3 Biotech (2018) 8:320
https://doi.org/10.1007/s13205-018-1352-6

ORIGINAL ARTICLE

Transcriptome analysis of abscisic acid induced 20E regulation in suspension *Ajuga lobata* cells

Yan-chen Wang[1] · Yue-yue Yang[1] · De-fu Chi[1]

Received: 20 May 2018 / Accepted: 11 July 2018 / Published online: 16 July 2018

Abstract

Ajuga lobata D. Don is a medicinal plant rich in 20-hydroxyecdysone (20E), alkaloids, and other active substances. In this study, the cell suspension was incubated for 7 days, followed by the analysis on the effects of abscisic acid (ABA) on the regulation of 20E synthesis. Then *A. lobata* suspension cells treated with 0.15 mg/l ABA were used as material, with the Illumina technology applied for transcriptome sequencing. Digital analysis on the gene expression profile was carried out on ABA treated and control samples, respectively. Finally, transcriptomics was applied to assess the molecular response of *A. lobata* induced by ABA through applying transcriptomics by evaluating differentially expressed genes. The results suggested that ABA promoted 20E accumulation, while longer processing time caused cell browning. A total of 154 genes were significantly regulated after ABA treatment, with 99 up-regulated and 55 down-regulated, respectively. In addition to 20E-related pathways, the genes belonged to the ko00900 (terpenoid backbone biosynthesis) pathway (six differentially expressed genes [DEGs]), ko00100 (steroid biosynthesis) pathway (four DEGs), and ko00140 (steroid hormone biosynthesis) pathway (six DEGs). Providing a better understanding of the 20E biosynthetic pathway and its regulation, in particular in plants, this study is necessary.

Keywords *Ajuga lobata* D. Don · Cell suspension · 20-Hydroxyecdysone · Transcriptomics · Gene expression

Abbreviations

20E	20-Hydroxyecdysone
MVA	Mevalonic acid
DOXP/MEP	5-Phosphate-D-deoxyxylulose/2-*C*-methy-D-erythritol-4-phosphate
DMAPP	Dimethylallyl pyrophosphate
IPP	Isopentenyl pyrophosphate
MS	Murashige and Skoog culture medium
2,4-D	2,4-Dichlorophenoxyacetic acid

Introduction

Ajuga lobata D. Don belongs to Lamiaceae in the order of Lamiales; Originating from the United States of America, it is mainly distributed in the southern part of China (Coll et al. 2007; Coll and Tandron 2008). *A. lobata* is a medicinal plant rich in sterones, alkaloids and other active substances; it is used for the treatment of inflammation, blood pressure, detoxification heat (Guo et al. 2005; Xiong et al. 2012; Cai et al. 2014). *Ajuga* contains 20-hydroxyecdysone (20E), which is the molting hormone of most arthropods, distributed in the plant kingdom and can be used as insecticide and insect growth regulator (Fekete et al. 2004; Rharrabe et al. 2009). It was demonstrated that 20E could cause deformity of larva or pupa in insects, resulting in death; in addition, it could impose antifeedant effects on insects as well (Ufimtsev et al. 2003; Marion-Poll et al. 2005; Selvaraj et al. 2007).

The commercial anabolic preparations and 20E supply for agricultural and therapeutic uses are mainly extracted from collected wild plants making it necessary to develop more reliable and sustainable means for 20E production and other bioactive ecdysteroids. Plant tissue and cell cultures can be alternatively used for the production of ecdysteroids (Lev et al. 1990; Wang et al. 2013).

The structure of 20-hydroxyecdysone is characterized by a *cis*-A/B ring junction, a 7-en-6-one conjugated system, and polyhydroxyl groups. Accumulated evidences in insects suggest that 20-hydroxyecdysone is biosynthesized from cholesterol via 7-dehydrocholesterol and 3β, 14α-dihydroxy-5β-cholest-7-en-6-one (5β-ketodiol) (Fujtmoto et al. 1997).

✉ De-fu Chi
chidefu@126.com; tony196015249@hotmail.com

[1] School of Forestry, Northeast Forestry University, No. 26, Hexing Road, Harbin 150040, China

It was recently proposed that 3β-hydroxy-5β-cholestan-6-one (5β-ketone) was involved in 20HE biosynthesis in the hairy roots of *Ajuga reptans* var. *atropurpurea* (Lamiaceae) (Fujimoto et al. 2015). Cholesterol was converted to the 5β-ketone with the migration of hydrogen from the C-6 to the C-5 position. These findings, along with the previous observation that the ketone was efficiently converted to 20-hydroxyecdysone, strongly suggest that the 5β-ketone is an intermediate immediately formed after cholesterol during 20-hydroxyecdysone biosynthesis in *Ajuga* sp. (Fujimoto et al. 2015).

It was shown that CYP71D443 has C-22 hydroxylation activity for the 5β-ketone substrate through a yeast expression system. The hydroxylated product, 22-hydroxy-5β ketone, had a 22*R* configuration in agreement with that of 20E. Furthermore, labeling experiments indicated that (22*R*)-22-hydroxy-5β-ketone could be converted to 20E in *Ajuga* hairy roots. Based on those results, a possible 20E biosynthetic pathway in *Ajuga* plants involved CYP71D443 is proposed (Tsukagoshi et al. 2016).

In contrast to the ability of synthesizing the cholesterol de novo of vertebrate animals and plant, insects must obtain steroid precursor from their diet, which is attributed to the absence of the enzymes involved in squalene synthesis (Kircher 1982; Iga and Kataoka 2012). Carnivorous insects can take cholesterol directly from their prey, whereas in phytophagous insects, phytosterols are the first dealkylated to cholesterol (Gilbert et al. 2002; Gilbert and Warren 2005).

However, it has been proposed that the mechanism of the construction of the characteristic 5β-*H*-7-en-6-one structure is different in insects and plants (Davies et al. 1980, 1981; Nagakari et al. 1994). It is generally accepted that 7-dehydrocholesterol is derived from cholesterol of insects (Sakurai et al. 1986). Accumulated evidences indicate that the biosynthesis of ecdysone in the prothoracic gland of insects proceeds via 7-dehydrocholesterol and 3β,14α-dihydroxy-5β-cholest-7-en-6-one (ketodiol), followed by the successive hydroxylation at C-25, C-22 and C-2 to yield ecdysone of Ketodiol, which is converted into 20-hydroxyecdysone in peripheral tissues such as the fat-body (Rees 1995; Huang et al. 2008).

The genes encoding the P450 enzymes that catalyze these hydroxylations have been characterized in insects (Huang et al. 2008). Conversion of cholesterol into 7-dehydrocholesterol (7dC) is mediated by a Rieske oxygenase Neverland (Yoshiyama et al. 2006; Yoshiyama-Yanagawa et al. 2011).

In *Drosophila melanogaster* and Bombyx mori, it has been proven that CYP307A1/A2 (Spook/Spookier, Spo/Spok) and CYP6T3 will be involved in the conversion of 7-dehydrocholesterol into 2,22,25-trideoxyecdysone (ketodiol). Moreover, a paralog Spookiest (Spot, CYP307B1) was also found in CYP307 family (Gilbert and Warren 2005; Namiki et al. 2005; Ono et al. 2006; Ou et al. 2011).

In contrast, the biosynthetic mechanism of 20-hydroxyecdysone in plants is much less acknowledged than that in insects (Festucci-Buselli et al. 2008). It was shown that hydroxylation of ecdysone at the C-20 position to form 20E is a cytochrome P450-dependent reaction occurring predominantly in the microsomal fraction in spinach (*Spinacia oleracea*) leaves (Grebenok et al. 1996). The early step mechanism in *Ajuga* hairy roots may distinct from that of insects (Lockley et al. 1975; Davies et al. 1981). *Ajuga* hairy roots are capable of introducing a double bond at the 7-position at the late stage of 20-hydroxyecdysone biosynthesis, suggesting the possibility of an alternative biosynthetic pathway without the involvement of 7-dehydrocholesterol as an obligatory intermediate (Hyodo and Fujimoto 2000).

20-Hydroxyecdysone biosynthesis is mainly through mevalonate (MVA) and DOXP/MEP pathways, in which the former pathway, as a major plant synthetic pathway begins with acetyl CoA condensation, followed by reduction to mevalonate, resulting in the production of steroid ketones and terpenes. Meanwhile, the latter pathway utilizes pyruvic acid and GA-3P as the starting substrates to produce terpenes. Although isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP) are intermediate products in both pathways, the mechanisms by which DMAPP and IPP are synthesized are different, so is the intracellular locations of metabolic end products (Adler and Grebenok 1995; Báthori and Pongrácz 2005; Ma et al. 2006).This synthetic pathway is complex with many compounds involved in it, but the specific synthesis pathway of 20E remains unclear (Dinan 2001).

Cholesterol and lathosterol are first metabolized into 7-dehydrocholesterol, followed by the conversion into 20-hydroxyecdysone via 7-dehydrocholesterol 5α, 6α-epoxide in the hairy roots of *A. reptans* var. *atropurpurea* (Ohyama et al. 1999). It is generally accepted, both in insects and plants, that the early stage of biosynthesis of 20-hydroxyecdysone from cholesterol yields an intermediate with a *cis*-A/B ring structure, which is then hydroxylated at the C-2, -20, -22 and -25 positions to furnish. It was reported that C-20, C-22 and C-25 hydroxylations were catalyzed by P450 mono-oxygenase enzymes, whereas a different type mono-oxygenase was responsible for C-2 hydroxylation (Nakagawa et al. 1997).

It was suggested that CYP307A1, CYP307A2 and non-molting glossy/shroud (a short-chain dehydrogenase/reductase) should be involved in the conversion of 7-dehydrocholesterol to 5b-ketodiol (Namiki et al. 2005; Rewitz et al. 2007; Niwa et al. 2010). Functioning as a carbon 25 hydroxylase CYP306A1 plays an essential role in ecdysteroid biosynthesis during insect development, which converts ketodiol into ketotriol via carbon 25 hydroxylation (Niwa et al. 2004). Cytochrome P450 enzymes catalyzing the final steps of ecdysteroid biosynthesis have been identified by mutations

in the ''Halloween'' gene family of *D. melanogaster*: phantom (CYP306a1; 25-hydroxylase), disembodied (CYP302a1; 22-hydroxylase); shadow (CYP315a1; 2-hydroxylase); and shade (CYP314a1; 20-hydroxylase) (Chavez et al. 2000; Gilbert et al. 2002; Warren et al. 2002, 2004; Petryk et al. 2003; Gilbert 2004; Niwa et al. 2004). The hydroxylation at C25 is mediated by CYP306A1 (Phantom: Phm) located in the endoplasmic reticulum, followed by hydroxylations at C22 and C2 carried out in the mitochondria by CYP302A1 (Disembodied: Dib) and CYP315A1 (Shadow: Sad), respectively (Gilbert and Warren 2005). Final conversion of E to 20E is catalyzed by a fourth hydroxylase, CYP314A1 (Shade: Shd, the ecdysone 20-monooxygenase), variously located in the endoplasmic reticulum and the mitochondria of peripheral target tissues (Petryk et al. 2003).

Abscisic acid could regulate plant dormancy, flowering, maturity, aging and stomatal movement (Cutler et al. 2009), exerting inhibitory effects on cell growth and extension (Hansen and Grossmann 2000). ABA is also considered a stress hormone, the content of which rises under environmental stress, initiating the defense system to enhance plant resistance (Anderson et al. 1994; Hauser et al. 2011; Ren et al. 2012). In recent years, studies have shown that ABA regulates and stimulates the production of secondary metabolites such as chlorogenic acid, phenols, flavonoids and terpenoid substances (Sandhu et al. 2011; Buran et al. 2012). The up-regulation expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) are involved in the biosynthesis under the treatment of various elicitors, such as methyl jasmonate (MeJA) or $AgNO_3$, which are responsible for 20E accumulation in cell cultures of *Cyanotis arachnoidea* (Wang et al. 2014).

In contrast, little is known about the last few steps of planting 20E biosynthesis, and no biosynthetic 20E gene about those steps has been reported thus far (Tsukagoshi et al. 2016). It is based on the establishment of a preliminary suspension culture system of *A. lobata* (Zhao et al. 2011; Li et al. 2013). Through identifying key genes of the 20E synthesis pathway and assessing their corresponding expression variability after adding exogenous ABA, the study aims to explore the mechanism by which exogenous ABA affects 20E synthesis.

A better understanding of the 20E biosynthetic pathway and its regulation, in particular in plants, is necessary. This is the first report about the isolation, characterization of a novel HMGR gene and the establishment of *A. lobata* cell suspension cultures for 20E production.

Materials and methods

Materials

The *A. lobata* cell suspension culture system was established by Insect laboratory of Northeast Forestry University and the suspension culture callus was used as the experimental material.

Methods

A. lobata suspension cell culture and screening for optimal ABA concentration

The basic liquid medium for *A. lobata* cell suspension culture was Murashige and Skoog culture medium (MS), 2,4-dichlorophenoxyacetic acid (2,4-D) 0.4 mg/l. The pH was adjusted to 5.8, with an inoculation ratio of 10% (5 g cells in 50 ml medium). Cells were cultured under a 16/8-h light/dark cycle with 2000-l× light intensity at 25 °C and 70% humidity, with culture flasks shaken at 120–130 rpm (Zhao et al. 2011; Li et al. 2013).

ABA was added to the suspension culture system at the seventh day after inoculation of *A. lobata* cells. Based on previous experiments, ABA concentrations in the suspension cultures were set at 0.05, 0.1, 0.15, and 0.2 mg/l, respectively, followed by the screen of the optimum concentration of ABA for 20E accumulation. Sampling was carried out every 24 h after ABA addition and 5 parallel samples were collected for each treatment.

Extraction and quantitation of 20E

20E content was assessed by high-performance liquid chromatography (HPLC). *A. lobata* cells were dried at 60 °C in a high temperature drying oven for 12 h, followed by the operation of soaking 0.2 g dry suspension cell in 5 ml methanol for 24 h, the sonicating for 1 h at 40 kHz (YH-200DH, produced by YUHAO in China), and digested with a microwave digestion system (WT-8000, Shanghai YaRong biochemical instrument factory, conditions: $T=50$ °C, $p=2$ P_0, $T=10$ min, $W=300\times2$). The digestion mixtures were filtered using organic filtration membrane. The filtrates were analyzed by HPLC (American, Waters Company) with the following conditions: UV–visible detection wave length range, 190–800 nm; detection wavelength, 242 nm; mobile phase, 1:1 formal dehyde: water; mobile phase flow rate, 0.8 ml/min; injection volume, 10 µl. Samples (5 per treatment group) were collected every 24 h; detection of each standard was repeated 3 times.

20E standards with concentrations of 0.8, 0.4, 0.2, 0.1, and 0.05 g/l were prepared. With average peak area as ordinate and mass concentration as abscissa, a regression equation for 20E was obtained ($y=18498+226.2x$, $R^2=0.999$).

cDNA library construction and transcriptome analysis

Suspension *A. lobata* cell scultured under the optimal ABA concentration, were screened as described above, with the

collection of samples at 48 h after addition of ABA, for of cDNA library construction and transcriptome analysis. In the control group, suspension *A. lobata* cells were cultured in basic liquid medium. Three samples were collected from untreated or treated suspension *A. lobata* cells, respectively, mixed and kept separately, and used in subsequent experiments.

Total RNA was isolated from mixed samples using TRIzol reagent according to the manufacturer's instructions. Transcriptome sequencing was carried out on Illumina. The Trimmomatic (v0.30) software was used to analyze raw sequencing data, in which adapter and low-quality sequences were removed to obtain clean data for further analysis (Grabherr et al. 2011). Sequencing data quality assessment was carried out with the Fast QC (v0.10.1) software, and TransDecoder was employed for ORF detection (Garg et al. 2011; Ness et al. 2011).

Functional annotation of transcripts was performed using BLASTX to compare nucleotide sequences in the NCBI's NR protein database (tHoen et al. 2008; Morrissy et al. 2008). All predicted genes were assigned to different functional categories using Blast2GO (http://www.blast2Go.org/) to facilitate global gene expression analysis (Conesa et al. 2005). A categorization into functional groups was performed automatically through the KEGG pathway database, the annotation of which was carried out with the online KEGG Automatic Annotation Server (KAAS), using the comparison method for bidirectional best hit (BBH). The functional classification by KEGG provided valuable information for investigating specific processes, functions, and pathways.

Screening of differentially expressed genes

Gene expression data were evaluated using the Rsemsoftware. The DESeq software was used to screen the differentially expressed genes (DEGs), with false discovery rate (FDR) corrected p value of 0.05, with the adoption of a cut off of two-fold regulation (Audic and Claverie 1997; Eisen et al. 1998; Saldanha 2004).

Analysis on differentially expressed genes

The identified DEGs were used for GO and KEGG pathway analysis. The GO enrichment analysis on functional significance was performed using the Gene Ontology database (http://www.geneontology.org/), evaluating gene numbers for every term and using a hypergeometric test to identify significantly enriched GO terms in DEGs. The following formula was used. The P-value of a significantly enriched GO term should be lower than 0.05; the P-value was corrected by Bonferroni

$$p = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

N = all genes with a GO annotation, n = DEGsin N, M = all genes annotated to certain GO terms, m = DEGsin M.

Compared with the whole-genome background, the KEGG pathway enrichment analysis identified significantly enriched metabolic pathways or signal transduction pathways in the DEGs (Kanehisa et al. 2015). The formula of the significantly enriched pathway was the same as that used for GO enrichment analysis.

Results

Effect of ABA on 20E content

The changes of 20E content are shown in Fig. 1. Treatment with 0.05 mg/l ABA showed no significant difference compared with that in the control group. Meanwhile, 0.1 mg/l ABA showed a significant difference at 48 h ($p < 0.05$); treatment with 0.15 mg/l ABA showed significant differences compared with that in the control group at 48 and 72 h, with 23.49 and 14.73% higher 20E contents compared with those in the control group respectively. Treatment with 0.2 mg/l ABA showed a trend of decline up to 20E content after an initial increase, with lower amounts than that in the control group at 72 h. Therefore, 0.15 mg/l ABA was selected as the optimal concentration for further experiments.

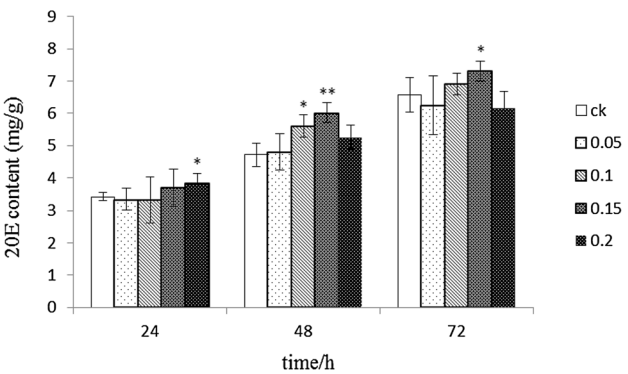

Fig. 1 20E contents in different ABA treatment groups. Note: **significant difference between ABA treatment and control groups

Effect of ABA on *A. lobata* suspension cell activity

At 72 h, suspension *A. lobata* cells grew well after the addition of 0.15 mg/l ABA. The suspension culture system was light yellow, uniform and loose. However, *A. lobata* suspension cells were brown at 96 h. After being treated with ABA, dry weight was increased during the non-browning period. Dry weights were significantly higher than those in the control group at 24 h ($p < 0.05$). There was no significant difference in the dry weight at 48 and 72 h between treated and control groups. However dry weights were significantly reduced compared with that in the control group at 96 h ($p < 0.05$). Growth status is shown in Fig. 2, while biomass changes are summarized in Table 1.

Illumina sequencing and data analysis

In this study, two cDNA libraries for *A. lobata* suspension cells treated with 0.15 mg/l ABA for 48 h and untreated, respectively, were generated on the Illumina HiSeq2000 platform. The statistics of raw data are shown in Table 2; clean reads were generated after removing adapter sequences, duplicate sequences, ambiguous reads, and low-quality reads (Table 3). Each library reached the saturation level of gene identification and a tag density sufficient for quantitative gene expression analysis. Of all the clean reads, 60.56% were matched to unique genomic locations; the uniquely matched reads were used for gene expression analysis. A comparison of clean reads and unigenesis is shown in Table 4. The distribution of the gene sequence length of all 119,359 genes detected by the RNA-Seq technology is shown in Fig. 3. For these genes, gene sequence length distribution was divided into five grades, along with the presentation of the total numbers and percentages of all genes. The proportion of long sequences was high among these genes, with 26,378 genes exceeding 1000 bp in length.

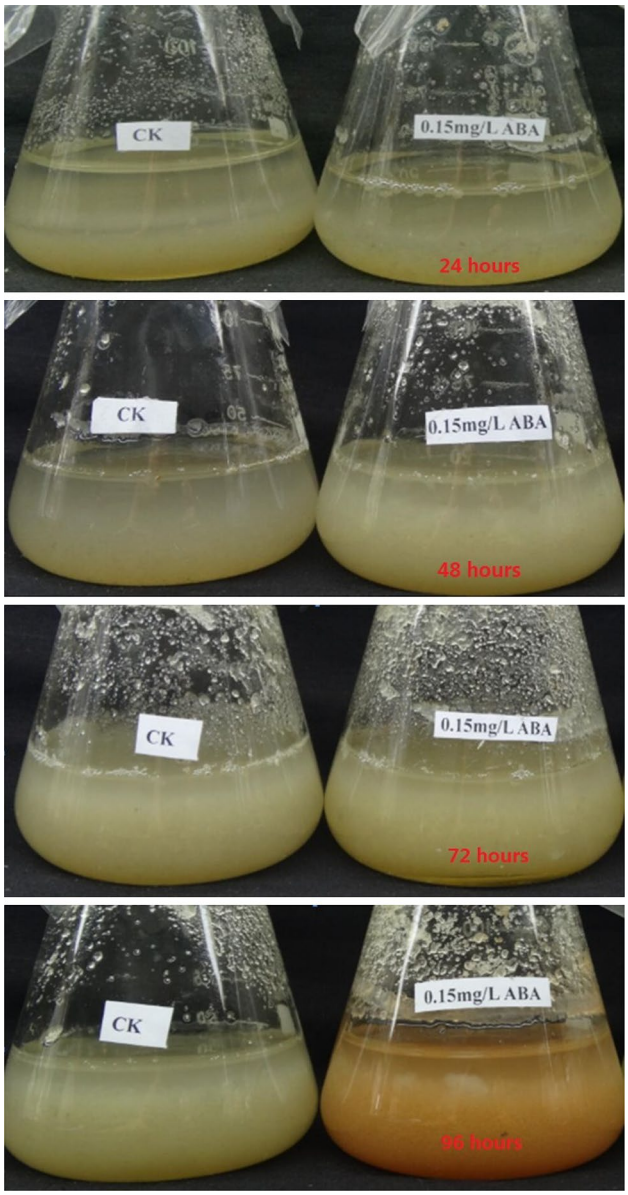

Fig. 2 Growth status of *A. lobata* suspension cells

Gene expression and functional classification of the detected genes

A total of 45,150 unigenes were annotated to the Nr protein database successfully. Owing to the fact that a gene could be matched to multiple proteins the 45,150 unigenes were matched to 265,156 proteins with data redundancy. A total of 173,520 matched proteins with high homology (E value $< 1E^{-30}$) making up 65.44% of all proteins. Meanwhile, 122,860 matched proteins showed higher homology (E value $< 1E^{-60}$), i.e. 46.36% of all proteins. The highest matched species *Vitisvinifera* accounted for 23.23%, followed by *Ricinuscommunis* (14.35%), *Solanumlycopersicum* (14.30%), *Populustrichocarpa* (8.78%), *Prunuspersica* (8.35%) and *Ricinuscommunis* (6.51%), *Glycine max* (6.34%), *Arabidopsis thaliana* (3.71%) and *Oryza sativa* (2.35%).

The annotations were verified manually and integrated based on the gene ontology (GO) classification. 24,026 of the detected genes had at least one GO annotation based on sequence similarity. These annotated genes were categorized based on the secondary classification of GO terms, assigning them to 69 functional groups of the three main GO classification categories (Fig. 4). Genes assigned to "cellular component" accounted for the majority of GO terms (24), followed by "biological process" (23) and "molecular function" (22). The terms "single-organism process" and "metabolic process", accounting for 24.98% and 13.28%,

Table 1 Cell biomass variations

	Dry cell weight/g			
	24th hours*	48th hours	72th hours	96th hours*
CK	0.480 ± 0.0071	0.542 ± 0.0192	0.567 ± 0.0157	0.635 ± 0.0212
ABA	0.505 ± 0.0141	0.555 ± 0.0096	0.577 ± 0.0149	0.512 ± 0.0068

*Significant difference between ABA treatment and control groups

Table 2 Statistical table showing raw data

Sample	Length	Reads	Bases	Q20 (%)	Q30 (%)	GC (%)	*N* (ppm)
CK	125	53,286,482	6,660,810,250	97.55	95.04	45.535	561.23
ABA	125	51,046,352	6,380,794,000	94.23	88.49	46.5654	137.15

Table 3 Statistical table of clean data

Sample	Length	Reads	Bases	Q20 (%)	Q30 (%)	GC (%)	*N* (ppm)
CK	123.43	48,405,691	5,968,647,065	99.32	98.11	45.34	1.14
ABA	119.95	41,896,665	5,023,735,715	98.235	95.21	46.155	0.455

Table 4 Comparison of clean reads and unigenes

Samples	Total clean reads	Total mapped	Unique mapped	Multi mapped	Mapped (%)	Unique (%)
CK	48,405,691	44,820,522	35,810,118	9,010,404	77.86	62.20
ABA	41,896,665	30,542,782	24,623,874	5,918,908	73.06	58.92

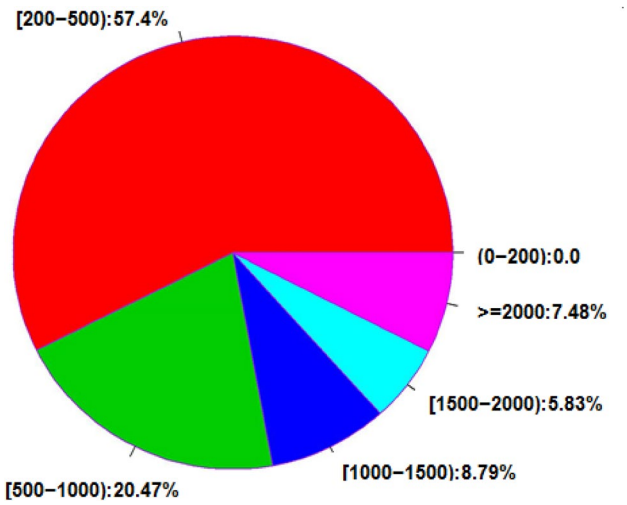

Fig. 3 Unigene interval distribution of different length

respectively, were the dominant GO terms. The terms "cellular process" (10.31%), "biological regulation" (8.39%) and "response to stimulus" (6.91%) were also common. Only a few genes were clustered in the way of "Channel regulator activity" and "cell killing".

Pathway-based analysis can be used to examine the biological functions and interactions of genes. We mapped the detected genes to reference canonical pathways in the KEGG pathway database to perform functional classification and pathway assignment. As a result, 8427 unigenes were assigned to 126 KEGG pathways (Table 5). among which the metabolism pathway was the largest category, followed by the ribosome, spliceosome and purine metabolism pathways. These results indicated that *A. lobata* cells were involved in the genetic and active metabolic processes. Moreover, in addition to the pathway related to 20E synthesis, terpenoid backbone biosynthesis (50 genes), steroid biosynthesis (21 genes) and steroid hormone biosynthesis (28 genes) pathways were identified.

Identification of differentially expressed genes

The analysis on differentially expressed genes (DEGs) between samples can be accomplished using RNA-SEq. In this study, DEGs were defined based on fold change of the normalized (RPKM) expression values, leading to the fact that there is a total of 154 genes exhibiting significant differential expression between the two samples. When comparing the control and ABA treatment group libraries, it can be found that 99 genes were up-regulated and 55 down-regulated (Fig. 5).

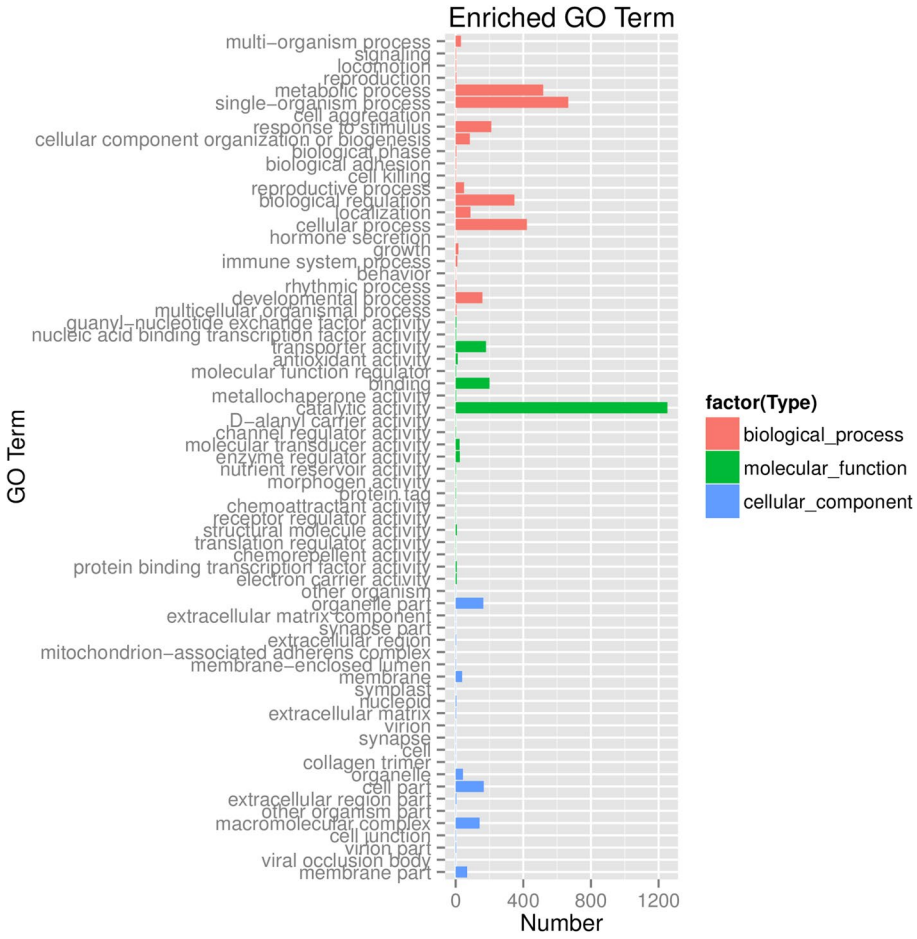

Fig. 4 GO function classification of unigenes

GO analysis of DEGs

All DEGs were mapped to GO terms in the database based on GO functional classification, followed by the comparison to the genomic background. The GO enrichment analysis on 81 genes is shown in Table 6 (agene could be mapped to multiple GO terms). We categorized the DEGs between the samples according to the secondary GO term classification. In all, 7, 10, and 8 functional groups were in the cellular component, molecular function, and biological process categories, respectively. In the biological process category, "metabolic process" was the dominant group, indicating that extensive metabolic activities occurred in ABA treated *A. lobata* cells. "Binding" and "catalytic activity" were highly represented in the molecular function category, while "cell part" was dominant in the cellular components category.

KEGG pathway analysis of DEGs

We mapped all DEGs to reference canonical pathways in the KEGG pathway database so as to identify the significantly enriched genes involved in metabolic or signal transduction pathways. By comparing with the whole transcriptome background, 18 pathways were significantly enriched, comprising 112 DEGs (Table 7). Pyrimidine metabolism and nitrogen metabolism pathways were significantly enriched with DEGs followed by RNA polymerase and biosynthesis of amino acids pathways. Among the pathways significantly enriched with DEGs, terpenoid backbone biosynthesis (6 DEGs), steroid biosynthesis (5 DEGs), steroid hormone biosynthesis (6 DEGs) pathways were identified.

KEGG pathway analysis of genes related to 20E synthesis

The terpenoid backbone biosynthesis can be completed through (ko00900) two sub-pathways, namely MVA and DOXP/MEP sub-pathways (Fig. 6). The former starts with acetyl CoA condensation, while the latter with Glyceraldehyde 3-phosphate (GA-3P). The terpenoid backbone biosynthesis pathway ends with IPP and moves into the next pathway (ko00100). Compared with the ko00900 pathway of untreated *A. lobata* suspension cells, the terpenoid backbone biosynthesis pathway had 5 genes up-regulated and 1 down-regulated after ABA treatment (Fig. 7).

Table 5 KEGG pathway analysis

Pathway ID	Pathway	Number of unigenes
ko01100	Metabolic pathways	2315
ko03010	Ribosome	346
ko03040	Spliceosome	208
ko00230	Purine metabolism	165
ko04141	Protein processing in endoplasmic reticulum	157
ko00190	Oxidative phosphorylation	157
ko03013	RNA transport	156
ko00240	Pyrimidine metabolism	140
ko00500	Starch and sucrose metabolism	133
ko04075	Plant hormone signal transduction	130
ko04120	Ubiquitin-mediated proteolysis	120
ko03015	mRNA surveillance pathway	110
ko04110	Cell cycle	110
ko00010	Glycolysis/gluconeogenesis	109
ko03018	RNA degradation	104
ko00520	Amino sugar and nucleotide sugar metabolism	101
ko04111	Cell cycle—yeast	91
ko04144	Endocytosis	90
ko03008	Ribosome biogenesis in eukaryotes	89
ko04626	Plant-pathogen interaction	89
ko04146	Peroxisome	85
ko00564	Glycerophospholipid metabolism	81
ko00330	Arginine and proline metabolism	77
ko04113	Meiotic division	75
ko00620	Pyruvate metabolism	73
ko00270	Cysteine and methionine metabolism	70
ko04145	Phagosome	69
ko00480	Glutathione metabolism	64
ko00970	Aminoacyl-tRNA biosynthesis	62
ko00860	Porphyrin and chlorophyll metabolism	61
ko00260	Glycine, serine, threonine metabolism	60
ko03420	Nucleotide excision repair	59
ko00680	Methane metabolism	58
ko00980	Metabolism of xenobiotics by cytochrome	58
ko00940	Phenylpropanoid biosynthesis	57
ko00195	Photosynthesis	55
ko00040	Pentose and glucuronateinterconversions	55
ko00510	*N*-Glycan biosynthesis	54
ko00710	Carbon fixation in photosynthetic organisms	54
ko00051	Fructose and mannose metabolism	52
ko03440	Homologous recombination	52
ko00030	Pentose phosphate pathway	52
ko00250	Alanine, aspartate and glutamate metabolism	51
ko00562	Inositol phosphate metabolism	51
ko04142	Lysosome	51
ko00630	Glyoxylate and dicarboxylate metabolism	51
ko00900	Terpenoid backbone biosynthesis	50
ko03030	DNA replication	48
ko00360	Phenylalanine metabolism	48
ko03022	Basal transcription factors	48

Table 5 (continued)

Pathway ID	Pathway	Number of unigenes
ko00020	Citrate cycle (TCA cycle)	47
ko00561	Glycerolipid metabolism	46
ko00053	Ascorbate and aldarate metabolism	45
ko03060	Protein export	44
ko04130	SNARE interactions in vesicular transport	43
ko00400	Phenylalanine, tyrosine and tryptophan biosynthesis	43
ko03430	Mismatch repair	43
ko03050	Proteasome	42
ko00052	Galactose metabolism	42
ko00563	Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	41
ko03410	Base excision repair	40
ko04010	MAPK signaling pathway	39
ko00071	Fatty acid metabolism	39
ko00280	Valine, leucine and isoleucine degradation	36
ko00640	Propanoate metabolism	35
ko00830	Retinol metabolism	33
ko00920	Sulfur metabolism	33
ko00600	Sphingolipid metabolism	33
ko00350	Tyrosine metabolism	32
ko00310	Lysine degradation	31
ko00061	Fatty acid biosynthesis	31
ko00410	beta-Alanine metabolism	30
ko00770	Pantothenate and CoA biosynthesis	30
ko00513	Various types of *N*-glycan biosynthesis	29
ko00130	Ubiquinone and other terpenoid-quinone biosynthesis	29
ko00380	Tryptophan metabolism	29
ko01040	Biosynthesis of unsaturated fatty acids	28
ko00140	Steroid hormone biosynthesis	28
ko04650	Natural killer cell mediated cytotoxicity	28
ko04122	Sulfur relay system	27
ko04712	Circadian rhythm—plant	27
ko02020	Two-component system	26
ko00790	Folate biosynthesis	26
ko00592	alpha-Linolenic acid metabolism	25
ko00910	Nitrogen metabolism	24
ko04140	Regulation of autophagy	24
ko00340	Histidine metabolism	24
ko00590	Arachidonic acid metabolism	22
ko00100	Steroid biosynthesis	21
ko00906	Carotenoid biosynthesis	20
ko00460	Cyano amino acid metabolism	20
ko00670	One carbon pool by folate	20
ko00760	Nicotinate and nicotinamide metabolism	20
ko00196	Photosynthesis antenna proteins	19
ko00511	Other glycan degradation	19
ko00780	Biotin metabolism	19
ko00290	Valine, leucine and isoleucine biosynthesis	19
ko00062	Fatty acid elongation	19
ko00624	Polycyclic aromatic hydrocarbon degradation	17
ko00730	Thiamine metabolism	17

Table 5 (continued)

Pathway ID	Pathway	Number of unigenes
ko02010	Transporters ABC	17
ko00960	Tropane, piperidine and pyridine alkaloid biosynthesis	16
ko00300	Lysine biosynthesis	15
ko00450	Selenocompound metabolism	14
ko00591	Linoleic acid metabolism	14
ko00904	Diterpenoid biosynthesis	11
ko00941	Flavonoid biosynthesis	11
ko00750	Vitamin B6 metabolism	11
ko00740	Riboflavin metabolism	10
ko00603	Glycosphingolipid biosynthesis - globo series	10
ko04710	Circadian rhythm	10
ko00531	Glycosaminoglycan degradation	10
ko00430	Taurine and hypotaurine metabolism	9
ko00440	Phosphonate and phosphinate metabolism	9
ko03450	Non-homologous end-joining	8
ko00908	Zeatin biosynthesis	7
ko00909	Sesquiterpenoid and triterpenoid biosynthesis	6
ko00073	Cutin, suberine and wax biosynthesis	6
ko00785	Lipoic acid metabolism	6
ko00514	Other types of *O*-glycan biosynthesis	6
ko00905	Brassinosteroid biosynthesis	5
ko00072	Synthesis and degradation of ketone bodies	4
ko00944	Flavone and flavonol biosynthesis	3
ko00965	Betalain biosynthesis	2
ko00942	Anthocyanin biosynthesis	1
ko00633	Nitrotoluene degradation	1

Corresponding to EC:1.1.1.34, the c49795_g1_i1gene was up-regulated, along with the identification as the Nicotinamide Adenine Dinucleotide Phosphate (NADPH) gene of the MVA pathway. NADPHisa, the key enzyme in the MVA pathway, converts HMF-CoA to mevalonate.

The c29419_g1_i1 gene (EC:4.6.1.12) was up-regulated, along with the identification as the 2C-methyl-D-erythritol

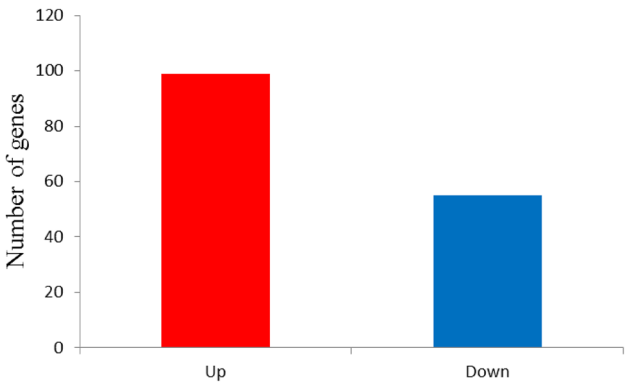

Fig. 5 Statistics of DEGs

2,4-cyclodiphosphate synthase (MCS) gene of the DOXP/MEP pathway. MCS gene function is to convert 2-phospho-4-(cytidine5′-diphospho)-2-*C*-methyl-D-erythritol to 2-*C*-methyl-D-erythritol2,4-cyclicphosphate.

The c44766_g1_i6 gene (EC:4.6.1.12,EC:1.8.3.5) was up-regulated, and identified as the Alpha farnesene synthesis (AFS) gene, which is the intermediate enzyme in farnesyl cysteine synthesis.

The c45098_g1_i1 and c73678_g1_i1 genes (EC:2.5.1.1,EC:2.5.1.10 and EC:2.5.1.29) genes, both corresponded to geranyl geranylpyrophosphate synthetase II (GGPPS) gene, were up-regulated. The enzymatic function of GGPPS is to produce geranyl pyrophosphate (GGPP).

The c81295_g1_i1gene (EC:3.4.24.84) was down-regulated, and identified as the ste24 endopeptidase gene, which is an intermediate enzyme in farnesyl pyrophosphate (FPP) synthesis.

Next to the ko00900 pathway was the steroid biosynthesis pathway (ko00100) beginning with IPP, with squalene as intermediate, and steroid compounds as end products (Fig. 8). The steroid biosynthesis pathway had 4 genes up-regulated and 1down-regulated (Fig. 9); only c27194_g1_i2

Table 6 Significant differences of the GO terms

Cluster	GO term	Cluster frequency
Cellular component	Cell part	24 out of 24 genes, 100%
	Intracellular part	22 out of 24 genes, 91.66%
	Cytoplasm	22 out of 24 genes, 91.66%
	Organelle	18 out of 24 genes, 75%
	Membrane-bounded organelle	16 out of 24 genes, 66.7%
	Plastid	6 out of 24 genes, 25%
	Chloroplast	6 out of 24 genes, 25%
Molecular function	Catalytic activity	9 out of 22 genes, 40.91%
	Hydrolase activity	8 out of 22 genes, 36.36%
	Hydrolase activity, hydrolyzing *O*-glycosyl compounds	6 out of 22 genes, 27.27%
	Binding	9 out of 22 genes, 40.91%
	Hydrolase activity, acting on glycosyl bonds	4 out of 22 genes, 18.18%
	Ion binding	3 out of 22 genes, 13.63%
	Small molecule binding	3 out of 22 genes, 13.63%
	Nucleotide binding	3 out of 22 genes, 13.63%
	Nucleoside phosphate binding	3 out of 22 genes, 13.63%
	Oxidoreductase activity	7 out of 22 genes, 31.81%
Biological process	Metabolic process	32 out of 35 genes, 91.425%
	primary metabolic process	27 out of 35 genes, 77.14%
	Organic substance metabolic process	20 out of 35 genes, 61.9%
	Response to stimulus	5 out of 35 genes, 14.28%
	Lipid metabolic process	5 out of 35 genes, 14.28%
	Carboxylic acid metabolic process	6 out of 35 genes, 17.14%
	Aromatic compound biosynthetic process	16 out of 35 genes, 45.71%
	Heterocycle biosynthetic process	14 out of 35 genes, 40%

Table 7 Pathways enriched by the differential expression genes

Pathway ID	Pathway	DEGs with pathway annotation		*p* value	*Q* value
ko00603	Glycosphingolipid biosynthesis—globo series	1	0.89%	0.01663	0.001326
ko00910	Nitrogen metabolism	10	8.93%	0.000403	0.002689
ko00591	Linoleic acid metabolism	1	0.89%	0.000221	0.003847
ko04142	Lysosome	4	3.57%	0.00183	0.004108
ko00710	Carbon fixation in photosynthetic organisms	5	4.46%	0.00205	0.004108
ko03430	Mismatch repair	5	4.46%	0.0013	0.004108
ko00030	Pentose phosphate pathway	6	5.36%	0.00191	0.004108
ko00051	Fructose and mannose metabolism	7	6.25%	0.00191	0.004108
ko03020	RNA polymerase	9	8.04%	0.0013	0.004108
ko00900	Terpenoid backbone biosynthesis	7	6.25%	0.000757	0.007405
ko00010	Glycolysis /Gluconeogenesis	7	6.25%	0.00818	0.01168
ko00240	Pyrimidine metabolism	14	12.50%	0.0133	0.015823
ko00230	Purine metabolism	8	7.14%	0.0182	0.020192
ko01220	Degradation of aromatic compounds	3	2.68%	0.01803	0.021453
ko01200	Carbon metabolism	6	5.36%	0.0257	0.027032
ko00100	Steroid biosynthesis	4	3.57%	0.01453	0.033462
ko00140	Steroid hormone biosynthesis	6	5.36%	0.019623	0.037926
ko01230	Biosynthesis of amino acids	9	8.04%	0.0335	0.047735

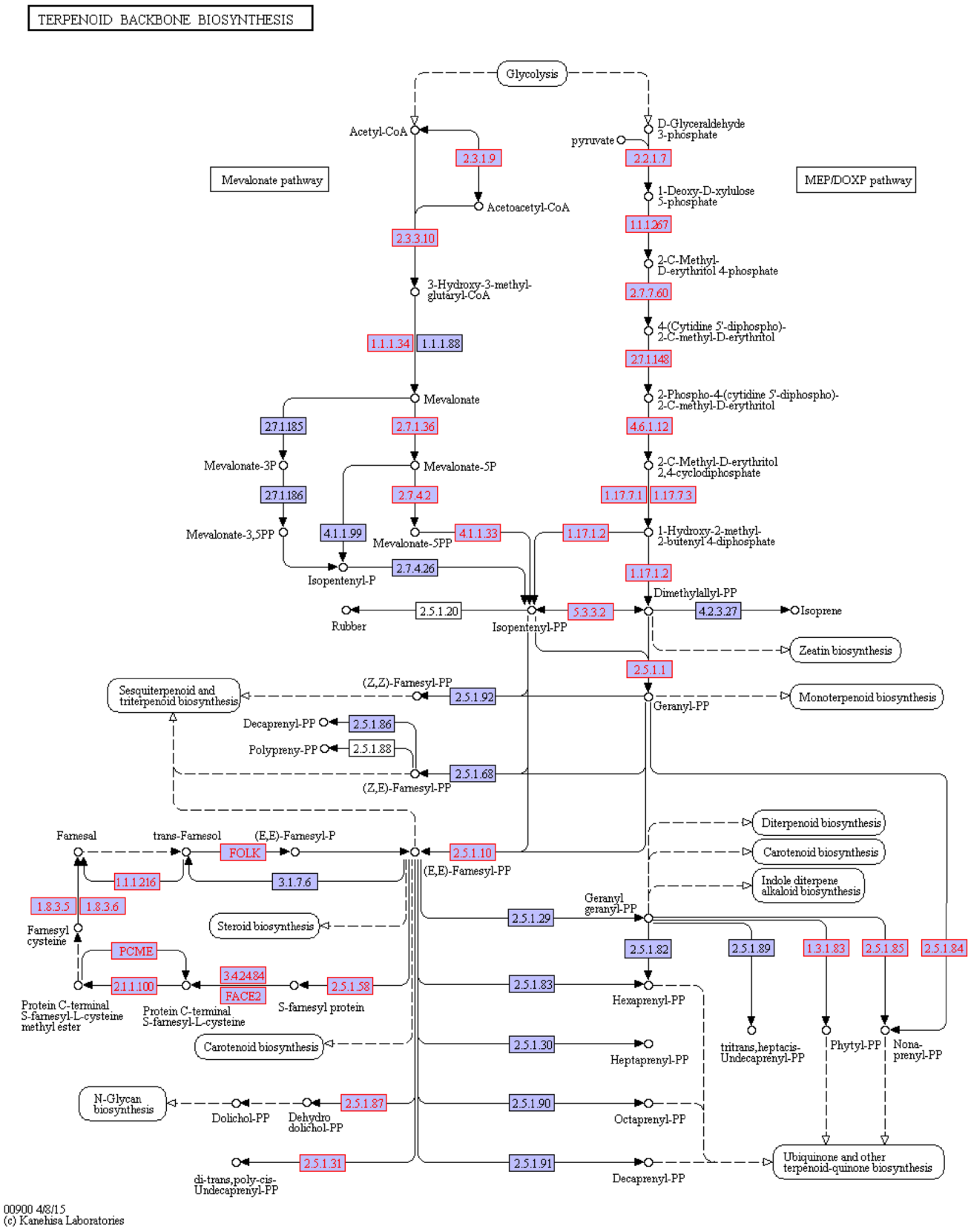

Fig. 6 The ko00900 pathway in untreated *A. lobata* suspension cells. Note: the genes in untreated *A. lobata* suspension cell cDNA library in the pathway are shown in red. Same below

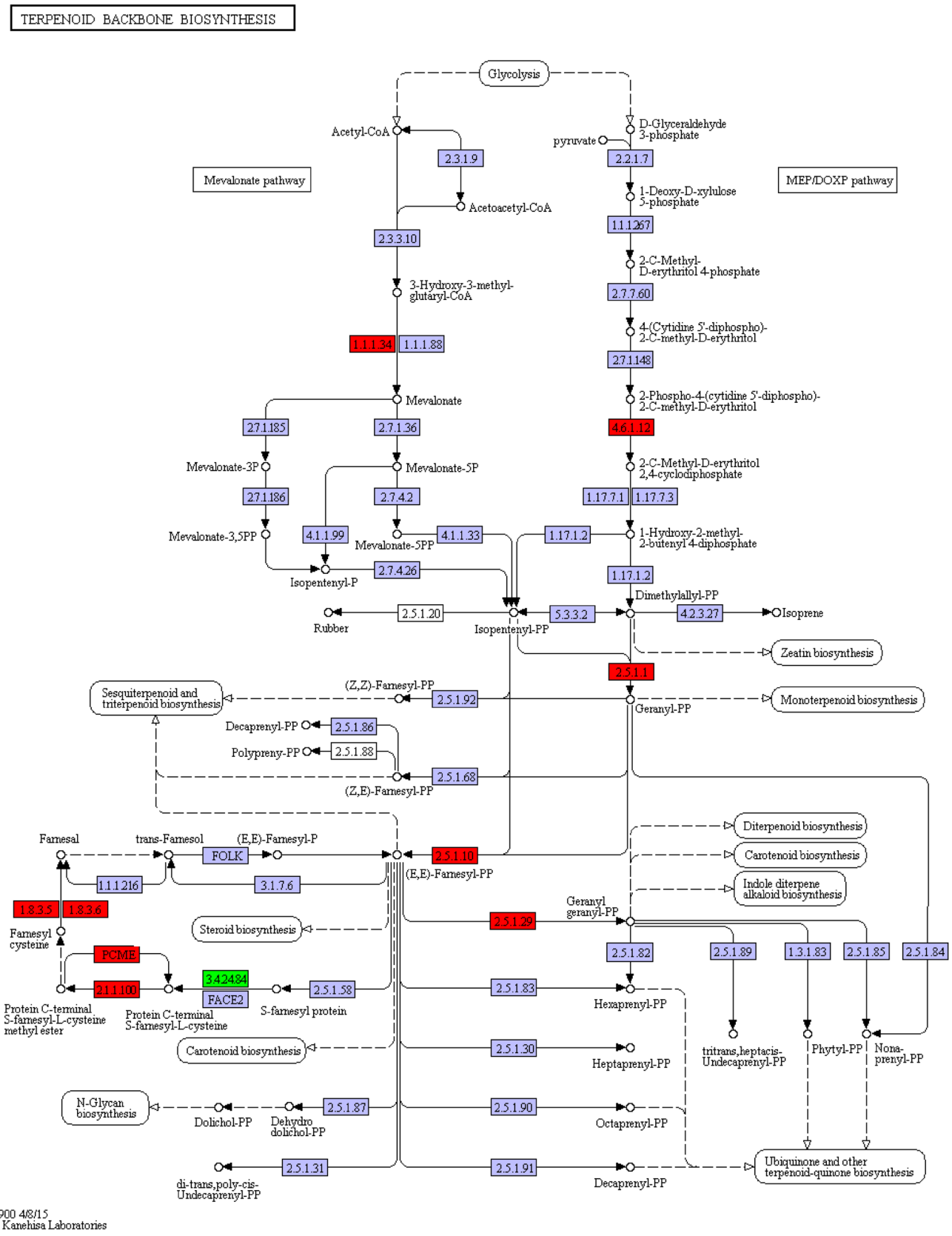

Fig. 7 DEGs were annotated to the ko00900 pathway. Note: red, up-regulated gene; green, down-regulated gene. Same below

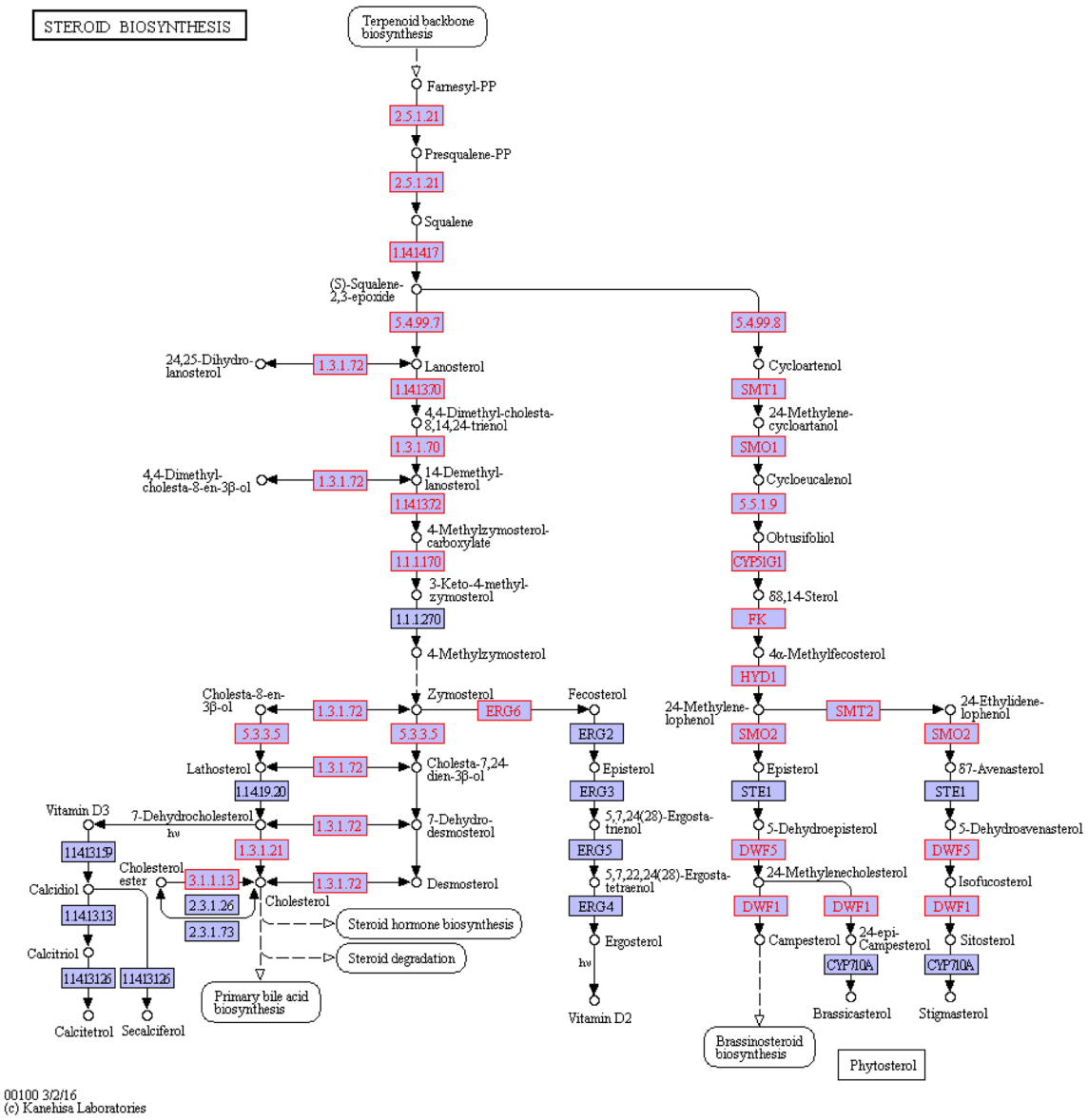

Fig. 8 The ko00100 pathway in untreated *A. lobata* suspension cells

(EC:1.1.1.170) and c34855_g1_i (EC:1.14.13.72) genes, both modifiers in the process of squalene related synthesis of steroid substances, were related to the synthesis of steroid hormones. Those two genes were both modifiers in the process of. The c27194_g1_i2 gene was identified as the 4α-carboxylic acid 3 dehydrogenase gene, while the c34855_g1_i gene was the methyl oxidase gene, which were associated with brassino steroid synthesis.

Next was the steroid hormone biosynthesis (ko00140) pathway (Fig. 10). After analysis, we found six DEGs that were all modifiers in the process of squalene related synthesis of steroid substances with a total of five genes up-regulated and one down-regulated (Fig. 11). Currently, the KEGG pathway database has not fully included the 20E pathway, and 20E synthetic steroid is similar to hormone synthesis included by ko00140; therefore, these genes were annotated to the ko00140 pathway

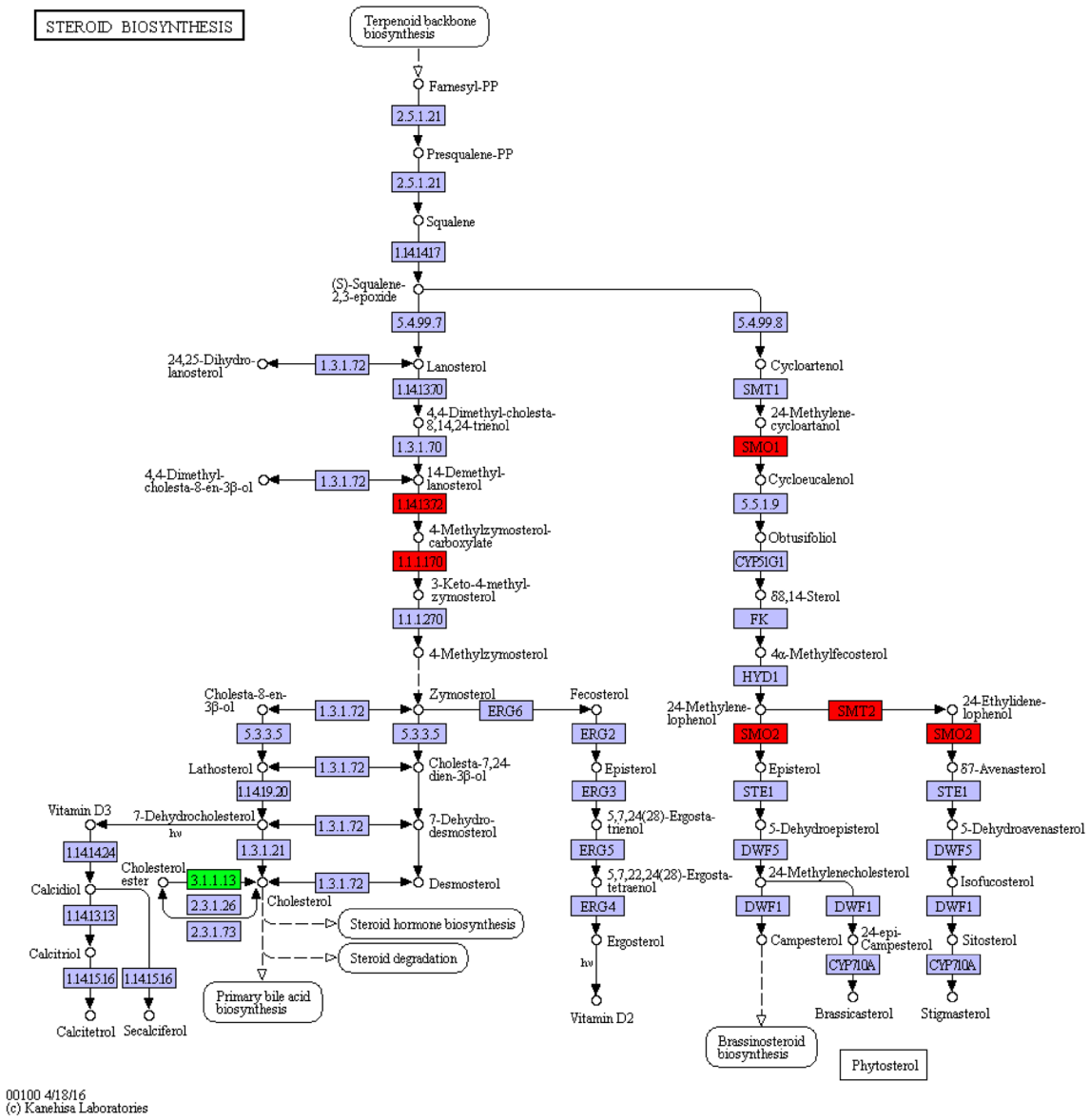

Fig. 9 DEGs annotated to the ko00100 pathway

with similar functions. Corresponded to the glucuronide transferase gene, the c47715_g1_i2,c47715_g1_i3, c47715_g1_i6 and c47715_g1_i7(EC:2.4.1.17) genes were up-regulated. The c21475_g1_i1 gene (EC:1.3.1.22 and1.3.1.94) was up-regulated, with identification as polyprenol reductase gene while the c40828_g1_i6gene (EC:2.4.1.17) was down-regulated, with the identification as the glucuronide transferase gene.

Discussion

Treatment with 0.15 mg/l ABA of *A. lobata* suspension cultures could promote 20E content increase in the short term but was not suitable for long-term culture, and browning was observed. In Wang et al. (2018) study, the browning also happened, which might be caused by the toxicity of ABA. In recent years, studies have shown that ABA can regulate and stimulate the production of secondary metabolites such as chlorogenic acid, phenols, flavonoids and terpenoid substances (Sandhu et al. 2011; Buran et al. 2012). The increase of these secondary metabolites is related to ABA stress inducing the defense system. It was also found that stimulus response genes differentially expressed after ABA treatment, but the relationship between stimulus response and 20E synthesis still needs further exploration.

ABA can inhibit growth, causing premature cell aging, which is responsible for the fact that *A. lobata* suspension

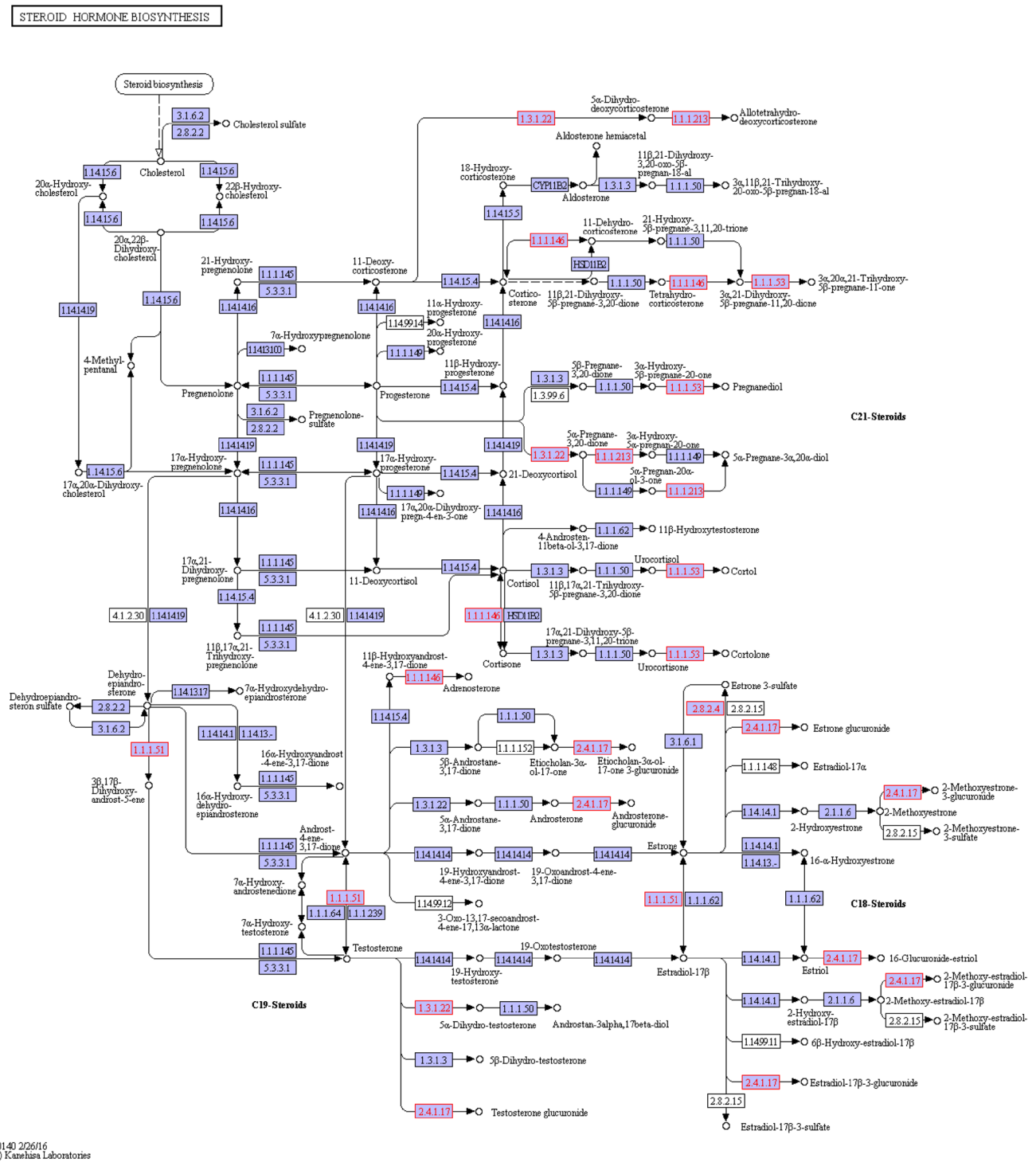

Fig. 10 The ko00140 pathway in untreated *A. lobata* suspension cells

cells turned brown after ABA treatment (Hansen and Grossmann 2000; Hunter et al. 2004). On the other hand, ABA produced stress stimulate the cells, and stress stimulation could promote cell browning (Dai et al. 2016). This might be one of the main causes of the browning phenomenon.

It was found in the studies 20E is mainly synthetized through the MVA and DOXP/MEP pathways. However, the full 20E synthesis pathway remains unclear; the pathways from squalene to 20E are not completely described (Adler and Grebenok 1995; Dinan 2001; Báthori and Pongrácz

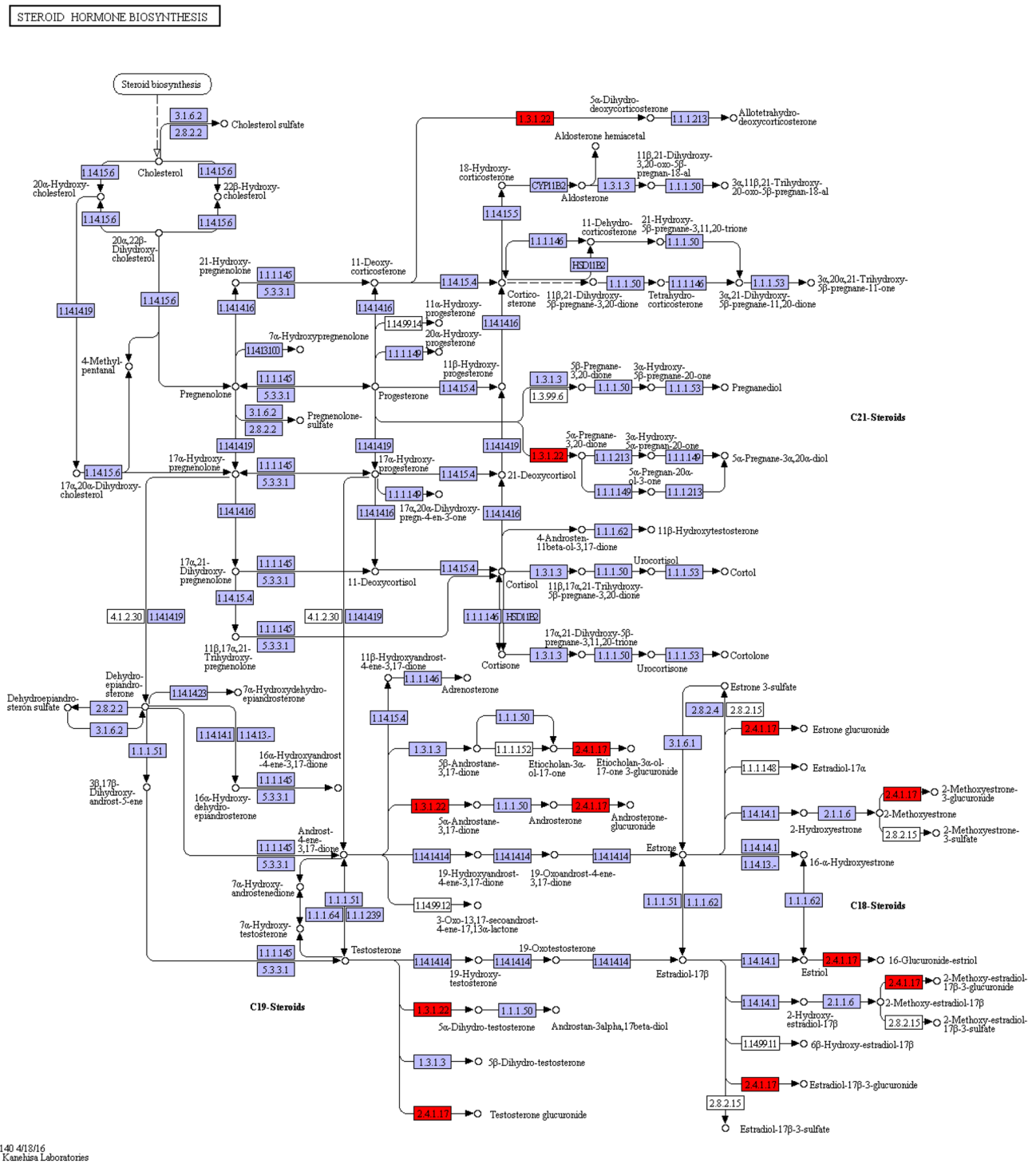

Fig. 11 DEGs annotated to the ko00140 pathway

2005; Ma et al. 2006). In the KEGG pathway database, we only found ko00900 and ko00100 pathways to produce squalene utilizing pyruvic acid and GA-3Pas starting points. Without including the pathway from squalene to 20E, the KEGG pathway database contains a pathway from squalene to other sterol hormones (ko00140). *A. lobata* cDNA library has similar genes in the ko00140 pathway. NADPH, MCS, AFS, GGPPS and STE24 endopeptidase genes in the ko00900 pathway of DEGs were all synthetase genes (Bach 1987; Adam and Zapp 1998; Kim et al. 2014; Liao et al.

2014). The 4α-carboxylic acid 3 dehydrogenase and methyl oxidase genes in the ko00100 pathway of DEGs were modifier genes. The DEGs similar to the genes in the ko00140 pathway were modifier genes widespread in plant secondary metabolism (glucuronide transferase genes). The function of those genesis glycosylation is to add sugar molecules on specific sites of metabolites (Mackenzie et al. 1997; Gachon et al. 2005; Asada et al. 2013). Glycosyl transferase has high specificity (Coutinho et al. 2003). Therefore, the relationship between glycosyl transferase gene modification and 20E synthesis also needs to be further explored.

Conclusions

The study found that ABA promoted 20E accumulation. A total of 154 genes were significantly regulated after ABA treatment, with 99 up-regulated and 55 down-regulated, respectively. In addition to 20E-related pathways, the genes belonged to the ko00900 (terpenoid backbone biosynthesis) pathway (six differentially expressed genes [DEGs]), ko00100 (steroid biosynthesis) pathway (four DEGs), and ko00140 (steroid hormone biosynthesis) pathway (six DEGs). It is hoped that these results can offer a strategy to explore a way to regulate the 20E accumulation from the molecular biological angle.

Acknowledgements This work was supported by the National Natural Science Foundation of China (NSFC) (Grant no.: 31370649).

Compliance with ethical standards

Conflict of interest No potential conflict of interest was reported by the authors.

References

Adam KP, Zapp J (1998) Biosynthesis of the isoprene units of chamomile sesquiterpenes. Phytochemistry 48(6):953–959

Adler JH, Grebenok RJ (1995) Biosynthesis and distribution of insect-molting hormones in plants—a review. Lipids 30(3):257–262

Anderson MD, Prasad TK, Martin BA, Stewart CR (1994) Differential gene expression in chilling-acclimated maize seedlings and evidence for the involvement of abscisicacid in chilling tolerance. Plant Physiol 105(1):331–339

Asada K, Salim V, Masada-Atsumi S, Edmunds E, Nagatoshi M, Terasaka K, Mizukami H, De Lucab V (2013) A 7-deoxyloganetic acid glucosyltransferase contributes a key step in secologanin biosynthesis in *Madagascar periwinkle*. Plant Cell 25(10):4123–4134

Audic S, Claverie JM (1997) The significance of digital gene expression profiles. Genome Res 7(10):986–995

Bach TJ (1987) Synthesis and metabolism of mevanoic acid in plants. Plant Physiol Biochem 35(25):163–178

Báthori M, Pongrácz Z (2005) Phytoecdysteroids from isolation to their effects on humans. Curr Med Chem 12(2):153–172

Buran TJ, Sandhu AK, Azeredo AM, Bent AH, Williamson JG, Gu LW (2012) Effects of exogenous abscisic acid on fruit quality, antioxidant capacities and phytochemical contents of southern high bush blueberrie. Food Chem 132(3):1375–1381

Cai ZY, Yi GQ, Li YY (2014) Nuclear magnetic resonance characteristics of neo-clerodane diterpene in Genus *Ajuga*. Cent South Pharm 12(11):1108–1112

Chavez VM, Marques G, Delbecque JP, Kobayashi K, Hollingsworth M, Burr J, Natzle JE, O'Connor MB (2000) The Drosophila disembodied gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. Development 127:4115–4126

Coll J, Tandron YA (2008) neo-Clerodane diterpenoids from *Ajuga*: structural elucidation and biological activity. Phytochem Rev 7(1):25–49

Coll J, Tandron YA, Zeng X (2007) New phytoecdysteroids from cultured plants of *Ajuga nipponensis* Makino. Steroids 72(3):270–277

Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21(18):3674–3676

Coutinho PM, Deleury E, Davies GJ, Henrissat B (2003) An evolving hierarchical family classification for glycosyltransferases. J Mol Biol 328(2):307–317

Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR (2009) Abscisicacid: emergence of a core signaling network. Annu Rev Plant Biol 61(1):651–679

Dai Y, Yang SH, Zhao HZ, Zhao LH (2016) Research progress on browning phenomenon in tissue culture of medicinal plants. Chin Tradit Herb Drugs 47(2):344–351

Davies TG, Lockley WJS, Boid R, Rees HH, Goodwin TW (1980) Mechanism of formation of the A/B cis ring junction of ecdysteroids in *Polypodium vulgare*. Biochem J 190:537–544

Davies TG, Dinan LN, Lockley WJS, Rees HH, Goodwin TW (1981) Formation of the A/B cis ring junction of ecdysteroids in the locust, *Schistocerca gregaria*. Biochem J 194:53–62

Dinan L (2001) Phytoecdysteroids: biological aspects. Phytochemistry 57(3):325–339

Eisen MB, Spellman PT, Brown PO, Botstein D, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 95(25):14863–14868

Fekete G, Polgár LA, Báthori M, Col J, Darvas B (2004) Per os efficacy of *Ajuga* extracts against sucking insects. Pest Manag Sci 60(11):1099–1104

Festucci-Buselli RA, Contim LAS, Barbosa LCA, Stuart J, Otoni WC (2008) Biosynthesis and potential functions of the ecdysteroid 20-hydroxyecdysone—a review. Botany 86:978–982

Fujimoto Y, Maeda I, Ohyama K, Hikiba J, Kataoka H (2015) Biosynthesis of 20-hydroxyecdysone in plants: 3β-hydroxy-5β cholestan-6-one as an intermediate immediately after cholesterol in *Ajuga* hairy roots. Phytochemistry 111:59–64

Fujtmoto Y, Kushiro T, Nakamura K (1997) Biosynthesis of 20-hydroxyecdysone in *Ajuga* hairy roots: hydrogen migration from C-6 to C-5 during cis. A/B ring formation. Tetrahedron Lett 38(15):2697–2700

Gachon CM, Langlois-Meurinn M, Saindrenan P (2005) Plant secondary metabolism glycosyl transferases: the emerging functional analysis. Trends Plant Sci 10(11):542–549

Garg R, Patel RK, Tyagi AK, Jain M (2011) De novo assembly of chickpea transcriptome using short reads for gene discovery and marker identification. DNA Res 18(1):53–63

Gilbert LI (2004) Halloween genes encode P450 enzymes that mediate steroid hormone biosynthesis in *Drosophila melanogaster*. Mol Cell Endocrinol 215:1–10

Gilbert LI, Warren JT (2005) A molecular genetic approach to the biosynthesis of the insect steroid molting hormone. In: Litwack G

(ed) Insect hormones—vitamins and hormones, vol 73. Elsevier, Amsterdam, pp 31–57
Gilbert LI, Rybczynski R, Warren JT (2002) Control and biochemical nature of the ecdysteroidogenic pathway. Annu Rev Entomol 47(47):883–916
Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng QD, Chen ZH, Mauceli E, Hacohen N, Gnirke A, Rhind N, diPalma F, Birren BW, Nusbaum C, Lindblad-Toh K, Friedman N, Regev A (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nature Biotechnology 29(7):644–652
Grebenok RJ, Galbraith DW, Benveniste I, Feyereisen R (1996) Ecdysone 20-monooxygenase, a cytochrome P450 enzymes from spinach, *Spinacia oleracea*. Phytochemistry 42:927–933
Guo XD, Huang ZS, Bao YD, An LK, Ma L, Gu LQ (2005) Chemical constituents of *Ajuga decumbens*. Chin Tradit Herb Drugs 36(5):646–648
Hansen H, Grossmann K (2000) Auxin-induced ethylene triggers abscisic acid biosynthesis and growth inhibition. Plant Physiol 124(3):1437–1448
Hauser F, Waadt R, Schroeder J (2011) Evolution of abscisic acid synthesis and signaling mechanisms. Curr Biol 21(9):346–355
Huang X, Warren JT, Gilbert LI (2008) New players in the regulation of ecdysone biosynthesis. J Genet Genom 35(1):1–10
Hunter DA, Ferrante A, Vernieri P, Reid MS (2004) Role of abscisic acid in perianth senescence of daffodil (*Narcissus pseudonarcissus* 'Dutch Master'). Physiol Plant 121(2):313–321
Hyodo R, Fujimoto Y (2000) Biosynthesis of 20-hydroxyecdysone in *Ajuga* hairy roots: the possibility of 7-ene introduction at a late stage. Phytochemistry 53(7):733–737
Iga M, Kataoka H (2012) Recent studies on insect hormone metabolic pathways mediated by cytochrome P450 enzymes. Biol Pharm Bull 35(6):838–843
Kanehisa M, Araki M, Goto S, Hattori M, Hirakawa M, Itoh M, Katayama T, Kawashima S, Okuda S, Tokimatsu T, Yamanishi Y (2015) KEGG for linking genomes to life and the environment. Nucleic Acids Res 36:53–72
Kim YJ, Lee OR, Oh JY, Jang MG, Yang DC (2014) Functional analysis of 3-hydroxy-3-methylglutaryl coenzyme a reductase encoding genes in riterpene saponin-producing ginseng. Plant Physiol 165(1):373–387
Kircher HW (1982) Sterols and insects. In: Dupont J (ed) Cholesterol systems in insects and animals. CRC Press, Inc., Boca Raton, pp 1–50
Lev SV, Zakirova RP, Saatov Z, Gorovits MB, Abubakirov NK (1990) Ecdysteroids from tissue and cell cultures of *Ajuga turkestanica*. Chem Nat Compd 26(1):40–41
Li X, Qian JJ, Li XC, Yan SC, Chi DF (2013) A study on edysterone production of *Ajuga lobata* D. Don cell lines in suspension culture. Chin Agric Sci Bull 29(34):127–133
Liao P, Wang H, Hemmerlin A, Nagegowda DA, Bach TJ, Wang M, Chye ML (2014) Past achievements, current status and future perspectives of studies on 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) in the mevalonate (MVA) pathway. Plant Cell Rep 33(7):1005–1022
Lockley WJS, Boid R, Lloyd-Jones GJ, Rees HH, Goodwin TW (1975) Fate of the C-4 hydrogen atoms of cholesterol during its transformation into ecdysones in insects and plants. J Chem Soc Chem Commun 9(9):346–348
Ma L, Ding P, Yang GX, He GY (2006) Advances on the plant terpenoid isoprenoid biosynthetic pathway and its key enzymes. Biotechnol Bull 1:22–30
Mackenzie PI, Owens IS, Burchell B, Bock KW, Bairoch A, Bélanger A, Fournel-Gigleux S, Green M, Hum DW, Iyanagi T, Lancet D, Louisot P, Magdalou J, Chowdhury JR, Ritter JK, Schachter H, Tephly TR, Tipton KF, Nebert DW (1997) The UDP glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. Pharmacogenet Genom 7(4):255–269
Marion-Poll F, Dinan L, Lafont R (2005) The role of phytoecdysteroids in the control of phytophagous insects. In: Regnault-Roger C, Philogène BJR, Vincent C (eds) Biopesticides of Plant Origin. Lavoisier Publishing, Paris France, pp 87–103
Morrissy AS, Morin RD, Delaney A, Zeng T, McDonald H, Jones S, Zhao Y, Hirst M, Marra MA (2008) Next-generation tag sequencing for cancer gene expression profiling. Genome Res 19(10):1825–1835
Nagakari M, Kushiro T, Yagi T, Tanaka N, Matsumoto T, Kakinuma K, Fujimoto Y (1994) 3β-Hydroxy-5β-cholest-7-en-6-one as an intermediate of 20-hydroxyecdysone biosynthesis in a hairy root culture of *Ajuga reptans* var. *atropurpurea*. Cheminform 25(51):1761–1762
Nakagawa T, Hara N, Fujimoto Y (1997) Biosynthesis of 20-hydroxyecdysone in *Ajuga* hairy roots: stereochemistry of C-25 hydroxylation. Tetrahedron Lett 38(15):2701–2704
Namiki T, Niwa R, Sakudoh T, Shirai K, Takeuchi H, Kataoka H (2005) Cytochrome P450 CYP307A1/Spook: a regulator for ecdysone synthesis in insects. Biochem Biophy Res Co 337(1):367–374
Ness RW, Siol M, Barrett SC (2011) De novo sequence assembly and characterization of the floral transcriptome in cross-and self-fertilizing plants. BMC Genom 12(1):298
Niwa R, Matsuda T, Yoshiyama T, Namiki T, Mita K, Fujimoto Y, Kataoka H (2004) CYP306A1, a cytochrome P450 enzyme, is essential for ecdysteroid biosynthesis in the prothoracic glands of Bombyx and Drosophila. J Biol Chem 279(34):35942–35949
Niwa R, Namiki T, Ito K, Shimada-Niwa Y, Kiuchi M, Kawaoka S, Kayukawa T, Banno Y, Fujimoto Y, Shigenobu S, Kobayashi S, Shimada T, Katsuma S, Shinoda T (2010) Non-molting glossy/shroud encodes a short-chain dehydrogenase/reductase that functions in the 'Black Box' of the ecdysteroid biosynthesis pathway. Development 137(12):1991–1999
Ohyama K, Kushiro T, Nakamura K, Fujimoto Y (1999) Biosynthesis of 20-hydroxyecdysone in *Ajuga* hairy roots: Fate of 6α- and 6β-hydrogens of lathosterol. Bioorg Med Chem 7:2925–2930
Ono H, Rewitz KF, Shinoda T, Itoyama K, Petryk A, Rybczynski R, Jarcho M, Warren JT, Marqués G, Shimell MJ (2006) Spook and Spookier code for stage-specific components of the ecdysone biosynthetic pathway in Diptera. Dev Biol 298(2):555–570
Ou Q, Magico A, King-Jones K (2011) Nuclear receptor DHR4 controls the timing of steroid hormone pulses during Drosophila development. Plos Biol 9(9):e1001160
Petryk A, Warren JT, Marques G, Jarcho MP, Gilbert LI, Kahler J, Parvy JP, Li Y, Dauphin-Villemant C, O'Connor MB (2003) Shade is the Drosophila P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. Proc Natl Acad Sci USA 100(24):13773–13778
Rees HH (1995) Ecdysteroid biosynthesis and inactivation in relation to function. Eur J Entomol 92:9–39
Ren F, Zhang RJ, Chen Q (2012) Progress in ABA and SA improving plant drought resistance and salt resistance. Biotechnol Bull 29(3):17–21
Rewitz KF, O'Connor MB, Gilbert LI (2007) Molecular evolution of the insect Halloween family of cytochrome P450s: phylogeny, gene organization and functional conservation. Insect Biochem Mol Biol 37(8):741–753
Rharrabe K, Sayah F, LafontLauber R (2009) Dietary effects of four phytoecdysteroids on growth and development of the Indian meal moth, *Plodia interpunctella*. J Insect Sci 10(2):259–272
Sakurai S, Yonemura N, Fujimoto Y, Hata F, Ikekawa N (1986) 7-Dehydrosterols in prothoracic glands of the silkworm *Bombyx mori*. Experientia 42(9):1034–1036

Saldanha AJ (2004) Java Treeview extensible visualization of microarray data. Bioinformatics 20(17):3246–3248

Sandhu AK, Gray DJ, Lu J, Gu LW (2011) Effects of exogenous abscisic acid on antioxidant capacities anthocyanins and flavonol contents of muscadine grape (Vitisrotundifolia) skins. Food Chem 126(3):982–988

Selvaraj P, John de Britto A, Sahayaraj K (2007) Phytoecdysone of *Pteridium aquilinum* (L) Kuhn (Dennstaedtiaceae) and its pesticidal property on two major pests. Arch Phytopathol Plant Prot 38(2):99–105

tHoen PA, Ariyurek Y, Thygesen HH, Vreugdenhil E, Vossen RH, de Menezes RX, Boer JMvanOmmen GJ, den Dunnen JT (2008) Deep sequencing-based expression analysis shows major advances in robustness, resolution and inter-lab portability over five microarray platforms. Nucleic Acids Res 36(21):e141

Tsukagoshi Y, Ohyama K, Seki H, Akashi T, Muranaka T, Suzuki H, Fujimoto Y (2016) Functional characterization of CYP71D443, a cytochrome P450 catalyzing C-22 hydroxylation in the 20-hydroxyecdysone biosynthesis of *Ajuga* hairy roots. Phytochemistry 127:23–28

Ufimtsev KG, Shirshova TI, Yakimchuk AP (2003) Effect of ecdysteroids of *Serratula coronata* L. on development of larvae of the Egyptian cotton leafworm. Rastitelnye Resursy 39(4):134–142

Wang QJ, Zheng LP, Sima YH, Yuan HY, Wang JW (2013) Methyl jasmonate stimulates 20-hydroxyecdysone production in cell suspension cultures of *Achyranthes bidentata*. Plant Omics 6(2):116–120

Wang QJ, Zheng LP, Zhao PF, Zhao YL, Wang JW (2014) Cloning and characterization of an elicitor-responsive gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase involved in 20-hydroxyecdysone production in cell cultures of *Cyanotis arachnoidea*. Plant Physiol Bioch 84:1–9

Wang YC, Zhao JJ, Chi DF (2018) β-ecdysterone accumulation and regulation in *Ajuga multiflora* bunge suspension culture. 3Biotech 8(2):87

Warren JT, Petryk A, Marques G, Jarcho M, Parvy JP, Dauphin-Villemant C, O'Connor MB, Gilbert LI (2002) Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of *Drosophila melanogaster*. Proc Natl Acad Sci USA 99(17):11043–11048

Warren JT, Petryk A, Marques G, Parvy JP, Shinoda T, Itoyama K, Kobayashi J, Jarcho M, Li Y, O'Connor MB, Dauphin-Villemant C, Gilbert LI (2004) Phantom encodes the 25-hydroxylase of *Drosophila melanogaster* and *Bombyx mori*: a P450 enzyme critical in ecdysone biosynthesis. Insect Biochem Mol Biol 34(9):991–1010

Xiong Y, Qu W, Liang J (2012) Progress on chemical constituents and biological activities of the genus *Ajuga*. Strait Pharm J 24(2):1–6

Yoshiyama T, Namiki T, Mita K, Kataoka H, Niwa R (2006) Neverland is an evolutionally conserved Rieske-domain protein that is essential for ecdysone synthesis and insect growth. Development 133(13):2565–2574

Yoshiyama-Yanagawa T, Enya S, Shimada-Niwa Y, Yaguchi S, Haramoto Y, Matsuya T, Shiomi K, Sasakura Y, Takahashi S, Asashima M (2011) The conserved rieske oxygenase DAF-36/neverland is a novel cholesterol-metabolizing enzyme. J Biol Chem 286(29):25756–25762

Zhao XJ, Li XC, Yu J, Chi DF (2011) Effects of growth regulator and culture methods on rooting of *Ajuga lobata* and content of β-ecdysone. Chin Tradit Herb Drugs 42(9):1828–1832