizhou Key (Engineering) Laboratory for Propagation and Cultivation of Medicinal Plants, Guiyang 550025, China*)

†E-mail: ewuwei@sicau.edu.cn

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Abstract: Transcriptome profiling has been widely used to analyze transcriptomic variation in plants subjected to abiotic or biotic stresses. Although gene expression changes induced by methyl jasmonate (MeJA) have been profiled in several plant species, no information is available on the MeJA-triggered transcriptome response of *Polygonum multiflorum* Thunb., a species with highly valuable medicinal properties. In this study, we used transcriptome profiling to investigate transcriptome changes in roots of *P. multiflorum* seedlings subjected to a 0.25 mmol/L-MeJA root-irrigation treatment. A total of 18 677 differentially expressed genes (DEGs) were induced by MeJA treatment, of which 4535 were up-regulated and 14 142 were down-regulated compared with controls. These DEGs were associated with 125 metabolic pathways. In addition to various common primary and secondary metabolic pathways, several secondary metabolic pathways related to components with significant pharmacological effects were enriched by MeJA, including arachidonic acid metabolism, linoleic acid metabolism, and stilbenoid biosynthesis. The MeJA-induced transcriptome changes uncovered in this study provide a solid foundation for future study of functional genes controlling effective components in secondary metabolic pathways of *P. multiflorum*.

Key words: *Polygonum multiflorum* Thunb., Methyl jasmonate, Transcriptome change, Differentially expressed genes
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1 Introduction

Methyl jasmonate (MeJA), the methyl ester derivative of jasmonic acid, is a signaling molecule in plant stress responses that regulates many aspects of plant development and growth including flowering, fruit ripening, and senescence (Mukkun and Singh, 2009; Rivera-Dominguez *et al*., 2012). MeJA is present in low concentrations in various parts of plants, including buds, shoots, leaves, flowers, fruits, and seeds, with the largest amounts present in fruits (Sayyari *et al*., 2011; Repčák and Suvak, 2013; Nopo-Olazabal *et al*., 2014). The effects of MeJA have been analyzed in different plant species and vary depending on the MeJA concentration, treated plant organ, and treatment duration (Takahashi and Hara, 2014). Exogenous application of MeJA involving quantities much higher than those found in nature induces synthesis of jasmonate-induced proteins (Li *et al*., 2014). These proteins activate the plant defense system in response to pathogen attack, mechanical wounding, insect herbivory, and general stress (Zhang *et al*., 2013; Sabater-Jara *et al*., 2014).

MeJA participates in the signal transduction pathway that induces particular enzymes to catalyze the formation of low molecular weight defensive compounds in plants (Sivanandhan *et al*., 2012; Ku and Juvik, 2013; Misra *et al*., 2014). Production of these compounds, such as alkaloids, terpenoids, and

‡ Corresponding author
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ORCID: Hong-chang LIU, http://orcid.org/0000-0002-0797-6095

phenylpropanoids, is affected through an extensive transcriptional reprogramming of plant metabolism (Sun G.L. *et al*., 2013; Zheljazkov *et al*., 2013). The induction of secondary metabolite accumulation is a stress response, which depends on MeJA to coordinate the activation of the expression of multiple biosynthetic genes and the activity of diversifying enzymes (Gaige *et al*., 2010). MeJA-induced transcriptome changes have been analyzed in many different plant species including wheat and sweet basil (Diallo *et al*., 2014; Misra *et al*., 2014).

Next generation sequencing (NGS) is a rapid and cost-effective means for analyzing the genome and transcriptome of non-model species. It is also powerful for quantifying gene expression because of its improvement in the *de novo* assembly of high-throughput sequencing data and relatively accurate estimation of gene expression levels (Sun G.L. *et al*., 2013). *Polygonum multiflorum*, a perennial plant in the Polygonaceae family, is used commercially to make a hair-darkening agent and a potent kidney tonic. The species has been widely used in traditional medicine for treating various ailments, and is known for its diverse pharmacological properties (Lv *et al*., 2010). Great efforts have been made to increase the production of stilbene glucoside, one of the major active ingredients of *P. multiflorum*, by using biotransformation (Yan *et al.*, 2007) and micropropagation (Lin *et al*., 2003) but current production processes suffer from low yields. Some compounds are known to stimulate the production of stilbenoids when added to the medium. Among them, MeJA has been found to elicit significantly the production of stilbenoids in cultured cells of grapevine (Jeandet *et al.*, 2002; Vannozzi *et al.*, 2012). Our experimental data showed that a 0.20 mmol/L-MeJA solution could significantly increase stilbene glucoside production in *P. multiflorum* (Fig. S1). However, the detailed biological mechanism of MeJA stimulation of stilbenoid production and concomitant transcriptome changes associated with the response to MeJA remains poorly understood. A detailed knowledge of the biosynthesis of stilbene glucoside and its regulation by MeJA is required before directed bioengineering can be implemented.

Diverse secondary metabolites with potent biological activities have been identified in roots of *P. multiflorum*, but the biosynthetic pathways producing these compounds are poorly understood. In addition, genomic and transcriptomic information for *P. multiflorum* is scarce in public databases. To identify genes involved in the biosynthesis of the biologically active materials, we used NGS to analyze transcriptomic changes in *P. multiflorum* roots following MeJA treatment.

2 Materials and methods

2.1 Plant materials and root-irrigation treatment of plantlets with MeJA solution

Plants were propagated from robust branches of cultivated *P. multiflorum* originally acquired from the He-Shou-Wu GAP cultivation base (Shibing County, Guizhou, China). These plants were cultivated in controlled conditions with a 16-h photoperiod, 25 °C/20 °C (day/night) temperature, and 75% relative humidity. Plants were watered once every three days with Hoagland nutrient solution (Hoagland and Arnon, 1950). The 45-d old plants with five to seven leaves were used for MeJA solution root-irrigation experiments. Our pre-experiment results showed that production of the main active component (stilbene glucoside) of *P. multiflorum* roots could be induced significantly 26 h after treatment with 0.25 mmol/L MeJA. Therefore, in this study, the final concentration of MeJA solution with wetting agent Triton X-100 at 0.1% was 0.25 mmol/L. The solution (500 ml) was added to each 10-L flowerpot containing 4 kg of vermiculite and expanded perlite (1:1) and two *P. multiflorum* plantlets. Control plantlets were irrigated with the Triton solution at 0.1%. Roots were collected 26 h after treatment, immediately frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. Treatment and control experiments were performed with 135 plants in triplicate, respectively, and all chemicals were purchased from Sigma Co., Ltd. (Guangzhou, China) unless otherwise stated.

2.2 Total RNA isolation, complementary DNA (cDNA) library construction and sequencing

Total RNA was extracted from 135 roots of *P. multiflorum* plantlets from control and treatment plants, respectively, following the protocol of the E.Z.N.A.™ Plant RNA Kit (OMEGA, GA, USA). A Thermo Scientific Multiskan GO spectrophotometer

(Thermo Fisher Scientific, USA) and gel electrophoresis were used to analyze the quality and quantity of the total RNA, respectively. For cDNA synthesis, equal quantities of high-quality RNA from control and treatment samples were pooled.

An mRNA-seq Sample Preparation Kit (Illumina Inc., Sandiego, CA, USA) was used for constructing an mRNA-seq library following the manufacturer's instructions, and then poly-(A) mRNA was isolated from total RNA samples using poly-T oligo-attached magnetic beads. The mRNA was cleaved into short fragments using an RNA Fragmentation Kit (Ambion, Austin, TX, USA), which were then used as templates to reverse-transcribe first-strand cDNA using random hexamer primers and reverse transcriptase (TaKaRa, Japan). Second-strand cDNA was synthesized in a reaction mixture containing buffer, DNA polymerase I, RNase H, and dNTPs (TaKaRa, Japan). A Genomic Sample Preparation Kit (Illumina, USA) was used for synthesizing a paired-end library according to the manufacturer's instructions. A MinElute polymerase chain reaction (PCR) Purification Kit (QIAGEN, Dusseldorf, Germany) was used for purifying short fragments, which were eluted in 10 μl of ethidium bromide (EB) buffer. Short fragments were connected using sequencing adaptors, and agarose gel electrophoresis and a Gel Extraction Kit (Axygen Biosciences, CA, USA) were used for separating and purifying desired fragments ((200±25) bp). Finally, the paired-end sequencing library was constructed by PCR amplification with 15 cycles and sequenced using an Illumina Solexa Genome Analyzer IIx sequencing platform (BGI-Huada Genomics Institute, Shenzhen, China). Illumina instrument software was used for performing data analysis and base calling.

2.3 Sequence analysis, assembly, and annotation

All raw reads were cleaned by removing reads with adapters or with unknown nucleotides larger than 5%, or reads in which the percentage of low quality bases (base quality ≤10) was more than 20%. Qualifying reads were extended into contigs using Trinity software (unpublished software of BGI-Huada Genomics Institute, Shenzhen, China) through the overlap between the sequences, and then contigs were connected to form transcript sequences according to paired-end information of sequences (Li *et al*., 2010; Grabherr *et al*., 2011). The overlap settings used for this assembly were 31 bp long and have 80% similarity, and the group pairs distance (the maximum length expected between a fragment pair) was set to 500 bp, with all the other parameters set to their default values. The longest transcripts were selected as unigene sequences of our samples. Transcript levels were quantified as reads per kilobase of exon model per million mapped reads (RPKM) (Mortazavi *et al*., 2008). Genes with high expression levels were screened and listed. Optimal assembly results were chosen according to the assembly evaluation, and then clustering analysis was performed to achieve a unigene database that comprised potential alternative splicing transcripts.

The assembled sequences were compared against the National Center for Biotechnology Information (NCBI) Nr, Nt, and Swiss-Prot databases using BLASTn (Version 2.2.14) with an *E*-value of 10^{-5}, and then each assembled sequence was assigned to gene names based on the highest scoring BLAST hit. For each query, to increase computational speed, searches were limited to the first 10 significant hits. The "getorf" program of EMBOSS software package (Rice *et al*., 2000) was used to predict open reading frames (ORFs), with the longest ORF extracted for each unigene. The Swiss-Prot BLAST results were imported into Blast2GO to obtain the gene ontology (GO) annotations of the unigenes (Conesa *et al*., 2005; Conesa and Gotz, 2008) for annotating the assembled sequences with GO terms. ANNEX (Myhre *et al*., 2006; Ye *et al*., 2006) was used for enriching and refining the obtained annotation. The unigene sequences were also aligned to the Clusters of Orthologous Group (COG) database for predicting and classifying functions (Tatusov *et al*., 2000). The online Kyoto encyclopedia of genes and genome (KEGG) automatic annotation server (http://www.genome.jp/kegg/kaas) was used to assign genes from the KEGG pathways to the assembled sequences. KEGG orthology assignment (Moriya *et al*., 2007) was obtained using the bi-directional best hit method. If results from different databases conflicted with each other, a priority order of Nr, Nt, Swiss-Prot, KEGG, and COG was followed when deciding the sequence direction of unigenes. When a unigene happened to be aligned to none of the above databases, ESTScan software (Iseli *et al*., 1999) was introduced to decide its sequence direction. For unigenes with

sequence directions, their sequences from the 5' end to 3' end were provided; for those without direction, their sequences were provided from the assembly software.

2.4 Unigene differential expression analysis

We performed this analysis to predict genes with different expression levels, and then carry out GO functional analysis and KEGG pathway analysis on them. The unigene expression was calculated using the fragments per kilobase of transcript per million fragments mapped (FPKM) method (Mortazavi *et al*., 2008). We have developed a rigorous algorithm to identify genes expressed differentially between two samples, which is based on "the significance of digital gene expression profiles" which has been cited hundreds of times (Audic and Claverie, 1997; Benjamini *et al*., 2001).

2.5 Real-time PCR and PCR product sequence validation

To validate RNA-seq gene expression results, quantitative real-time PCR (qRT-PCR) was run using SYBR® Premix Ex Taq™ II (Perfect Real Time; TaKaRa, Japan) and a real-time PCR thermal cycler (qTower 2.2, Analytikjena, Germany) using the same cDNA samples as used in the RNA-seq experiment. A first-strand cDNA fragment was synthesized from total RNAs using the TaKaRa PrimerScript™ RT Reagent Kit with gDNA eraser (Perfect Real Time; TaKaRa, Japan). Gene-specific primers were designed for target transcript sequences and *UBQ14*, *UBQ4-1*, and *SAMS* were selected as reference genes (RGs) in our experiments (Tables S1–S3). The comparative threshold cycle method was used to calculate the relative gene expression (Livak and Schmittgen, 2001). Each qRT-PCR was carried out three times. qRT-PCR products of 13 target transcripts were sequenced for comparing sequence similarity with RNA-seq data.

Primers of RGs and genes of interest (GOIs), which putatively coded for key enzymes in the stilbenoid biosynthesis pathway, were designed using Primer Premier 6.0 software (Premier Biosoft, CA, USA) and assessed with Oligo 7.0 software (Molecular Biology Insights, CO, USA), based on the following criteria: 55–65 °C annealing temperature, 40%–60% GC content, 18–24-bp primer length, and 75–300-bp amplicon length. The products of PCR amplification were sequenced to confirm the specificity of RG primer annealing. Reaction mixtures contained 10.0 μl of 2× SYBR Premix Ex Taq II, 0.4 μmol/L of each primer, and 2.0 μl cDNA as a template in a total volume of 20 μl. The following amplification program was used in all qRT-PCR reactions: 40 cycles of 95 °C for 5 s, 55 °C for 30 s, and 72 °C for 2 min. The specificity of each amplification reaction was verified by dissociation curve (melting curve) analysis by heating the amplicon from 55 to 95 °C at the end of the 40-cycle reaction. Non-template controls were included for each primer pair. Three biological samples were used for each RG and GOI, with expression levels in each sample based on three technical replicates. Root samples subjected to the non-MeJA root-irrigation treatment were used to normalize the values obtained from MeJA root-irrigation-treated seedlings.

A standard curve based on a five-fold dilution series was calculated according to the equation $(1+E)=10^{\text{slope}}$, which was used to determine PCR efficiencies (E) of the RGs (Vandesompele *et al*., 2002). Mean E values were obtained for each biological replicate. RG and GOI transcript abundances in all samples were determined according to the cycle quantity (C_q) value in the exponential phase of the PCR. C_q values were converted using the formula $Q=E^{\Delta C_q}$ (Huggett *et al*., 2005), where ΔC_q= the lowest C_q value of the calibrator (corresponding to the sample with the highest expression)−the C_q value of the sample tested. Data obtained from each biological replicate were analyzed independently.

3 Results

3.1 Effect of MeJA on gene expression

We identified 79 565 consensus sequences from non-elicited and elicited root transcriptome data of *P. multiflorum* seedlings, with 89 440 and 78 409 sequences detected from non-elicited and elicited root transcriptome data, respectively. The total number and length (nt) of consensus sequences and the number of distinct clusters and singletons were smaller under MeJA treatment conditions, suggesting that MeJA may inhibit the expression of certain genes (Table S4). The entire set of consensus sequences

was subjected to differential expression analysis (false discovery rate≤0.001 and $|\log_2 \text{Ratio}| \geq 1$) and then processed to eliminate putative redundancies (Fig. S2). A total of 18 677 differentially expressed genes (DEGs) were identified, with 4535 genes up-regulated and 14 142 down-regulated compared with the controls.

3.2 GO functional classification of DEGs

DEGs were classified into three main GO categories, namely biological process, cellular component, and molecular function. Genes differentially regulated upon exogenous MeJA treatment were most heavily represented in the biological process category, followed by cellular component and molecular function. With respect to biological processes, most DEGs were related to cellular and metabolic processes, indicating the greater sensitivity of these genes to MeJA, followed by response to stimulus, biological regulation, and single-organism process. Gene expression related to cells and cell parts dominated activity in the cellular component category; genes associated with cells and cell parts were also responsible for the highest expression observed in any of the three main GO categories. Other important categories were organelles as well as membranes and organelle parts. In regard to molecular function, the most predominant category was binding and catalytic activity, followed by transporter activity, structural molecule activity, and nucleic acid-binding transcription factor activity as shown in Fig. 1 (Liu *et al*., 2015).

3.3 Pathway enrichment analysis of DEGs

MapMan software using a specifically designed mapping file was used for the functional analysis of DEGs (Thimm *et al*., 2004). We functionally classified all genes from the transcriptome sequences into 125 major metabolic pathways to build the mapping file (Table 1). Genes involved in the 125 major pathways of the KEGG database were found to be significantly up- or down-regulated by MeJA treatment. Only one pathway, "thiamine metabolism", was found to be fully up-regulated, while 12 of 125 major pathways, including "synthesis and degradation of ketone bodies", "other glycan degradation", and "glycosaminoglycan degradation", were completely down-regulated (Table 1). These results indicate that 0.25 mmol/L MeJA can completely inhibit gene activity in these pathways, thereby likely inhibiting the activity of their corresponding enzymes.

3.3.1 Effect of MeJA on the "plant-pathogen interaction" pathway

Different functional categories were specifically adjusted after MeJA treatment in *P. multiflorum* roots,

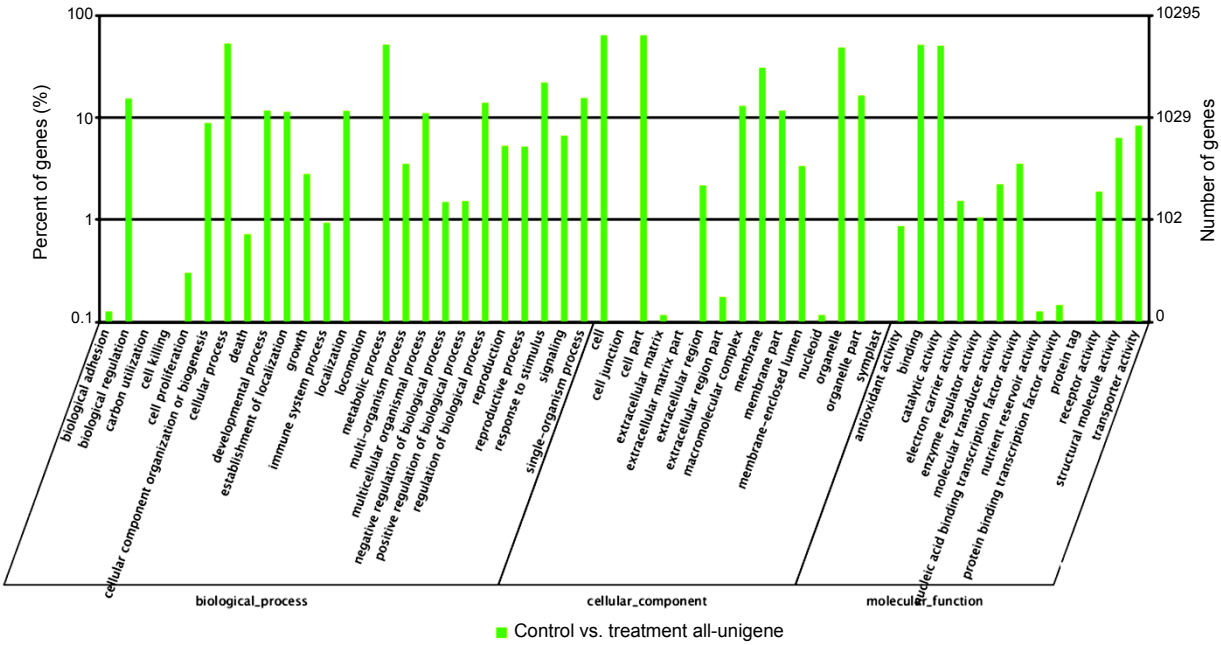

Fig. 1 GO function classification of DEGs (Liu *et al*., 2015)

Table 1 Significantly modulated pathways in *P. multiflorum* root transcriptome subjected to MeJA treatment

No.	Pathway	Up-regulated genes	Down-regulated genes	Genes with pathway annotation	
				DEGs (8936)	All (32 790)
1	Glycolysis/gluconeogenesis	25	74	99 (1.11%)	460 (1.40%)
2	Citrate cycle (TCA cycle)	7	20	27 (0.30%)	151 (0.46%)
3	Pentose phosphate pathway	19	34	53 (0.59%)	171 (0.52%)
4	Pentose and glucuronate interconversions	58	145	203 (2.27%)	457 (1.39%)
5	Fructose and mannose metabolism	7	45	52 (0.58%)	217 (0.66%)
6	Galactose metabolism	9	62	71 (0.79%)	284 (0.87%)
7	Ascorbate and aldarate metabolism	25	59	84 (0.94%)	189 (0.58%)
8	Fatty acid biosynthesis	7	13	20 (0.22%)	77 (0.23%)
9	Fatty acid elongation	8	9	17 (0.19%)	59 (0.18%)
10	Fatty acid metabolism	8	40	48 (0.54%)	181 (0.55%)
11	Synthesis and degradation of ketone bodies		4	4 (0.04%)	29 (0.09%)
12	Cutin, suberine and wax biosynthesis	19	18	37 (0.41%)	115 (0.35%)
13	Steroid biosynthesis	1	30	31 (0.35%)	111 (0.34%)
14	Ubiquinone and other terpenoid-quinone biosynthesis	6	18	24 (0.27%)	93 (0.28%)
15	Oxidative phosphorylation	84	34	118 (1.32%)	609 (1.86%)
16	Photosynthesis	4	12	16 (0.18%)	114 (0.35%)
17	Photosynthesis-antenna proteins	4	1	5 (0.06%)	24 (0.07%)
18	Purine metabolism	59	202	261 (2.92%)	919 (2.80%)
19	Pyrimidine metabolism	52	205	257 (2.88%)	895 (2.73%)
20	Alanine, aspartate and glutamate metabolism	3	34	37 (0.41%)	208 (0.63%)
21	Glycine, serine and threonine metabolism	10	44	54 (0.60%)	167 (0.51%)
22	Cysteine and methionine metabolism	28	64	92 (1.03%)	290 (0.88%)
23	Valine, leucine and isoleucine degradation	5	50	55 (0.62%)	174 (0.53%)
24	Valine, leucine and isoleucine biosynthesis	3	21	24 (0.27%)	76 (0.23%)
25	Lysine biosynthesis	4	7	11 (0.12%)	40 (0.12%)
26	Lysine degradation	4	22	26 (0.29%)	86 (0.26%)
27	Arginine and proline metabolism	9	51	60 (0.67%)	268 (0.82%)
28	Histidine metabolism	1	21	22 (0.25%)	71 (0.22%)
29	Tyrosine metabolism	19	26	45 (0.50%)	167 (0.51%)
30	Phenylalanine metabolism	27	87	114 (1.28%)	251 (0.77%)
31	Tryptophan metabolism	17	35	52 (0.58%)	117 (0.36%)
32	Phenylalanine, tyrosine and tryptophan biosynthesis	6	21	27 (0.30%)	134 (0.41%)
33	Benzoxazinoid biosynthesis	4	10	14 (0.16%)	46 (0.14%)
34	β-Alanine metabolism	7	30	37 (0.41%)	205 (0.63%)
35	Taurine and hypotaurine metabolism	2	8	10 (0.11%)	29 (0.09%)
36	Selenocompound metabolism	6	19	25 (0.28%)	72 (0.22%)
37	Cyanoamino acid metabolism	12	35	47 (0.53%)	137 (0.42%)
38	Glutathione metabolism	34	33	65 (0.73%)	268 (0.82%)
39	Starch and sucrose metabolism	75	243	318 (3.56%)	897 (2.74%)
40	*N*-glycan biosynthesis	3	19	22 (0.25%)	137 (0.42%)
41	Other glycan degradation		67	67 (0.75%)	214 (0.65%)
42	Other types of *o*-glycan biosynthesis	3	3	6 (0.07%)	37 (0.11%)
43	Amino sugar and nucleotide sugar metabolism	31	89	120 (1.34%)	450 (1.37%)
44	Glycosaminoglycan degradation		28	28 (0.31%)	132 (0.40%)

To be continued

Table 1

No.	Pathway	Up-regulated genes	Down-regulated genes	Genes with pathway annotation	
				DEGs (8936)	All (32 790)
45	Glycerolipid metabolism	21	24	45 (0.50%)	146 (0.45%)
46	Inositol phosphate metabolism	20	80	92 (1.03%)	299 (0.91%)
47	Glycosylphosphatidylinositol-anchor biosynthesis	10	45	55 (0.62%)	141 (0.43%)
48	Glycerophospholipid metabolism	169	334	503 (5.63%)	1345 (4.10%)
49	Ether lipid metabolism	138	311	449 (5.02%)	1165 (3.55%)
50	Arachidonic acid metabolism	2	3	5 (0.06%)	30 (0.09%)
51	Linoleic acid metabolism	12	11	23 (0.26%)	55 (0.17%)
52	α-Linolenic acid metabolism	36	36	72 (0.81%)	168 (0.51%)
53	Sphingolipid metabolism	1	47	48 (0.54%)	141 (0.43%)
54	Glycosphingolipid biosynthesis-globo series		4	4 (0.04%)	24 (0.07%)
55	Glycosphingolipid biosynthesis-ganglio series		26	26 (0.29%)	79 (0.24%)
56	Pyruvate metabolism	16	59	75 (0.84%)	321 (0.98%)
57	Glyoxylate and dicarboxylate metabolism	4	46	50 (0.56%)	206 (0.63%)
58	Propanoate metabolism	11	24	35 (0.39%)	146 (0.45%)
59	Butanoate metabolism	2	18	20 (0.22%)	92 (0.28%)
60	C_5-branched dibasic acid metabolism	3	4	7 (0.08%)	14 (0.04%)
61	One carbon pool by folate	2	10	12 (0.13%)	53 (0.16%)
62	Carbon fixation in photosynthetic organisms	13	38	51 (0.57%)	284 (0.87%)
63	Thiamine metabolism	7		7 (0.08%)	27 (0.08%)
64	Riboflavin metabolism	3	13	16 (0.18%)	55 (0.17%)
65	Vitamine B_6 metabolism	11	4	15 (0.17%)	41 (0.13%)
66	Nicotinate and nicotinamide metabolism	3	3	6 (0.07%)	63 (0.19%)
67	Pantothenate and CoA biosynthesis	2	23	25 (0.28%)	99 (0.30%)
68	Lipoic acid metabolism		2	2 (0.02%)	5 (0.02%)
69	Folate biosynthesis		7	7 (0.08%)	26 (0.08%)
70	Porphyrin and chlorophyll metabolism	15	60	75 (0.84%)	179 (0.55%)
71	Terpenoid backbone biosynthesis	16	57	73 (0.82%)	245 (0.75%)
72	Indole alkaloid biosynthesis	4	4	8 (0.09%)	24 (0.07%)
73	Monoterpenoid biosynthesis	4	1	5 (0.06%)	25 (0.08%)
74	Limonene and pinene degradation	35	54	89 (1.00%)	215 (0.66%)
75	Diterpenoid biosynthesis	38	22	60 (0.67%)	146 (0.45%)
76	Brassinosteroid biosynthesis	4	19	23 (0.26%)	65 (0.20%)
77	Carotenoid biosynthesis	14	55	69 (0.77%)	183 (0.56%)
78	Zeatin biosynthesis	13	63	76 (0.85%)	215 (0.66%)
79	Sesquiterpenoid and triterpenoid biosynthesis	38	4	42 (0.47%)	78 (0.24%)
80	Nitrogen metabolism	6	31	37 (0.41%)	136 (0.41%)
81	Sulfur metabolism: reduction and fixation	13	21	34 (0.38%)	79 (0.24%)
82	Phenylpropanoid biosynthesis	68	148	216 (2.42%)	622 (1.90%)
83	Flavonoid biosynthesis	75	83	158 (1.77%)	340 (1.04%)
84	Anthocyanin biosynthesis	6	1	7 (0.08%)	17 (0.05%)
85	Isoflavonoid biosynthesis	4	11	13 (0.15%)	29 (0.09%)
86	Flavone and flavonol biosynthesis	20	41	61 (0.68%)	144 (0.44%)
87	Stilbenoid, diarylheptanoid and gingerol biosynthesis	44	72	116 (1.30%)	282 (0.86%)
88	Isoquinoline alkaloid biosynthesis	2	8	10 (0.11%)	42 (0.13%)

To be continued

Table 1

No.	Pathway	Up-regulated genes	Down-regulated genes	Genes with pathway annotation	
				DEGs (8936)	All (32 790)
89	Tropane, piperidine and pyridine alkaloid biosynthesis	2	9	11 (0.12%)	45 (0.14%)
90	Glucosinolate biosynthesis	3	23	26 (0.29%)	43 (0.13%)
91	Aminoacyl-tRAN biosynthesis	9	17	26 (0.29%)	243 (0.74%)
92	Biosynthesis of unsaturated fatty acids	5	12	17 (0.19%)	85 (0.26%)
93	ABC transporters	52	45	97 (1.09%)	387 (1.18%)
94	Ribosome biogenesis in eukaryotes	24	152	176 (1.97%)	750 (2.29%)
95	Ribosome	74	570	644 (7.21%)	1402 (4.28%)
96	RNA transport	87	257	354 (3.96%)	1240 (3.78%)
97	mRNA surveillance pathway	66	136	202 (2.26%)	800 (2.44%)
98	RNA degradation	24	133	157 (1.76%)	744 (2.27%)
99	RNA polymerase	51	150	201 (2.25%)	572 (1.74%)
100	Basal transcription factors (eukaryotes)	3	21	24 (0.27%)	186 (0.57%)
101	DNA replication	1	48	49 (0.55%)	191 (0.58%)
102	Spliceosome	57	156	213 (2.38%)	1085 (3.31%)
103	Proteasome	3	15	18 (0.20%)	143 (0.44%)
104	Protein export		19	19 (0.21%)	179 (0.55%)
105	Base excision repair	1	27	28 (0.31%)	127 (0.39%)
106	Nucleotide excision repair	2	59	61 (0.68%)	261 (0.80%)
107	Mismatch repair		29	29 (0.32%)	122 (0.37%)
108	Homologous recombination	4	45	49 (0.55%)	265 (0.81%)
109	Non-homologous end-joining		6	6 (0.07%)	45 (0.14%)
110	Phosphatidylinositol signaling system	35	55	90 (1.01%)	303 (0.92%)
111	Plant hormone signal transduction	156	465	621 (6.95%)	1746 (5.32%)
112	Ubiquitin mediated proteolysis	18	97	115 (1.29%)	589 (1.80%)
113	Sulfur relay system		4	4 (0.04%)	19 (0.06%)
114	Snare interactions in vesicular transport	11	21	32 (0.36%)	131 (0.40%)
115	Regulation of autophagy	11	21	32 (0.36%)	198 (0.60%)
116	Protein processing in endoplasmic reticulum	30	168	198 (2.22%)	895 (2.73%)
117	Endocytosis	154	378	532 (5.95%)	1517 (4.63%)
118	Phagosome	18	92	110 (1.23%)	438 (1.34%)
119	Peroxisome	14	27	41 (0.46%)	289 (0.88%)
120	Plant-pathogen interaction	232	395	627 (7.02%)	1990 (6.07%)
121	Natural killer cell mediated cytotoxicity	14	24	38 (0.43%)	121 (0.37%)
122	Circadian rhythm		10	10 (0.11%)	105 (0.32%)
123	Circadian rhythm-plant	28	80	108 (1.21%)	293 (0.89%)
124	Metabolic pathways			2441 (27.32%)	8071 (24.61%)
125	Biosynthesis of secondary metabolites			1079 (12.07%)	3557 (10.85%)

being either induced or repressed. MeJA treatment had the strongest inductive effect on the "plant-pathogen interaction" pathway, which had the greatest number of expressed genes identified in the *P. multiflorum* root transcriptome (Table 1). Of the 1990 genes, 627 were differentially expressed by MeJA treatment, with 232 up-regulated and 395 down-regulated. Genes coding for disease-resistance proteins such as RPS2, RPS3, RPS5, and (pathogenesis-related protein 1) PR1 were heavily represented in this pathway (Fig. S3). The effect of MeJA on the induction of these biotic/abiotic stress associated-genes suggests that they could be exploited to improve plant resistance to pathogens and pests.

3.3.2 Effect of MeJA on the "phenylpropanoid biosynthesis" pathway

One of the most effective protective mechanisms against potential damage due to induction of senescence by MeJA treatment is the reduction of MeJA penetration into plant tissues (Malladi *et al*., 2012). Studies of the biosynthesis of diverse secondary metabolites, especially the biosynthesis of flavonoids and other phenolic compounds, may provide insights into the molecular events underlying MeJA induction in plants (Nopo-Olazabal *et al*., 2014). Treatment of *P. multiflorum* roots with MeJA had a significant inductive effect on genes in the "secondary metabolism" functional category, particularly those involved in the "phenylpropanoid biosynthesis" pathway (Fig. S4) and subcategories of "phenylpropanoid general pathway-flavonoid biosynthesis", "anthocyanin biosynthesis", "isoflavonoid biosynthesis", "flavone and flavonol biosynthesis", and "phytoalexins-stilbenoid, diarylheptanoid and gingerol biosynthesis". Genes coding for key enzymes in the "phenylpropanoid biosynthesis" pathway, including phenylalanine ammonialyase (EC 4.3.1.24), *trans*-cinnamate 4-monooxygenase (EC 1.14.13.11), 4-coumarate-CoA ligase (EC 6.2.1.12), cinnamate 4-hydroxylase (EC 1.14.13.11), and stilbene synthase (EC 2.3.1.95), were up- or down-regulated in response to MeJA (Fig. 2). Of 17 genes expressed in the "anthocyanin biosynthesis" pathway, mainly those encoding anthocyanidin 3-*O*-glucosyltransferase (EC 2.4.1.115) and anthocyanidin 3-*O*-glucoside 5-*O*-glucosyltransferase (EC 2.4.1.298), seven were differentially expressed under MeJA treatment: six were up-regulated and one down-regulated (Fig. S5).

Stilbene synthase also can use chalcone synthase substrates for the production of stilbenes which are classified as phytoalexins because of their roles in plant defense mechanisms against fungal pathogens (Lin *et al*., 2015). Many genes assigned to the functional category "stilbenoid, diarylheptanoid and gingerol biosynthesis" were affected by MeJA (Table 1). Among them, genes encoding mainly stilbene synthase and resveratrol synthesis were significant in this functional category (Fig. 2). The enhanced representation of the "phenylpropanoid general pathway" and "stilbenoid, diarylheptanoid and gingerol biosynthesis" categories demonstrates that MeJA promotes the expression of defense system genes.

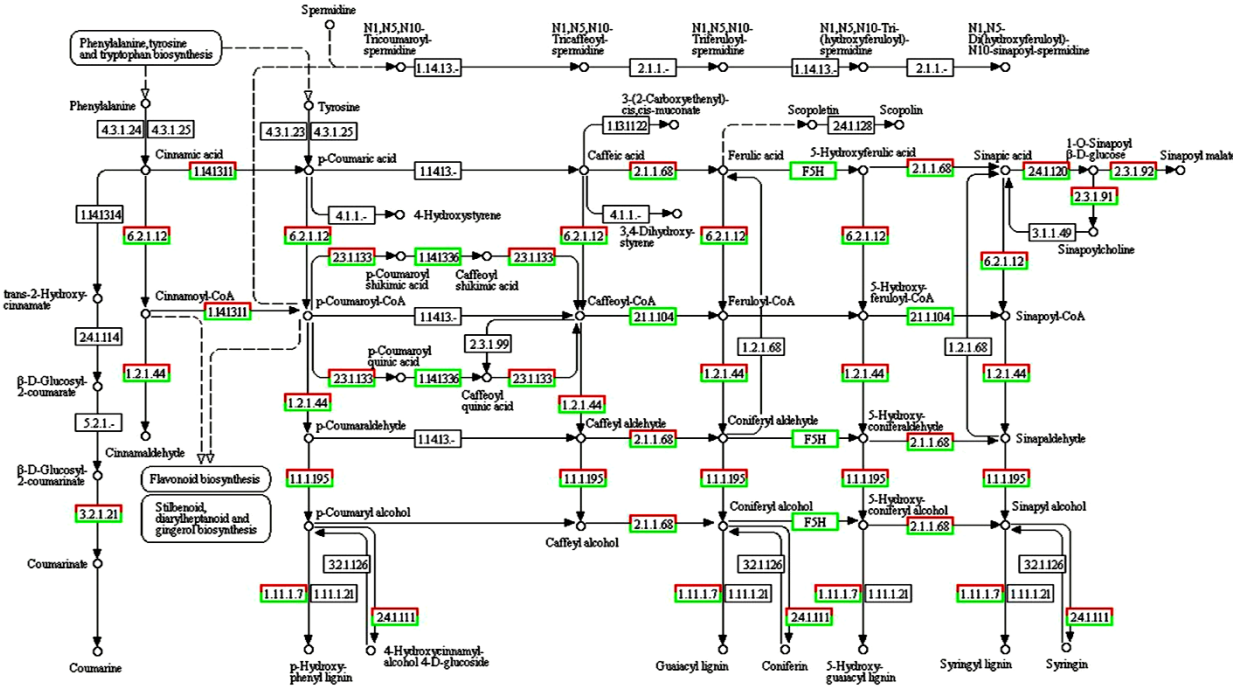

Fig. 2 "Stilbenoid, diarylheptanoid and gingerol biosynthesis" pathway induced by MeJA in root transcriptome of *P. multiflorum*

3.3.3 Effect of MeJA on the "plant hormone signal transduction" pathway

Plant hormones play a vital role in various growth and developmental processes in response to abiotic/biotic stresses. Some plant hormones, such as auxins, abscisic acid, and ethylene, have been associated with MeJA-induced phenotypes (Mukkun and Singh, 2009). The number of genes expressed in the "plant hormone signal transduction" pathway was second only to that of genes in the "plant-pathogen interaction" pathway, which comprised 1746 genes, including 621 DEGs (Table 1). Among the DEGs, 156 genes were up-regulated and 465 were down-regulated. Many genes related to plant hormones, including genes encoding gibberellin receptor, ethylene receptor (EC 2.7.13.–), and mitogen-activated protein kinase 6 (EC 2.7.11.24), were up- or down-regulated to variable degrees (Fig. S6).

The functional categories of "brassinosteroid biosynthesis" (Fig. S7) and "zeatin biosynthesis" (Fig. S8) were significantly over-represented under MeJA treatment. We found that a large number of genes, such as those encoding brassinosteroid insensitive 1-associated receptor kinase 1 (EC 2.7.10.1 and 2.7.11.1), protein brassinosteroid insensitive 1 (EC 2.7.10.1 and 2.7.11.1), protein brassinosteroid insensitive 2 (EC 2.7.11.1), brassinosteroid-6-oxidase 1 (EC 1.14.–.–), and brassinosteroid-6-oxidase 2 (EC 1.14.–.–), were induced under MeJA treatment. Genes encoding cytokinin dehydrogenase (EC 1.5.99.12) and cytokinin-*N*-glucosyltransferase (EC 2.4.1.–) were also differentially expressed.

3.3.4 Effect of MeJA on the vitamin pathway

Vitamins play key roles in many plant physiological processes, including promotion of plant growth and development, adjustment of physiological processes, and enhancement of production and quality (Miret *et al*., 2014). Our study found that the biosynthesis of eight types of vitamins was affected by MeJA, with many genes up- or down-regulated to different degrees. Among these biosynthetic pathways, 189 genes involved in the "ascorbate and aldarate" pathway (Fig. S9) were expressed, including 84 DEGs (25 up-regulated and 59 down-regulated) mainly encoding GDP-D-mannose-3',5'-epimerase (EC 5.1.3.18 and 5.1.3.–), L-ascorbate oxidase (EC 1.10.3.3), and L-ascorbate peroxidase (EC 1.11.1.11). A total of 99, 63, 55, and 41 genes were induced in pathways associated with "thiamine metabolism" (Fig. S10), "riboflavin metabolism" (Fig. S11), "nicotinate and nicotinamide metabolism" (Fig. S12), and "vitamin B_6 metabolism" (Fig. S13), respectively. Genes encoding cysteine desulfurase (EC 2.8.1.7), thiamine pyrophosphokinase (EC 2.7.6.2), riboflavin synthase (EC 2.5.1.9), riboflavin kinase (EC 2.7.1.26), nicotinamide-nucleotide adenylyltransferase (EC 2.7.7.1), nicotinate-nucleotide adenylyltransferase (EC 2.7.7.18), pyridoxal phosphatase (EC 3.1.3.74), and pyridoxine 4-dehydrogenase (EC 1.1.1.65) were up- or down-regulated to different degrees following MeJA treatment.

3.3.5 Effect of MeJA on the "starch and sucrose metabolism" pathway

Starch and sucrose metabolism provides energy for plant growth and development and other metabolic activities. In our experiment, 897 genes related to starch and sucrose metabolism were expressed in roots subjected to exogenous MeJA application. Among them, 318 genes were DEGs, with 75 genes up-regulated and 243 genes down-regulated (Fig. S14) including genes encoding pectinesterase (EC 3.1.1.11), sucrose synthase (EC 2.4.1.13), starch phosphorylase (EC 2.4.1.1), β-amylase (EC 3.2.1.2), α-amylase (EC 3.2.1.1), glycogen debranching enzyme (EC 2.4.1.25), and β-glucosidase (EC 3.2.1.21).

3.3.6 Effect of MeJA on the terpenoid biosynthesis pathway

Plant terpenoids, which play an important role in the basic life activities of plants, are increased by foliar applications of MeJA (Han *et al*., 2007; Chang *et al*., 2013; Misra *et al*., 2014). We found that genes involved in eight types of terpenoid biosynthesis pathways were induced. Among them, 245 genes related to the "terpenoid backbone biosynthesis" pathway (Fig. S15) were expressed, including 73 DEGs (16 up-regulated and 57 down-regulated). Among genes encoding key enzymes in this metabolic pathway, hydroxymethylglutaryl-CoA synthase (EC 2.3.3.10), hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34), mevalonate kinase (EC 2.7.1.36), phosphomevalonate kinase (EC 2.7.4.2), and farnesyl diphosphate synthase (EC 2.5.1.1 and 2.5.1.10) were differentially expressed. The next most-enriched

pathway was the "limonene and pinene degradation" pathway (Fig. S16), with 215 expressed genes including 89 DEGs, of which 35 genes were up-regulated and 54 were down-regulated. A total of 183 genes associated with the "carotenoid biosynthesis" pathway (Fig. S17) were expressed, of which 69 were DEGs, with 14 and 55 genes up- and down-regulated, respectively, under MeJA treatment. In the "diterpenoid biosynthesis" pathway (Fig. S18), 146 genes were expressed, including 60 DEGs, with 38 genes up-regulated and 22 genes down-regulated (Table 1).

3.3.7 Effect of MeJA on alkaloid biosynthesis pathways

Genes involved in three alkaloid pathways were expressed under exogenous MeJA application. Eleven of 45 expressed genes participating in the "tropane, piperidine and pyridine alkaloid biosynthesis" pathway (Fig. S19) were differentially expressed under MeJA treatment, with 2 genes up-regulated and 9 genes down-regulated. These genes mainly encoded the key enzymes such as primary-amine oxidase (EC 1.4.3.21), tropinone reductase 1 (EC 1.1.1.206), aspartate aminotransferase (EC 2.6.1.1), and tyrosine aminotransferase (EC 2.6.1.5). Of the 42 genes identified in the "isoquinoline alkaloid biosynthesis" pathway (Fig. S20), 10 were differentially expressed (2 up-regulated and 8 down-regulated), including genes encoding polyphenol oxidase (EC 1.10.3.1) and tyrosine decarboxylase (EC 4.1.1.25). Many key enzymes, such as EC 2.6.1.1, EC 2.6.1.5, and EC4.1.1.25, are common to these two metabolic pathways. Eight of 24 expressed genes related to the "indole alkaloid biosynthesis" pathway (Fig. S21) were differentially expressed under exogenous MeJA treatment; 4 genes were up-regulated and 4 genes were down-regulated. They encoded mainly strictosidine synthase (EC 4.3.3.2) and polyneuridine-aldehyde esterase (EC 3.1.1.78).

3.3.8 Effect of MeJA on the basic physiological metabolic pathways

"Endocytosis", a basic physiological metabolic process, was the third most significantly expressed pathway. Among the 1517 genes associated with this pathway, 532 were DEGs (154 up-regulated and 378 down-regulated) (Table 1, Fig. S22). Other basic physiological metabolic pathways, such as "ribosome", "glycerophospholipid metabolism", "RNA transport", "ether lipid metabolism", "RNA polymerase", "ubiquitin mediated proteolysis", and "RNA degradation", were differentially up- or down-regulated, with the number of affected genes varying from 572 to 1402. Genes in common plant pathways such as "glycolysis/gluconeogenesis", "pentose phosphate pathway", and "pentose and glucuronate interconversions", were also significantly induced by MeJA treatment (Table 1).

3.4 Validation results

To validate the results of the transcriptome sequencing analysis, we carried out qRT-PCR assays on 70 genes, 50 up-regulated and 20 down-regulated, using gene-specific primers and RGs (Tables S1 and S2) based on the corresponding consensus sequences. Linear regression analysis revealed highly significant correlations between RG transcriptome sequencing and qRT-PCR results. The validation results showed that the expression profile of the 70 genes was consistent with corresponding RNA-seq data (Table S3). In addition, PCR products of 13 genes were sequenced to validate the accuracy of raw sequences. The similarity of sequences varied from 91.05% to 100% between the two methods (Table S5). These results further support the reliability of the RNA-seq data.

4 Discussion

Down-regulation of gene expression associated with active plant growth is an important effect of MeJA treatment. The concentration of MeJA partially determines whether genes are up- or down-regulated (Sun J.W. *et al*., 2013; Misra *et al*., 2014). In our study, some metabolic pathways were characterized by strong down-regulation due to 0.25 mmol/L MeJA. It is well known that MeJA can accelerate chlorophyll degradation, stimulate ethylene synthesis, improve hydrolase activity, and ultimately promote plant senescence when the exogenous MeJA concentration is higher than the plant's physiological concentration (Matsuoka *et al*., 2015). Consistent with this reported activity, annual cutting seedlings of *P. multiflorum* treated with 0.25 mmol/L MeJA in our study started

wilting after 4 d and then slowly began to defoliate, with tender plant parts affected first. Similar symptoms have been observed in rabbiteye and southern highbush blueberry (Malladi *et al*., 2012). However, plants did not die, and new leaf growth recommenced after 20 d. This result indicates that MeJA concentrations above the plant physiological concentration have adverse effects on plant growth and development. Under these circumstances, it is likely advantageous for *P. multiflorum* plants to temporarily halt physiological activities, allowing DNA damage to be repaired and preventing the introduction of MeJA molecules into daughter cell DNA (Matsuoka *et al*., 2015), but the mechanism needs further research.

5 Conclusions

The root transcriptome of *P. multiflorum* was differentially affected by root-irrigation treatment with MeJA. Three times as many genes were down-regulated than up-regulated. Analysis of the functional categories differentially represented under MeJA treatment revealed the existence of common stress responses. General protective responses, such as the induction of pathways regulating the synthesis of alkaloids, terpenoids, and stilbene compounds, the induction of the plant-pathogen interaction system, and the activation of the plant hormone signal transduction pathway associated with biotic or abiotic stress responses, played critical roles. Some common physiological metabolic pathways, such as "amino acid metabolism", "glucose metabolism", "fatty acid metabolism", "citrate cycle", "photosynthesis", and "oxidative phosphorylation", were also greatly induced. Furthermore, exogenous application of 0.25 mmol/L MeJA caused down-regulation of a large number of genes involved in numerous primary and secondary metabolic processes, especially "ribosome", "plant hormone signal transduction", "plant-pathogen interaction", and "endocytosis" metabolic pathways. Down-regulation of a large number of genes under high MeJA concentrations may be an advantageous protective mechanism in plants. In addition, a large number of DEGs associated with various metabolic pathways were identified in the *P. multiflorum* root transcriptome, which will serve as a useful reference for future studies of functional genes controlling effective components of metabolic pathways in *P. multiflorum*. More importantly, these findings provide a preliminary functional genomics framework to understand the complexity of the response of *P. multiflorum* to MeJA treatment.

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Compliance with ethics guidelines

Hong-chang LIU, Wei WU, Kai HOU, Jun-wen CHEN, and Zhi ZHAO declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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List of electronic supplementary materials

Table S1 Validation of up- or down-regulation of 70 genes of interest (GOIs) obtained from transcriptome sequencing of MeJA root-irrigation-treated samples of *P. multiflorum*

Table S2 Statistical comparison of contigs and unigenes between control and treatment root transcriptome data of *P. multiflorum* seedlings

Table S3 Description of the three reference genes (RG) and their amplified sequences

Table S4 Specific primers of genes of interest (GOIs) used to validate the RNA-seq data

Table S5 Validation of raw sequences of 13 genes of interest (GOI) by sequencing of their PCR products

Fig. S1 Effects of MeJA on stilbene glucoside content in *P. multiflorum*

Fig. S2 Expression levels of unigenes in control vs. treatment

Fig. S3 "Plant-pathogen interaction" metabolic pathway induced by MeJA in *P. multiflorum* Thunb. root transcriptome

Fig. S4 "Phenylpropanoid biosynthesis" pathway induced by MeJA in *P. multiflorum* root transcriptome

Fig. S5 "Anthocyanin biosynthesis" pathway induced by MeJA in *P. multiflorum* root transcriptome

Fig. S6 "Plant hormone signal transduction" pathway induced by MeJA in *P. multiflorum* root transcriptome

Fig. S7 "Brassinosteroid biosynthesis" pathway induced by MeJA in *P. multiflorum* root transcriptome

Fig. S8 "Zeatin biosynthesis" pathway induced by MeJA in *P. multiflorum* root transcriptome

Fig. S9 "Ascorbate and aldarate" pathway induced by MeJA in *P. multiflorum* root transcriptome

Fig. S10 "Thiamine metabolism" pathway induced by MeJA in *P. multiflorum* root transcriptome

Fig. S11 "Riboflavin metabolism" pathway induced by MeJA in *P. multiflorum* root transcriptome

Fig. S12 "Nicotinate and nicotinamide metabolism" pathway induced by MeJA in *P. multiflorum* root transcriptome

Fig. S13 "Vitamin B_6 metabolism" pathway induced by MeJA in *P. multiflorum* root transcriptome

Fig. S14 "Starch and sucrose metabolism" pathway induced by MeJA in *P. multiflorum* root transcriptome

Fig. S15 "Terpenoid backbone biosynthesis" pathway induced by MeJA in *P. multiflorum* root transcriptome

Fig. S16 "Limonene and pinene degradation" pathway induced by MeJA in *P. multiflorum* root transcriptome

Fig. S17 "Carotenoid biosynthesis" pathway induced by MeJA in *P. multiflorum* root transcriptome

Fig. S18 "Diterpenoid biosynthesis" pathway induced by

MeJA in *P. multiflorum* root transcriptome

Fig. S19 "Tropane, piperidine and pyridine alkaloid biosynthesis" pathway induced by MeJA in *P. multiflorum* root transcriptome

Fig. S20 "Isoquinoline alkaloid biosynthesis" pathway induced by MeJA in *P. multiflorum* root transcriptome

Fig. S21 "Indole alkaloid biosynthesis" pathway induced by MeJA in *P. multiflorum* root transcriptome

Fig. S22 "Endocytosis" pathway induced by MeJA in *P. multiflorum* root transcriptome

中文概要

题　目：茉莉酸甲酯处理对何首乌根转录组的影响

目　的：研究外源茉莉酸甲酯（MeJA）处理对中药何首乌（*Polygonum multiflorum*）根转录组的影响，分析在外源 MeJA 刺激下何首乌根各代谢途径中差异表达基因的变化，为寻找影响何首乌根主要药效成分合成的关键酶基因奠定基础。

创新点：首次在中药何首乌上利用外源 MeJA 处理研究何首乌根转录组的变化，且获得了根中各代谢途径的大量差异表达基因。

方　法：将何首乌植株分为对照组（Hoagland 营养液灌根）和实验组（含 0.25 mmol/L MeJA 的 Hoagland 营养液灌根）。灌根处理 26 h 后，分别提取对照组和实验组根中的总 RNA，利用新一代测序技术分析对照组和实验组根的转录组变化并进行测序。同时，利用美国国家生物技术信息中心（NCBI）的蛋白质数据库和基因数据库信息，对所测 cDNA 序列进行基因功能注释和代谢途径归类，寻找 MeJA 刺激下何首乌根各条代谢途径中的差异表达基因。

结　论：外源 MeJA 刺激处理可影响中药何首乌根转录组的表达变化（表 S1）。与对照相比，外源 MeJA 处理可导致何首乌根中 4535 个基因上调，14 142 个基因下调变化，并分别在 125 个代谢途径中富集（表 1）。代谢途径差异表达基因分析的结果表明，可在相应代谢途径中找到影响何首乌某些主要药效成分合成的关键酶基因（图 2）。差异表达基因的代谢途径归类分析结果提示今后可对何首乌根各代谢途径中的关键酶基因分别进行研究（表 1）。综上所述，利用外源 MeJA 处理后进行转录组测序对寻找何首乌中主要有效成份合成的关键酶基因有重要价值。

关键词：何首乌；茉莉酸甲酯；转录组变化；差异表达基因