Identification of early jasmonate-responsive genes in *Taxus × media* cells by analyzing time series digital gene expression data

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Abstract Jasmonate, an effective elicitor, can induce the biosynthesis of paclitaxel, a well-known anticancer drug, in *Taxus* cell culture. The jasmonate signaling pathway has been well studied in *Arabidopsis*, and many early jasmonate-responsive genes have been found to be involved in signaling pathway. In *Taxus*, only a few late jasmonate-responsive genes related to paclitaxel biosynthesis were identified. So, identification of early responsive genes and knowledge of the jasmonate signaling pathway are essential for understanding the effects of jasmonate on paclitaxel biosynthesis and for improving paclitaxel production in *Taxus* cells. In this study, total RNA of *Taxus × media* cells cultured in liquid medium was extracted after 0, 0.5, 3, and 24 h of methyl jasmonate treatment. Three biological independent repetitions were performed. The 12 extracted RNA samples were integrated and sequenced on an Illumina HiSeq 2500 platform using the paired-end method. A total of 45,583 transcript clusters were obtained by de novo assembly of the sequenced reads. Based on the transcriptome data, the digital gene expressions of each RNA sample were investigated. We found that after 0.5, 3, and 24 h of methyl jasmonate treatment; 134, 1008, and 987 unigenes were differentially expressed. For the secondary metabolism pathways, phenylalanine pathway unigenes were responsive to jasmonate after 3 h of treatment,

while genes related to paclitaxel biosynthesis were induced after 0.5 h of treatment. The digital gene expression levels of candidate genes related to paclitaxel biosynthesis were confirmed by qRT-PCR. Transcriptome sequencing and digital gene expression profiling identified early jasmonate-responsive genes in cultured *Taxus × media* cells. The comprehensive time series jasmonate-responsive gene expression data have provided transcriptome-wide information about the mechanism of paclitaxel biosynthesis regulation by jasmonate signaling.

Keywords Jasmonate signaling · Paclitaxel · Digital gene expression · Transcriptome sequencing · Jasmonate early responsive gene · *Taxus × media*

Introduction

Paclitaxel is a well-known anti-cancer pharmaceutical that has been used since 1992 to treat ovarian cancer (Wani and Horwitz 2014). Demand for this chemical has increased in recent years and, as a result the natural sources are being depleted. *Taxus* cell cultures have been proposed as an alternative sustainable source of paclitaxel; however, large-scale production is difficult because of the low paclitaxel content of these cells.

Many efforts have been made to improve paclitaxel production in *Taxus* cell cultures (Wilson and Roberts 2012). One approach is to manipulate genes in the paclitaxel biosynthesis pathway. Most of the genes involved in paclitaxel biosynthesis in *Taxus* have been identified. However, direct manipulation of the expression of these genes is not simple, because the paclitaxel biosynthesis pathway is complicated, and involves at least 19 genes (Sheludko 2010). Transcription factors (TFs) that can

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regulate the expressions of genes in the biosynthesis pathway may be alternative candidates for elevating paclitaxel production. In addition, the phytohormones, jasmonate and its derivatives were shown to be powerful elicitors that could increase paclitaxel biosynthesis in cell cultures by dozens of folds (Yukimune et al. 1996). In *Taxus* cells, most of the paclitaxel biosynthesis pathway genes were found to be induced by jasmonates treatment (Laskaris et al. 1999; Lenka et al. 2012; Li et al. 2012). These findings showed that jasmonate signaling was essential for regulating the expression of paclitaxel biosynthesis genes, and suggested that upstream TFs that could regulate its biosynthesis may be involved in the jasmonate signaling pathway. The phytotoxin coronatine was reported as mimic of jasmonyl-L-isoleucine, the jasmonate signaling perception molecule (Thines et al. 2007), and was found to be more effective than methyl jasmonate (MeJA) in inducing paclitaxel biosynthesis (Onrubia et al. 2013). These results further indicate that a similar jasmonate-signaling pathway may be present in *Taxus* cells.

Jasmonate-signaling pathways have been elucidated in different model plants, such as *Arabidopsis*, *Nicotiana tabacum*, *Lycopersicon esculentum* and *Oryza sativa*, and were found to share similar signaling perception and regulation mechanisms (Chini et al. 2007; Ishiga et al. 2013; Seo et al. 2011; Shoji et al. 2008; Thines et al. 2007). In these plants, one jasmonate derivative, jasmonyl-L-isoleucine was reported to mediate the interaction of jasmonate ZIM domain-containing protein (*JAZ*) and coronatine-insensitive protein 1 (*COI1*), which is the component of E3 ubiquitin-ligase protein complex (*SCFCOI1*), leading to ubiquitin ligation with *JAZ* (Chini et al. 2007; Thines et al. 2007). In *Arabidopsis*, the labeled *JAZ* was found to be degraded by a proteinase and TFs such as *MYC2*, *MYB24*, *MYB75* and *MYB51* were released and involved in the regulation of the expressions of genes related to downstream secondary metabolite biosynthesis (Chini et al. 2007; Frerigmann and Gigolashvili 2014; Qi et al. 2011; Thines et al. 2007). Interestingly, the expressions of *JAZ*, *MYC2*, *MYB24*, *MYB75*, and *MYB51* could be induced by short (less than 3 h) exogenous jasmonate treatment (Chini et al. 2007; Frerigmann and Gigolashvili 2014; Qi et al. 2011; Song et al. 2011; Thines et al. 2007). Not only in model plant but also in chinese medicines, such as *Artemisia annua* and *salvia miltiorrhiza*, *AaMYC2* and *SmMYC2* activated the transcript levels of secondary metabolite biosynthesis genes and increased the content of artemisinin and tanshinones (Shen et al. 2016; Zhou et al. 2016).

Most of the previous studies that explored the genes involved in jasmonate-mediated paclitaxel biosynthesis focused mainly on genes that were induced after at least 6 h of jasmonate treatment. For example, the transcriptome

of *Taxus chinensis* was sequenced after 16 h of MeJA treatment and over 10,000 genes were reported to be differentially expressed compared with a control (Li et al. 2012). The differentially expressed genes in *Taxus × media* after 6 h of jasmonate treatment were analyzed by subtractive suppression hybridization and microarray and over 200 jasmonate-responsive genes were identified (Lenka et al. 2012). After 8 days of MeJA treatment, the *Taxus × media* transcriptome was sequenced and over 6000 differentially expressed genes that were stably expressed after long-term jasmonate treatment were identified (Sun et al. 2013). Genes that show an early response to jasmonate treatment have rarely been identified in previous studies (Lenka et al. 2012; Li et al. 2012; Sun et al. 2013).

In this study, we performed a time-series investigation of the expressions of early jasmonate-responsive genes in *Taxus × media* cells by transcriptome sequencing to identify early jasmonate-responsive genes that might be involved in jasmonate signaling as candidate TFs that regulate paclitaxel biosynthesis. Digital gene expression in *Taxus × media* suspension culture cells after 0, 0.5, 3, and 24 h of MeJA treatment was analyzed based on integrated transcriptome sequencing data. Over 1000 genes were found to be differentially expressed ($p < 0.01$) at the three time point (0 h was the control), including newly identified early jasmonates-responsive genes in the jasmonate signaling pathway.

Materials and methods

Cell culture, jasmonate treatment, and RNA isolation

The *Taxus × media* cell line Zike used in this study was induced from sapling leaf purchased in Beijing. The cells were subcultured using the modified SSS medium as previously described (Y and ZG 1999). For jasmonate treatment, 25 μ L 100 mM methyl jasmonate (MeJA) dissolved in ethanol was added to 2 g cells that were suspension cultured for 14 days in 50 mL medium after subculture. Samples were collected after 0, 0.5, 3, and 24 h MeJA treatment using a Büchner funnel. Three times biological independent experiments were performed and a total of 12 samples were obtained. Total RNA was isolated from each of the sample as described before (Chen et al. 2015). Generally, the cells were frozen in liquid nitrogen and ground to powder. Every 2 g of cell powder was lysed in 10 mL GTC buffer (62.5 mM Tris–HCl, 12.5 mM EDTA, and 5 M guanidinium isothiocyanate, pH 8.0), then incubated on ice for 15 min and centrifuged at 15,000 $\times g$ for 15 min. The supernatant was added to an equal volume of

isopropanol to precipitate the total RNA. The total RNA was purified using an RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's instructions.

RNA sequencing and data analysis

The 12 total RNA samples were analyzed by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and had RNA integrity numbers (RIN) larger than 8.8. For transcriptome sequencing, 1 µg total RNA from every sample (12 µg total RNA) was mixed for sequencing on the Illumina HiSeq 2500 system with the paired-end mode according to the manufacturer's instructions. Briefly, mRNA was purified from the 12 µg total RNA using oligo (dT) beads. After purification, the mRNA was fragmented and used for first-strand cDNA synthesis using reverse transcriptase and random primers. Second-strand cDNA was synthesized using DNA polymerase I and RNaseH. Adaptors were added to the cDNA fragments and the ligation products were purified and amplified by PCR to create the final cDNA library for sequencing.

After removing low quality reads and adaptor contaminated reads, the clean reads were assembled with the Trinity, which uses the de Bruijn graph algorithm (Haas et al. 2013). First, all overlapping k-mers are extracted from the clean reads, then each unique k-mer is examined in decreasing order of abundance, and transcript contigs are generated using a greedy extension based on (k – 1)-mer overlaps. Next, Trinity clusters related contigs into scaffolds, using the clean reads to group transcripts on the basis of shared read support and paired read links. Finally, Trinity processes the individual graphs in parallel, ultimately reporting full-length transcripts for alternatively spliced isoforms and teasing apart transcripts that correspond to paralogous genes (Haas et al. 2013). Here, the transcripts were clustered and defined as unigenes for further annotation using BLAST in Trinotate (<http://trinotate.github.io/>) against the UniProt/Swiss-Prot, GO, and KEGG databases with a cut-off E-value of 10^{–5} (Haas et al. 2013).

Digital gene expression libraries sequencing

For digital gene expression, 5 µg total RNA from every sample was prepared. The cDNA libraries were constructed using the method (described above) that was used to construct the library for transcriptome sequencing. The libraries were sequenced on a Illumina HiSeq 2500 system using the SE50 strategy to get reads that were at least 50 bases in length. After filtering out low quality reads, the clean reads were mapped to the transcriptome (constructed as described above) with TopHat 2.0.9 (<http://ccb.jhu.edu/software/tophat/index.shtml>). The expression levels of the

transcripts were estimated by calculating their average RPKM values using the DESeq package (<http://www.biocconductor.org/packages/release/bioc/html/DESeq.html>) under R version 3.1.1. The unigene expression levels in the three times biological independent experiments were accessed based on the negative binomial distribution. Unigenes that showed differential expression levels of more than 2-fold compared with the control (0-h MeJA treatment) were identified as significant when the *p* value was < 0.01. For the GO and KEGG pathway enrichment analyses of all differentially expressed genes, Blast2go (<https://www.blast2go.com/>) or WEGO (Ye et al. 2006) was used in this study.

Quantitative real time PCR validation

We selected 14 paclitaxel biosynthesis pathway genes for validation by qRT-PCR according to the disciplines described by Bustin et al. (2009). The cDNA samples of the selected genes were synthesized using the PrimeScript RT reagent kit (Takara Bio Inc, Japan) from 0.5 µg total RNA. qRT-PCR was carried out using SYBR Premix Ex Taq II (Tli RNaseH Plus) with the ROX Plus kit (Takara Bio Inc, Japan) on an ABI 7300 Real Time PCR system with three technical replicates. The primers were designed using Primer3 software according to the instructions supplied by Takara Bio Inc. The primers used in this study are listed in Table S1. The expression levels of the genes were normalized to the expression of the elongation factor (ELF)-like unigene (comp66568_c0), which showed stable RPKM values in all 12 samples.

Result

Illumina sequencing, de novo transcriptome assembly, and annotation

To generate cDNA libraries, *Taxus × media* cell suspension cultures were treated with 50 µM MeJA for 0, 0.5, 3, and 24 h (hereafter referred to as MeJA_0 h, MeJA_0.5 h, MeJA_3 h, MeJA_24 h). Three biological independent repetitions (hereafter referred to as samples MeJA_0h_1, MeJA_0h_2, MeJA_0h_3, and MeJA_0.5h_1... etc.) were performed for each time course. Total RNA was extracted from the 12 samples, mixed in equal quantities, and sequenced using an Illumina HiSeq 2500 system in the paired-end mode to obtain integrated transcriptome data for digital gene expression (DGE) analysis. A total of 136,573,020 raw reads (Q30 = 87.68%, Table S2) were obtained. After removing low quality reads and adaptor contaminated reads, 114,941,906 clean reads of 100 bps remained (Q30 = 93.9%; GC% = 42.3%, Table S2) and

was submitted into the SRA of NCBI (SRA accession: SRP133890). The clean reads were assembled into 81,563 transcripts using Trinity (Haas et al. 2013). The average length, median length, N50 length, and maximum transcript length of the transcripts were 687, 440, 1026, and 9527 bps respectively (Table 1). The distribution of the transcript lengths is shown in Fig. 1. The gradual change in the lengths of the transcripts indicates good sequencing and assembly quality. These transcripts were clustered into 45,583 unigenes using Trinity, and all the unigene sequences are presented in Table S3. The unigenes were annotated by BLAST searches against the UniProt/Swiss-Prot database with an E-value < 0.00001. We retained 18,264 (40.1%) of the top BLAST hits (Table S4).

Gene ontology (GO) assignments were made to annotate the 18,264 unigenes and the GO terms were classified using WEGO (Ye et al. 2006). The results showed that the terms ‘cellular process’, ‘cell’, and ‘binding’ were the most abundant terms in the three main GO categories, biological process, cellular component, and molecular function, respectively (S1 Fig.). The annotated unigenes were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and 4881 unigenes were assigned to 842 KEGG pathways (Table S5).

Sequencing and digital gene expression analysis after jasmonate treatment

To analyze the gene expression levels at 0, 0.5, 3, and 24 h, 12 mRNA samples was enriched from 5 µg total RNA and fragmented as templates and cDNAs were synthesized by reverse transcription separately for each sample. The cDNA samples were sequenced using an Illumina HiSeq 2500 system. Ten million or more clean reads were obtained for each sample (Q30 > 97%, Table S2). All the reads were mapped to the de novo transcriptome sequence, which was assembled as described above. The hit rate was 100%, and 37,842, 37,991, 37,952, and 37,852 unigenes (a

Fig. 1 Transcripts length distributions of assembled *Taxus* transcriptome

total of 38,399 unique unigenes) from the 0, 0.5, 3, and 24-h samples, respectively, were mapped to the *Taxus × media* transcriptome (Table S6). The DGE clean reads was also submitted into the SRA of NCBI (SRA accession: SRP133888). The expression levels of these unigenes were evaluated by calculating RPKM (reads per Kb per million reads) values according to the numbers of mapped reads. The expression levels of the unigenes and their annotations are shown in Table S6. Correlation coefficients of the expression levels of the unigenes in the 12 samples were calculated based on the RPKM values and are presented in Table S7. All the coefficients were > 0.8. The correlation coefficients between samples from the same batch or same treatment were no less than 0.90, indicating good data quality and good repeatability between the three replicates. Average RPKM values were calculated for the data from the three samples of each of the biological replicates. Differentially expressed genes (DEGs) in the MeJA-treated samples compared with the controls (0-h samples) were also identified ($p < 0.01$) (Table S8). A total of 1493 unigenes were found to be differentially expressed by more than twofold ($p < 0.01$) within 24 h of treatment (Fig. 2). Their expression profiles were clustered based on the RPKM values and are shown in Fig. 3. We found that of the 1493 unigenes, 135, 1008, and 987 were differentially expressed at 0.5, 3, and 24 h respectively; 135 (100%), 934 (92.7%), and 758 (76.8%) of them were up-regulated. More unigenes were up-regulated at 3 h than at 24 h, indicating that MeJA induced their

Table 1 Summary of *Taxus × media* transcriptome assembly

Name of transcriptome	Tm_MeJA_Z14
Clean reads number	136,573,020
Total transcripts	81,563
Total components	45,583
GC percent	42.43
Transcript N50	1026
Maximum transcript length	9527
Median transcript length	440
Average transcript length	687
Total assembled bases	56,060,228

Fig. 2 Summary of unigenes and transcription factors differentially expressed at three time courses. Number of unigenes (**a**) and transcription factors (**b**) with digital gene expression levels that were differentially expressed or up-regulated by more than two-fold ($p < 0.01$) after 0.5-, 3-, 24-h MeJA treatment compared to control.

Each circle indicates total number of differentially expressed unigenes or TFs in the samples at one time course. Numbers of differentially expressed unigenes are presented in dark; and numbers of up-regulated unigenes are presented in parentheses in red (colour figure online)

Fig. 3 Heatmap of DEGs expressions in twelve samples. The DEG expression levels based on RPKM values, calculated in the twelve samples (S1_0–S3_24) at four time courses (0, 0.5, 3, and 24 h of MeJA treatments), were centered by average RPKM values and scaled by the standard deviation of the RPKM values in all samples. The DEGs were clustered by the Pearson's correlation coefficient between them

expression quite early compared with the down-regulated genes that were induced later.

Among the 1493 differentially expressed unigenes, 1080 (71.7%) had BLAST hits against the NCBI nr database and UniProt/Swiss-Prot database (Fig. 2, Table S8), which is higher than the BLAST hit rate for the whole transcriptome (40.1%), reflecting the high number of studies that have reported jasmonate-responsive genes in *Taxus*. GO distribution analysis showed that the top ten most abundant GO

terms assigned to the 1493 DEGs were cell, cell part, cellular process, metabolic process, binding, catalytic, organelle, pigmentation, response to stimulus, and biological regulation (S2 Figure). More DEGs were classified as biological regulation, cellular process, metabolic process, pigmentation, response to stimulus, organelle, and binding in the 0.5-h samples compared with the 3 or 24-h samples, but fewer DEGs were classified as catalytic in the 0.5-h samples. The KEGG metabolic pathway mapping results

showed that the DEGs at 0.5, 3, and 24 h are assigned to 3, 42, and 69 metabolic pathways (Table S9). The most abundant pathways were diterpenoid biosynthesis and linoleic acid metabolism at 0.5 h; and diterpenoid biosynthesis, phenylalanine metabolism, and alpha-linolenic acid metabolism at 3 and 24 h.

Early jasmonate-responsive genes are involved in the paclitaxel biosynthesis pathway

Paclitaxel is probably the most valuable secondary metabolite in *Taxus*, and the biosynthesis of its derivatives has been well studied, as a result, most of the genes responsible for paclitaxel biosynthesis have been identified (Croteau et al. 2006). The genes involved in paclitaxel biosynthesis include eight terpenoid backbone biosynthesis-related enzymes and 13 downstream backbone modification genes (including the branch pathway gene 14-beta hydroxylase). The sequences of these genes were obtained from the NCBI nr database. These sequences were used in BLAST searches against the transcriptome database and the alignments were checked manually. Unigenes that shared more than 90% similarity were identified as paclitaxel biosynthesis pathway genes. The RPKM values of unigene sequences identified as the same gene were added together to estimate their expression levels and fold increases (Fig. 4, Table S10). For terpenoid backbone biosynthesis genes, only sequences annotated as 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (IDS) and geranylgeranyl diphosphate synthase (*GGPPS*) were up-regulated by more than twofold ($p < 0.01$) in samples after 3 (for *GGPPS*) or 24 (for *IDS* and *GGPPS*) hours of MeJA treatment. The expression of other terpenoid backbone biosynthesis genes showed no increase after the 24 h of MeJA treatment. However, for the 13 sequences identified as downstream terpenoid backbone modification genes, the expression levels were up-regulated by more than twofold ($p < 0.01$) after MeJA treatment for 24 h. Most genes expression levels were highest after 3 h of treatment compared with the levels at the other three times; an exception was the sequences identified as 5 α -hydroxylase, the enzyme responsible for the first terpenoid backbone modification step that showed the highest expression level at 24 h. Of the early jasmonate-responsive genes (after the 0.5-h treatment), the expression levels of four of them (*DBTNBT*, *TBT*, *TS*, and *TAT*) increased by more than twofold ($p < 0.01$) compared with the control. After the 3-h treatment, the expression levels of all the DEGs improved by more than twofold ($p < 0.01$) compared with the control, they also had RPKM values that were higher than the values after the 0.5-h treatment. However, the expression levels of all the DEGs, except 5 α -hydroxylase, were down-regulated at 24 h, and the expression level of

Fig. 4 Paclitaxel biosynthesis pathway genes expression level after jasmonate treatment. The expression levels of differentially expressed paclitaxel biosynthesis pathway genes at 0.5, 3, and 24 h of MeJA treatments compared with the control (0 h) were evaluated by RPKM values. Genes responsible for the paclitaxel backbone biosynthesis: 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (IDS), 2-C-methyl-D-erythritol 4-phosphate cytidyl transferase (MCT), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), 1-deoxy-D-xylulose-5-phosphate synthase (DXS), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS), hydroxymethylglutaryl-CoA synthase (HMGS), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), and geranylgeranyl diphosphate synthase (GGPPS). Genes responsible for the paclitaxel biosynthesis pathway: taxadiene synthase (TS), taxadiene 5- α hydroxylase (5 α OH), taxadienol-10- β -hydroxylase (10 β OH), taxane 14 β -hydroxylase (14 β OH), taxa-4(20),11(12)-dien-5 α -ol-O-acetyl transferase (TAT), 3'-N-debenzoyltaxol N-benzoyl transferase (DBTNBT), taxane 2- α -O-benzoyl transferase (TBT), 10-deacetylbaicatin III-10-O-acetyl transferase (DBAT), taxane 13- α -hydroxylase (13 α OH), phenylpropanoyl transferase (BAPT), taxoid 2- α -hydroxylase (2 α OH), taxoid 7- β -hydroxylase (7 β OH), and phenylalanine aminomutase (PAM)

only one gene was twofold lower than in the control. These results showed that MeJA induced taxane biosynthesis genes mainly via modification-related genes, but not terpenoid backbone biosynthesis-related genes. MeJA improved the expressions of paclitaxel biosynthesis genes from an extremely early time course (0.5 h), when TFs

were induced. The expression levels of these early jasmonate-responsive genes were down-regulated at 24 h compared with their levels after 3 h of treatment.

Interestingly, apart from the DEGs that shared more than 90% similarity with previously identified genes, another 49 DEGs shared similarities from 52.5% to 78.3% with known paclitaxel biosynthesis pathway genes (Table S11) and were annotated as taxanoid modification-related hydroxylase, benzoyltransferase, and acetyltransferase (Table S8). In addition to the DEGs identified as biosynthesis-related genes, seven DEGs were annotated as ABC transporters; five and three were up-regulated by more than twofolds ($p < 0.01$) after 3 h and 24 h of MeJA treatment, respectively.

Phenylalanine metabolism pathway genes were induced by MeJA at later time course

Jasmonates were reported to up-regulate the expression of genes in the phenylalanine metabolism (PAL) pathway and improve anthocyanin biosynthesis in *Arabidopsis thaliana* (Qi et al. 2011). Based on the KEGG mapping results, we detected 111 unigenes that were predicted to be involved in the phenylalanine metabolism pathway (Table S5) and 29 of them were differentially expressed by more than twofold ($p < 0.01$) in the 24-h MeJA treated samples (Table S8). Interestingly, unlike the DEGs in the diterpenoid biosynthesis pathway, no DEGs in the PAL pathway genes were differentially expressed in the early 0.5-h samples. 14 and 22 PAL pathway jasmonate-responsive unigenes were differentially expressed by more than twofold ($p < 0.01$) after 3 and 24 h of treatment, respectively (Table S12). These results indicate that MeJA induced the expression of phenylalanine metabolism pathway genes from 3 h, but not from 0.5 h, when we found that the expressions of diterpenoid biosynthesis pathway genes were induced.

Early jasmonate-responsive genes regulate jasmonate metabolism and the jasmonate signaling pathway

In *Arabidopsis*, the expression of the jasmonate biosynthesis pathway genes, lipoxygenase (LOX), allene oxide synthase (AOS), and 12-Oxophytodienoate reductase 3 (OPR3) was induced by jasmonate treatment to improve the endogenous biosynthesis of jasmonate for jasmonate signal amplification (Sasaki et al. 2001) and transduction (Koo et al. 2009). And later, the cytochrome P450 *CYP94B3* modified jasmonyl-isoleucine to inactivate jasmonate signaling in *Arabidopsis* (Kitaoka et al. 2011). Among the DEGs in our study, we identified 5, 3, 2, and 3 unigenes that shared > 50% similarities with *LOX*, *AOS*, *OPR3*, and *CYP94B3*, respectively (Table 2). The average

RPKM values of these DEGs are presented in Table 2. After the 0.5-h MeJA treatment, one *LOX*-like (comp77544_c0) and all three *CYP94B3*-like sequences were up-regulated by twofold ($p < 0.01$), while after the 3-h treatment, all these unigenes were up-regulated by more than twofold compared with their expressions in the control and after the 0.5-h treatment. At 24 h, expressions of most of these unigenes were down-regulated compared with their expressions at 3 h, but, except for one *AOS*-like unigene, all the unigenes still showed higher expression levels than in the control. These findings indicate that the early jasmonate-responsive genes could induce both jasmonate biosynthesis and inactivation in *Taxus*, which is similar to the results reported for *Arabidopsis*.

The jasmonate signaling pathway is well established in *Arabidopsis* (Gfeller et al. 2010). Twelve JAZ proteins and six *MYC2*-like proteins were found to be regulated by jasmonate signaling and these proteins play pivotal roles in the signaling pathway (Fernandez-Calvo et al. 2011). To analyze the genes involved in the *Taxus* jasmonate signaling pathway, we aligned the *Arabidopsis* *JAZ1* and *MYC2* gene sequences against the DEG sequences using BLAST and identified eight JAZ-like unigenes and three *MYC2*-like unigenes among the DEGs (Table 2). The RPKM values of these unigenes showed that they were up-regulated by more than twofold ($p < 0.01$) in the 3-h samples. The expressions of nearly all the JAZ-like unigenes were highly up-regulated at 0.5 h, 3 h, and then down-regulated at 24 h compared with their expressions at 3 h, which is similar to the expressions of the JAZ genes reported in *Arabidopsis*. The expressions of the *MYC2*-like sequences were highly up-regulated at 3 h, then down-regulated at 24 h, when their expression levels were similar to their expressions in the control. Another important protein for jasmonate signaling perception, coronatine insensitive 1 (*COI1*), which is a component of the E3 ubiquitin-ligase protein complex (*SCFCOI1*), was not induced in *Arabidopsis* by jasmonate treatment (Sasaki et al. 2001). Two homologs of *COI1* were found in the *Taxus* transcriptome; their RPKM values indicated that they were not induced after the 24-h MeJA treatment (Table 2), in agreement with their expressions in *Arabidopsis*. These results implied that *Arabidopsis* and *Taxus* might share a similar jasmonate signaling pathway.

Early jasmonate-responsive transcription factors in the *Taxus × media* transcriptome

In addition to the *MYC2*- and *JAZ*-like unigenes that serve important roles in jasmonate signaling, many other TFs were found to be induced by jasmonate in *Taxus*. BLAST searches against the UniProt/Swiss-Prot database identified 423 unigenes as TFs in the transcriptome (Table S13); 63

Table 2 Expression levels of unigenes related to jasmonate signaling and jasmonate metabolism

Annotation	Unigene ID	RPKM_0 h	RPKM_0.5 h	RPKM_3 h	RPKM_24 h
LOX	comp71297_c0	0.6	1.1	17.0**	8.6*
	comp77544_c0	0.3	0.6**	9.1**	4.4**
	comp45421_c0	0.0	0.0	3.7**	2.3*
	comp73946_c0	28.1	36.1	230.6**	249.1*
	comp77544_c1	0.9	1.4	16.7**	9.6**
AOS	comp78534_c0	6.3	12.5	138.4**	128.0**
	comp57565_c0	0.9	1.6	4.0**	0.6
	comp73571_c0	32.1	43.8**	98.2**	59.3**
OPR3	comp66828_c0	422.9	436.9	796.1*	1449.1**
	comp77240_c0	4.2	11.4**	62.2**	27.2**
CYP94B3	comp68030_c0	1.6	10.2*	69.0**	30.8**
	comp58947_c0	0.0	3.1*	28.2**	4.0**
	comp78715_c0	0.6	5.7*	95.0**	35.5**
JAZ	comp65899_c0	0.9	9.1**	131.5**	81.3**
	comp66635_c0	0.1	1.2*	25.5**	2.9**
	comp66717_c0	40.4	92.2**	419.4**	220.0**
	comp73780_c0	17.6	42.9**	177.5**	85.9**
	comp73885_c0	20.5	28.8*	111.3**	116.0**
	comp75328_c0	0.3	14.0**	101.3**	2.7*
	comp76274_c0	2.6	10.1*	122.8**	11.1*
	comp76841_c0	36.9	49.0**	140.3**	71.3**
	comp77131_c0	5.7	8.8**	26.5**	9.4**
MYC2	comp77888_c0	13.5	24.0**	46.5**	25.8**
	comp78752_c0	2.8	4.4**	9.2**	4.5*
	comp78658_c0	1.7	1.8	1.4	1.4
COI1	comp69536_c0	7.6	7.7	5.7	7.0

Annotations are based on the BLAST alignments between the unigene and corresponding Arabidopsis sequences. RPKM_0 h, RPKM_0.5 h, RPKM_3 h, and RPKM_24 are the RPKM values for the unigenes after the 0, 0.5, 3 and 24-h methyl jasmonate treatments

LOX Lipxygenase, *AOS* allene oxide synthase, *OPR3* 12-Oxophytodienoate reductase 3, *JAZ* Jasmonate Zim domain containing protein, *COI1* coronatine insensitive 1

p* value < 0.05; *p* value < 0.01

of them were differentially expressed after 24 h of MeJA treatment and were present among the 1493 DEGs. Twelve, 57, and 35 of these TFs were differentially expressed by more than twofold ($p < 0.01$) compared with the control at 0.5, 3, and 24 h respectively (Fig. 2, Table S13). A larger portion of the differentially expressed TFs (0.5 h: 12/135, 3 h: 57/1008, 24 h: 35/987) were induced early in the time course (0.5 h); however, more TFs were up-regulated at 3 h ($p < 0.01$), and most of them were down-regulated at 24 h compared with their expressions at 3 h. The top five TF families induced were members of the ethylene-responsive (*ERF*), basic helix-loop-helix (*bHLH*), including three *MYC2*-like unigenes), *MYB* domain-containing, *NAC* domain-containing, and *WRKY* domain-containing families.

Validation of DGEs by real-time quantitative reverse transcriptase PCR (qRT-PCR)

To validate the quality of the DGE data, qRT-PCR was performed to determine relative gene expression levels. We selected 14 paclitaxel biosynthesis pathway genes for qRT-PCR. The relative expression levels of the 14 genes at the four time courses are presented in Table S14 and the expression levels obtained using both the average RPKM values and the qRT-PCR method are presented in Fig. 5. The variation trends of 13 of the 14 paclitaxel biosynthesis genes after MeJA treatment evaluated by both methods were similar; the exception was 5 α -hydroxylase, which showed much higher expression levels by qRT-PCR compared with those estimated based on the RPKM values. The relative expression levels of 11 of the 14 genes had high correlation coefficients (> 0.947) with the

Fig. 5 Comparison of paclitaxel biosynthesis genes expression levels evaluated by qRT-PCR and RPKM values. The RPKM values and qRT-PCR expression levels are both presented as mean $\pm$ SD at the different time courses with three times repetitions. The qRT-PCR values are relative expression levels compared with the expression

level of the elongation factor (ELF)-like unigene, and the values were normalized against the RPKM values at 3 h of MeJA treatment for comparison. The original RPKM values, qRT-PCR data, and *p* values are presented in Table S14

corresponding average RPKM values at the four time points (Table S14) and exhibited good coordination (Fig. 5). These results indicate that the average RPKM value was an effective way of evaluating gene expression levels in this study.

Discussion

Jasmonate-responsive unigenes in *Taxus*

Jasmonate was shown to induce paclitaxel biosynthesis pathway genes expression and thereafter increase its content in *Taxus* cell cultures (Jennewein and Croteau 2001). To increase the yield of taxanoids in *Taxus* cell cultures, many studies have investigated the jasmonate induction mechanism and many attempts have been made to isolate paclitaxel biosynthesis pathway genes (Croteau et al. 2006; Lenka et al. 2012). With the development of the next generation sequencing technologies, the effects of jasmonates on the expression levels of *Taxus* genes have been investigated and, for example, large-scale RNA sequencing data have been published by Li et al. (2012) and Sun et al. (2013). In these two studies, more than 10,000 unigenes (20% of the total number of unigenes) were differentially expressed by more than twofold after 16-h or 8-day jasmonate treatments. This finding was quite different from

the jasmonate effects reported in *Arabidopsis* where about 1000 genes were differentially expressed after 24-h jasmonate treatment (Sasaki-Sekimoto et al. 2003). We also found more than 10,000 unigenes in our RNA sequencing data that were differentially expressed in one single experiment; about 3000 were up-regulated and 7000 were down-regulated; however, in the three times biological repetitions, we found that only 135, 1008, and 984 unigenes were differentially expressed by more than twofold ($p < 0.01$) after the 0.5-, 3-, and 24-h jasmonate treatments. Overall, a total of only 1493 differentially expressed unigenes were identified in the *Taxus* transcriptome data, which is similar to the numbers reported in *Arabidopsis* (Sasaki-Sekimoto et al. 2003). We identified known jasmonate-responsive paclitaxel biosynthesis-related genes (Croteau et al. 2006) among the 1493 *Taxus* DEGs. These results indicate that most of the jasmonate-responsive genes identified in *Taxus* by RNA sequencing without the biological repetitions may not be related to the jasmonate signal. In particular, the unigenes that were found to be down-regulated in the single experiment after MeJA treatment showed no significant differential expression in the data from the three times independent biological repetitions. The *Taxus* expression data presented here show a clear relation between the MeJA treatments and the jasmonate-responsive gene expression levels, which is very important for the further analyses of jasmonate-signaling genes.

Jasmonate regulates early gene expressions in *Taxus*

Interestingly, we found that more unigenes were differentially expressed after the 3-h MeJA treatment (1008) compared with the numbers after the 24-h MeJA treatment (987); unsurprisingly, a smaller number of unigenes were differentially expressed after the 0.5-h MeJA treatment (135) (Table S6). Most of the DEGs had the highest expression levels after the 3-h treatment, and some of them were down-regulated at 24 h compared with at 3 h (Table 2, Table S6), but all of them still showed higher expression levels than the control. These results indicated that jasmonate signaling acts fast in the *Taxus* cell cultures, which is similar to the effects of jasmonate in *Arabidopsis* where many genes were reported to be up-regulated from 3 h. Some genes expression levels were up-regulated from 0.5 h in *Taxus*; for example, the JAZ-like and jasmonate biosynthesis pathway genes (Table 2), which were also found to be induced by jasmonate treatment in *Arabidopsis* from 0.5 h (Sasaki et al. 2001). The expression levels of genes related to the synthesis of secondary metabolites, for example genes in the jasmonate-responsive PAL pathway, increased from 3 h (Table S10), as was reported previously for genes in the PAL pathway in *Arabidopsis*, implying that the regulation mechanisms may be similar. However, based on the qRT-PCR results, 10 known paclitaxel biosynthesis genes (seven based on their average RPKM values) were induced by more than twofold from 0.5 h, before genes in the secondary metabolite biosynthesis pathway were induced. At this early time in the jasmonate signaling pathway, only genes responsible for the regulation of other genes and genes in the jasmonate metabolism pathway were induced; for example, JAZ and OPR3. In *Arabidopsis*, the early up-regulation of these genes was found because of the direct degradation of the JAZ proteins that inactivate their transcription (Chini et al. 2007; Thines et al. 2007). The expression of the early jasmonate-responsive genes in *Taxus* suggested that transcription of the paclitaxel biosynthesis pathway genes might be suppressed directly by JAZ proteins.

Expressions of jasmonate-responsive genes in the paclitaxel biosynthesis pathway

All the known paclitaxel biosynthesis-related genes were identified in our *Taxus × media* transcriptome data (Table S8), including eight terpenoid backbone synthesis-related genes and 13 paclitaxel biosynthesis-related genes (Croteau et al. 2006). Only two of the previous identified terpenoid backbone synthesis genes, *GGPPS* and *IDS*, were induced by the 24-h MeJA treatment by more than twofold ($p < 0.01$) (Table S8), although the expressions of all eight were induced slightly by the MeJA treatment (Fig. 4). In a

previous study, Sun et al. reported that DXR expression was induced by an 8-day jasmonate treatment (Sun et al. 2013), suggesting that the treatments may have different effect at the early stage and at the later steady-state stage. Unlike the terpenoid backbone genes, the 13 paclitaxel biosynthesis-related genes were induced by the 24-h MeJA treatment and at 3 h, all 13 genes were differentially expressed by more than twofolds ($p < 0.01$) based on both the average RPKM values and the qRT-PCR results (Table S8, Fig. 4). This finding implied that the paclitaxel biosynthesis pathway genes were responsive to jasmonate faster than was expected based on previous studies that focused mainly on gene expression levels after at least 6 h of jasmonate treatment (Lenka et al. 2012; Li et al. 2012; Sun et al. 2013). Interestingly, Li et al. (Li et al. 2012) found that the expression levels of *TBT*, *13αOH*, *DBAT*, *BAPT*, and *DBTNBT* in *Taxus chinensis* showed less than twofold up-regulation at 16 h after treatment by qRT-PCR. Conversely, Sun et al. (Sun et al. 2013) found that, except *TBT* and *BAPT*, the genes expression levels of the other three genes showed more than twofold up-regulation in *Taxus × media* after 8 days jasmonate treatment, and attributed this finding to different induction times for these genes. In *Taxus × media*, we found that these five genes were highly up-regulated by the 24 h MeJA treatment. These results indicated that different time courses may not explain the different gene expressions that were observed after jasmonate treatment, but may instead be caused by different genes expression responses to jasmonate treatment in different *Taxus* species. We found that another less up-regulated gene after 16-h jasmonate treatment reported by Li et al. (2012), *PAM*, which catalyzes the first step of paclitaxel side chain formation, was induced at 3 h, and then down-regulated dramatically at 24 h compared with its expression at 3 h. This finding may explain the lower expression level of *PAM* at 16 h reported by Li et al. (2012). The expression levels of genes in the paclitaxel biosynthesis pathway (*PAM*, *13αOH*, *2βOH*, *7βOH*, and *DBTNBT*) identified in our study showed similar trends to the expression levels of these genes after coronatine treatment as reported by Onrubia et al. (2013). The expression levels of these genes after coronatine treatment were up-regulated at 3 h, then down-regulated at 24 h. These results further suggested that all paclitaxel biosynthesis pathway-related modification genes were early jasmonate-responsive genes that were induced quickly by jasmonate. At later time courses, a suppression mechanism works to regulate jasmonate signaling and down-regulate the expression of the paclitaxel pathway genes, especially in the *Taxus* cell line, which had no paclitaxel-producing potential. After 24 h, the expressions of these genes were induced again by an as yet unidentified mechanism to elevate taxane biosynthesis (Onrubia et al. 2013; Sabater-

paclitaxel biosynthesis pathway. Three hydroxylases, *13αOH*, *2αOH*, and *7βOH*, had relatively lower expression levels, and may either be located downstream of paclitaxel biosynthesis pathway or be a time-limited step for paclitaxel synthesis. The expression levels of four acetyl transferases were intermediate among the 14 genes. The expression levels of *TAT* and *DBAT* were lower than the expression levels of *5αOH* and *10βOH*, but higher than the expression levels of *2αOH*, *13αOH* and *7βOH*, implying that the acetyl transfer step may occur after *5α* and *10β* hydroxylation, but earlier than other genes. *TBT* and *DBTNBT* showed relatively higher expression levels than *2αOH* and *BAPT*, suggesting that they were not the time-limiting step in paclitaxel side chain formation. The

expression levels of *14βOH* obtained from the average RPKM values and qRT-PCR data were inconsistent; this important taxanoid biosynthesis branch pathway could not be analyzed in the present study.

Jasmonate signaling pathway genes are early response genes

In *Arabidopsis*, the *JAZ*- and *MYC2*-like genes and *COI1* are well known jasmonate signaling pathway genes that play important roles in the jasmonate signaling pathway. In the *Taxus* transcriptome, we identified eight *JAZ*-like and three *MYC2*-like jasmonate-responsive unigenes, the expression levels of all these unigenes peaked at 3 h MeJA treatment (Table 2). Two *COI1*-like unigenes were also identified, but they were different from those identified in a previous study (Li et al. 2012); their expressions were not induced by jasmonate treatment, which is similar to the *COI1* expression reported in *Arabidopsis* (Sasaki et al. 2001). The three types of genes identified here suggested that the jasmonate signaling pathway in *Taxus × media* was similar to the pathway in *Arabidopsis*. The extremely high induction effect of coronatine (Onrubia et al. 2013) indicated that the active jasmonate may be jasmonyl-isoleucine, which mediates the binding of the *COI1* and *JAZ* proteins; however, more evidence is required to confirm the existence of a similar jasmonate signaling pathway in *Taxus*. The connection between jasmonate-induced paclitaxel biosynthesis and jasmonate signaling is still unclear. Whether *COI1*-*JAZ*-*MYC2* mediated jasmonate signaling can regulate the expression levels of the paclitaxel biosynthesis genes and the paclitaxel content of the cells, need to be clarified in future studies. This information is important for understanding the effects of jasmonates in *Taxus*, and may provide the basis for improving paclitaxel production in cell cultures via jasmonate signaling regulation.

Conclusions

We generated 45,583 transcripts clusters by sequencing mixed total RNA samples from *Taxus × media* cell cultures and identified DEGs in samples from four time courses of MeJA treatment. We detected 134, 1008, and 987 unigenes (a total of 1493 unique unigenes), including 63 TFs, that showed differential expression levels of more than twofold ($p < 0.01$) after 0.5-, 3-, and 24-h MeJA treatments, respectively, compared with the control. Analysis of the DEGs at the early time (0.5 h) revealed that they included genes related to jasmonate signaling and jasmonate metabolism, including *JAZ*-like and *MYC*-like unigenes that were identified previously in *Arabidopsis*,

indicating that similar jasmonate signaling systems may operate in *Arabidopsis* and *Taxus*. Systemic investigation of the expression levels of paclitaxel biosynthesis-related genes at the early time after the MeJA treatments prompted us to propose a hypothetical paclitaxel biosynthesis process. We also found that only the downstream backbone modification-related genes were induced by MeJA and were essential for improving paclitaxel production. Early induction of the jasmonate-responsive genes indicated that there may be a direct connection between jasmonate signaling and paclitaxel-related genes expression. Furthermore, paclitaxel biosynthesis pathway gene similarities which presented induced expression after MeJA treatment may identify candidate genes for the as yet unknown enzymes involved in paclitaxel biosynthesis. In this study, using a combination of transcriptome sequencing and high quality time series digital gene expression profile analysis, we identified early jasmonate-responsive genes in *Taxus* and produced in-depth information about the mechanism of paclitaxel biosynthesis regulation by jasmonate signaling. These findings should provide a base of further research on *Taxus*.

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Author's contribution ZG designed research: RM and YC performed research: JC analyzed the data: RM and JC wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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