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ANALYSIS OF GENE EXPRESSION AND QUALITY OF AGARWOOD USING AGAR-BIT IN *AQUILARIA SINENSIS*

ZQ Wu[1], S Liu[2], JF Li[2], MC Li[3], HF Du[3], LK Qi[3] & L Lin[3, *]

[1]*College of Pharmacy, Xinxiang Medical University, Xinxiang, Henan 453003, People's Republic of China*
[2]*College of Sports and Health, Guangzhou University of Chinese Medicine, Guangzhou, Guangdong 510006, People's Republic of China*
[3]*College of Chinese Materia Medical, Guangzhou University of Chinese Medicine, Guangzhou, Guangdong 510006, People's Republic of China*

linlitcm@126.com

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Agarwood, precious resinous trunks or branches derived from *Aquilaria* plants, is widely used in incense and perfume. However, due to its low yield and high production cost, a novel stimulating method is required to produce agarwood rapidly and effectively. We studied the biologically agarwood-inducing technique (Agar-Bit) that achieved high yield and qualified production. In this study the gene expressions and chemical compositions in agarwood obtained from *Aquilaria sinensis* trees using Agar-Bit were characterised. Mechanically-stimulated agarwood was used as control. Some characteristic synthase genes expressed degrees and times differently compared with control. For Agar-Bit group expressions of biological synthase genes were upregulated in 12 hours, then downregulated except CHS1 (chalcone synthase genes) after stimulation. For mechanically-stimulated agarwood they were upregulated in 24 hours, then downregulated except HMGR (3-hydroxy-3-mythlglutaryl-CoA reductase). The chemical compositions of agarwood by Agar-Bit and control were investigated by total chromone and essential oil contents. Therefore, we concluded that Agar-Bit can markedly motivate some characteristic synthase gene expressions and produce unique and identical chromone chemicals in agarwood. These findings may supplement information on agarwood formation theories and provide genetic varieties during the process.

Keywords: Stimulating method, biological synthase genes, chromones, essential oil

INTRODUCTION

Agarwood, precious resinous trunks or branches derived from *Aquilaria* plants, is widely used in perfume, incense and traditional Chinese medicines. Agarwood also plays an important role in Chinese traditional medicine for use as sedative, carminative and to relieve gastric problems, coughs, rheumatism and high fever (Liu et al. 2013). However agarwood is obtained only from wounded trees. That means a healthy *Aquilaria* tree is worthless, while highly qualified agarwood from wounded trees can be worth almost USD100,000 kg^{-1} (Naef 2011). The high economic value and heavy demand for agarwood products have resulted in over-exploitation and habitat loss of *Aquilaria* spp. and eventually causing their population to decline to the point where they are categorised as threatened. Since 2005, all *Aquilaria* species have been listed in the Appendix II of the CITES. Great efforts have been made to preserve natural *Aquilaria* trees including cultivation of the species. Many *Aquilaria* plantation has been established in South-East Asia.

Aquilaria sinensis is the only certified source for agarwood listed in the *China Pharmacopoeia* (Anonymous 2015). Almost 20 million *A. sinensis* trees grow in Hainan, Guangdong and Yunnan provinces, and more than one-fourth of them are more than 5 years old, providing abundant resources for agarwood production. There are many methods to stimulate yield of agarwood from *Aquilaria* trees but they cost time and money which do not commensurate the low yields. These methods include partial trunk prunning (Liu et al. 2013), hammering of nails into tree trunks, burning-chisel-drilling, fungi-inoculation, wounding or rotting parts of trunk and microbial invasion. Natural events such as lightning strike, animal grazing and insect attack can also induce agarwood but yield is extremely low. The process

of agarwood formation takes at least 3 or 4 years to get the general quality agarwood. For high quality agarwood, the process takes longer, i.e. 10 years or more. In view of the low yield and high resources, more efficient and convenient techniques for inducing qualified agarwood for wide use are required. Towards this end, several new methods have been developed such as cultivated agarwood kits (CA-Kits) and the whole-tree agarwood inducing technique (Agar-Wit). CA-Kits, a chemical treatment, provide easy evaluation of discoloration area in the heartwood (Blanchette & Heuveling 2009). The yield and quality of this method were satisfactory, but the procedures were somehow traditional. Agar-Wit, combining chemical stimulation with water transportation, forms an overall wound (i.e. from the root to the top of the tree) in the tree and agarwood is developed around the wound not long after that (Liu et al. 2013). However, this method needs improvement in reducing decay throughout the tree. Decayed wood contain chromones, a specific type of compounds in agarwood, but the quality is not certain because the analyses used for identification are not elaborated (Mei et al. 2013, Liao G et al. 2016).

Two main kinds of chemicals found in agarwood are the essential oil and 2-(2-phenylethyl)chromones. The essential oil consists of characteristic sesquiterpenes and armonic chemicals, which have sedative and analgesic effects (Okugawa et al. 1996, 2000). More than 70 sesquiterpenes and 60 2-(2-phenylethyl)chromones have been isolated from agarwood (Espinoza et al. 2014). The determination of their concentrations is regarded to be an effective method in quality control of agarwood. GC-MS, HPLC, LC-MS and time-of-flight mass spectrometry have been applied in distinguishing qualified agarwood. Results revealed that the analysis for contents of total chromones of 2-(2-phenylethyl)chromones were promising to distinguish cultivated from wild harvested agarwood (Espinoza et al. 2014).

In addition to determining the mechanism of agarwood formation it is also important to understand the biosynthesis and regulation of sesquiterpenes and chromone in *Aquilaria* spp. Unfortunately, the biosynthesis pathway of chromone derivatives is still unknown, but similar structure of mother nucleus of flavonoids can provide comparative information to some extent. The biosynthesis of sesquiterpenes can occur via the mevalonic acid (Gardner & Hampton et al. 1999) and 1-deoxy-d-xylulose-5-phosphate synthase (DXS1) (Rohmer 1999) pathways. Resin and terpenoids in other plants are produced in response to biotic and abiotic stress (Bohlmann et al. 1998, Martin et al. 2002). Little information is available on the biosynthesis of fragrant compounds at the molecular level because all agarwood-producing trees require considerable time to grow. As the resinous portions are in the heartwood, the cells that contain relevant enzymes are quite difficult to obtain. In order to uncover the mechanism of agarwood production, it is necessary to study the stress response of *Aquilaria* trees especially in the variation of gene expression level after stimulations. The applications of RNA-seq and digital gene expression profiling (DGE) in *Aquilaria* spp. provide huge molecular information in biosynthesis pathways. Transcriptome library was established between healthy and wounded *A. sinensis* in different times, and the relevant genes involving signal transduction were selected. Currently, more than 20 relevant genes have been cloned in *A. sinensis* from the GeneBank. They play vital roles in the syntheses of key enzymes in the pathways of sesquiterpenes and flavonoids.

For gene expression analysis at the transcriptional level, northern blotting and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) are two most commonly used approaches with high accuracy and specificity. The latter, with its superiority in speed, sensitivity, reproducibility and wide dynamic range, is becoming the method of choice (Van Guilder et al. 2008). The results of gene expression analysis are highly dependent on the choice of reference genes.

Various hypotheses have been discussed regarding agarwood formation. Autoinducers such as methyl jasmonate, salicylic acid and yeast extract induce agarwood formation. Fungi, chemicals, physical wounds are considered as essential reasons or inducers to explain the molecular mechanism for agarwood formation. In this study, we evaluated a novel method that can induce *A. sinensis* to produce agarwood within 6 months (Lin et al. 2015) and revealed the mechanism involved. Gene expression in sesquiterpenes and flavonoids biosynthesis pathways were observed using qRT-PCR. The agarwood quality was evaluated by total chromone and essential oil contents.

MATERIALS AND METHODS

Plant materials and chemicals

Five-year-old *A. sinensis* trees cultivated in Huizhou City, Guangdong Province, China, were used for Agar-Bit and wound treatments. For Agar-Bit, liquid reagents including plant hormones, penetration enhancers and bacteria extractives (100 mL) were injected into the stems of the tree 30 cm above the ground. It is assumed that the reagents would be transported to the whole tree due to water transport thus forming a larger scale of injury in the tree than the wounded ones. Physical treatment was carried out 30 min after injection. Physical stimulated trees samples were taken as negative control. These were not injected with reagents and samples of their heartwood were collected with a chisel at varying times (0, 3, 12, 24 and 48 hours) after injury for analysis of gene expression. Genetic samples were taken from the xylem at 1.5 to 2.0 cm depth and stored in liquid nitrogen immediately. Six months after stimulation, discoloured parts in the centre of the stem were collected and compared with natural agarwood for quality analysis. Cultivated *A. sinensis* trees and natural agarwood purchased from Guangdong Qingping Market were identified by CM Pan from the Guangzhou University of Chinese Medicine, Guangzhou, China. Specimens (voucher number CX20140122) were deposited at the Experimental Management Center, Chinese Medicine College. Plant materials of treatment were shown in Figure 1.

Gene expression

Total RNA isolation and cDNA synthesis

Total RNA was extracted from all samples using the optimised method by Gao (2012). The

Figure 1 (a) Agar-Bit method and sample collection and (b) formation of agarwood in *Aquilaria sinensis* stems after 6 months

quantity and quality of each RNA sample were examined by a microplate spectrophotometer to confirm that the OD260/OD280 value was between 1.8 and 2.2. RNA integrity was measured by 1% agarose gel electrophoresis. An amount of 5 µL RNA for each sample was used in the 20 µL reverse-transcription reaction system with PrimeScript™ RT reagent Kit and gDNA Eraser following the manufacturer's instruction. The cDNA was diluted with water at 1:10 for use in real-time PCR.

PCR primer design

A total of seven genes of synthetase that were selected from the National Center for Biotechnology Information nucleotide of *A. sinenesis* were used for primer design. The sequence of farnesyldiphosphate synthase (FPS) was designed according to previously cloned gene in *A. sinenesis*. Other primers were designed with Primer Primer 5.0 software from genomic BLAST databases. Sequences of histone primers were obtained from Kumeta and Ito (2010). Detailed information of these genes is described in Table 1.

Real-time PCR

Real-time PCR reaction was performed using SYBR® Premix Ex Taq™ and run on 96-wells plates with the thermocycler. A 2-µL template cDNA diluted with water at 1:5 from the reverse-transcripted DNA was added to the 10 µL PCR reaction mixture containing 0.8 µL of each primer. Double-distilled water was used to compensate the reaction system to a final volume of 20 µL. PCR was initiated with 30 s incubation at 95 °C and followed by 40 cycles of 95 °C for 5 s and 55 °C to 60 °C for 30 s and 72 °C for 30 s. qRT-PCR reactions for evaluation of all genes were carried out in biological duplicate. Each PCR was repeated three times. The specificity of the qRT-PCR reaction was determined by melt curve analysis of the amplified products with heating procedure from 55 to 65 °C in 0.5 °C steps. Negative controls with water instead of cDNA were included for each gene target under assay. Standard curves of histone and target gene were obtained after the PCR procedures by the determination of gradient dilution from original sum of cDNA at all times.

Data analysis

Raw expression data were analysed by Bio-Rad CFX Manager Software version 2.0. All amplification plots were analysed with a threshold fluorescence value of 0.1 to obtain cycle threshold (Ct) values. The expression levels were evaluated by $2^{-\Delta\Delta Ct}$ method and analysed using Excel 2010.

Table 1 Primer sequences for real-time polymerase chain reaction in *Aquilaria sinensis*

Gene bank registration ID	Gene name	Primer sequence	Length (bp)
KF135951.1	TPS	F:5'-TGCATGAGGCTGCATGTGTA-3' F:5'-AGCCTTGGGATGTTCTTCGG-3'	172
JQ990217.1	HMGR	F:5'-GCCGTTCCATCTTACTCGCTG-3' R:5'-CATCTCACAGCACTGACCCAAT-3'	159
-	FPS	R:5'-GCCAATGGTGATCCCCAGAA-3' F:5'-AGGATAACGCAGAAAATGTAGCC-3' R:5'-CACTGATTGAACCGCTTTGCT-3'	145
JX860326.1	DXS1	F:5'-TCATGCCAATCCTGCAACCT-3' R:5'-GCTCTCCCTGATAGTGACGC-3'	156
JQ712682.1	ASS1	F:5'-CCGTTTGGTTTCGGTTGCTT-3' R:5'-AGTCCTCGCATCCTCAGTCT-3'	107
EF103196.1	CHS1	F:5'-AAGAATCCCGCCATTGCTGA-3' R:5'-ACTCCTTGATGGCCTTGTCG-3'	115
KF134785.1	CHI1	F:5'-CACCGATGCTGAAGCCAAAG-3' R:5'-CTGCCTTCCCAACTTCAGGT-3'	158

TPS = terpene synthase, HMGR = HMG-CoA reductase, FPS = farnesyldiphosphate synthase, DXS1 = 1-deoxy-d-xylulose-5-phosphate synthase, ASS1 = sesquiterpene synthases, CHI1 = chalcone isomerase 1, CHS1 = chalcone synthase

Essential oil content

Samples were powdered, passed through 20-mesh sieve and their moisture contents determined using toluene method according to *China Pharmacopeia* (Liu et al. 2013). Then samples (10 g) were soaked in diethyl ether overnight and filtered twice at room temperature (25 °C). The filter liquor was collected and volatilised to concrete at room temperature. Methylene dichloride was added as the solvent and left standing for 48 hours. The mixture was filtered, volatilised and weighed. The remaining liquor was dried over hydrous sodium sulfate and stored in a freezer at -20 °C until analysis. Essential oil contents were calculated by the weight of oil extraction in crude material (w/w%).

Total chromone content

Aquilarone E as the reference compound was accurately weighed and dissolved in methanol and then diluted to 1.518 mg L^{-1}. The stock and working standard solutions were stored at 4 °C until used for analysis. Coarsely powdered dried sample (0.1 g) was heated twice at 80 °C with 85% methanol (20 mL) for 1.5 hours. The resultant suspension was cooled to room temperature. Methanol was added to the original weight extract to keep the total liquor 50 mL. An amount of 0.5 mL of the liquor was added to scaled test tubes with H_2O_2 (1 mL) and sodium carbonate-sodium bicarbonate buffer (pH 11.4, 2mL). Phosphomolybdiumtungstic acid (0.5 mL) was added after being left standing for 30 min. Sodium carbonate (5%, 1.5 mL) was added into the liquor after 5 min. After 10 min, the solution was heated at 50 °C for 5 min and cooled to room temperature. The final solution underwent full wave scanning at wavelength 760 nm (maximum absorption) using UV-Vis spectrophotometry.

RESULTS AND DISCUSSION

Gene expression

RNA isolation

High-quality RNA was obtained through the protocol described previously. RNA integrity was assessed by the sharpness of ribosomal RNA (rRNA) bands visualised on a denaturing 1.2% agarose gel. For all RNA samples tested, well-resolved 28S and 18S rRNA bands were observed with no visible signs of degradation (Figure 2) of gDNA bands. The elimination of gDNA was a precondition for further molecular analysis. The A260/A280 ratios ranged from 1.90 to 1.99 (average = 1.94, coefficient of variation (CV) = 1%), indicating low or no protein contamination. The yield of total RNA was ~22–342 μg g^{-1} FW. For the whole set of samples extracted, the average yield was 310 μg RNA g^{-1} FW with a CV of 21%. The A260/A230 ratio was greater than 2.0 for all of samples (average = 2.3, CV = 6.1%). Overall, these data demonstrated that the extraction protocol described here was efficient in yielding high quality, integrity and quantity of total RNA from *A. sinensis* stems.

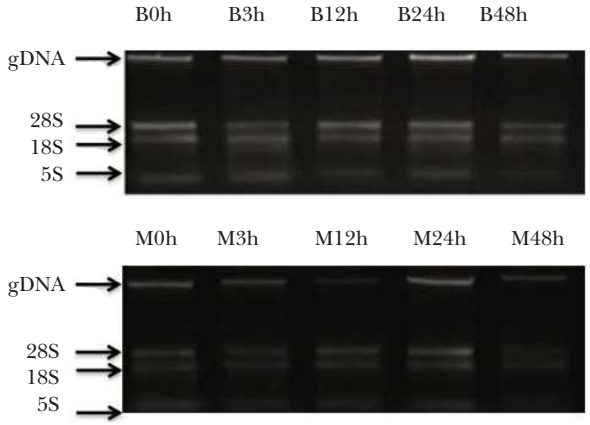

Figure 2 Total RNA from *Aquilaria sinensis* stems at different times, separated on an agarose gel (1.2%). B 0h, B 3h, B 12h, B 24h, B 48h stand for samples in Agar-Bit group at 0, 3, 12, 24 and 48 hours; M 0h, M 3h, M 12h, M 24h, M 48h stand for samples in mechanically stimulated group at 0, 3, 12, 24 and 48 hours

Gene expression

Gene expression was monitored using qRT-PCR with histone as the reference gene. Melting curve analysis of the amplification products confirmed that the primers amplified a single product. A fivefold dilution series of one of the samples was used to prepare a standard curve from which primer efficiency was calculated using the formula $E = 10^{-1/SLOPE}$. Proper temperature was selected for different genes. The results were shown in Table 2.

After the establishment of optimal qRT-PCR conditions, all samples from different groups at

Table 2 Amplification efficiencies (E), melting temperature (T_m) and regression coefficient measured from different genes

	TPS	HMGR	FPS	ASS1	DXS1	CHS1	CHI1	Histone		
T_m (°C)	59	61.4	61.4	64.5	64.5	61.4	64.5	59	61.4	64.5
E(%)	89.1	98.7	91.3	101.9	104.4	98.4	101.4	88.4	88.7	109.8
Slope	0.991	0.997	0.998	0.996	0.997	0.999	0.999	0.999	1.000	0.998
Regression coefficient	-3.615	-3.352	-3.549	-3.278	-3.221	-3.360	-3.290	-3.637	3.661	-3.108

TPS = terpene synthase, HMGR = HMG-CoA reductase, FPS = farnesyldiphosphate synthase, ASS1 = sesquiterpene synthases, DXS1 = 1-deoxy-d-xylulose-5-phosphate synthase, CHI1 = chalcone isomerase 1, CHS1 = chalcone synthase

different times were analysed using real-time qPCR system for gene expression with control (at 0 hour). Results are shown in Figures 3 and 4. DXS1, HMGR and FPS were the synthetase genes in upstream of sesquiterpenes pathway. These three genes upregulated after stimulations and at 3 hours their expressions reached maximum except for FPS which was expressed greatest at 12 hours. After 24 hours the level of three gene expressions in both groups returned to normal. Of the three, the upregulation ranges of HMGR were the largest in both groups. Moreover at 24 and 48 hours the genes in Agar-Bit group maintained high levels of expression.

ASS1 (sesquiterpene synthases) and TPS (terpene synthase) are quite specific in sesquiterpenes pathway. Results demonstrated that expression of ASS1 obviously increased after stimulation, including the maximal expression at 3 hours in Agar-Bit group and 12 hours in mechanical group, and turned back to normal after 24 hours. Expression of TPS varied at different stimulations. After mechanical stimulation its expression raised to the top which was almost 80 times more than the original value at 24 hours. In the Agar-Bit group, expression was 6 times higher at 3 hours and 10 times at 48 hours (Figure 3).

CHS1 (chalcone synthase) and CHI1 (chalcone isomerase 1) are key synthetase genes in flavonoid pathway. The expression of CHS1 climbed to the top at 48 hours in the Agar-Bit group which was much higher than the mechanical group. Maximum expression of CHI1 was at 24 hours in the mechanical group which was almost 10 times higher than the rest. Expression of CHI1 in the biophysical group reached maximum at 3 hours, which was similar to CHS1 (Figure 4).

More genes of synthetase in terpenes pathway of *A. sinensis* were found according to the GeneBank. They were TPS, HMGR, FPS, ASS1 and DXS1. TPS family in plants encodes enzymes that use similar substrates and give similar products, but have clearly diverged in different lineages (Bohlmann et al. 1998). FPS is one of the key rate- limiting enzymes in sesquiterpene metabolic pathways. Tissue expression analysis indicated that FPS gene in *A. sinensis* was mainly expressed in root and stem and induced by mechanical wound as well as chemical liquid induction. Expressions of HMGR and DXS1 in the Agar-Bit and mechanical groups increased to maximum at 3 hours. HMGR was strongly expressed in both groups which revealed that HMGR is the key gene in terpene pathways and played an important role in agarwood formation, which is consistent with the previous study by Xu et al. (2014). DXS1 in Agar-Bit group was expressed quite differently whereby its expression was twice that of the mechanical group (Figure 3), indicating the different genetic motivating mechanism in former group. Both Agar-Bit and mechanical stimulation induced the expression of FPS and in the former, the expression reached maximum at 12 hours and in the latter group, 3 hours. This result was in agreement with the expression of FPS in *A. sinensis* induced by mechanical wound as well as chemical liquid induction (Yang et al. 2013). Expression of ASS1 showed obvious distinction between the two groups. In the Agar-Bit group, highest ASS1 was expressed at 3 hours, while in the mechanichal group it was at 12 hours. The expression level in the former group was much higher than the latter. These results demonstrated that ASS1 was expressed in very different mechanism in time and intensity after biophysical stimulation. In the methyl jasmonate-treated calluses of *A.*

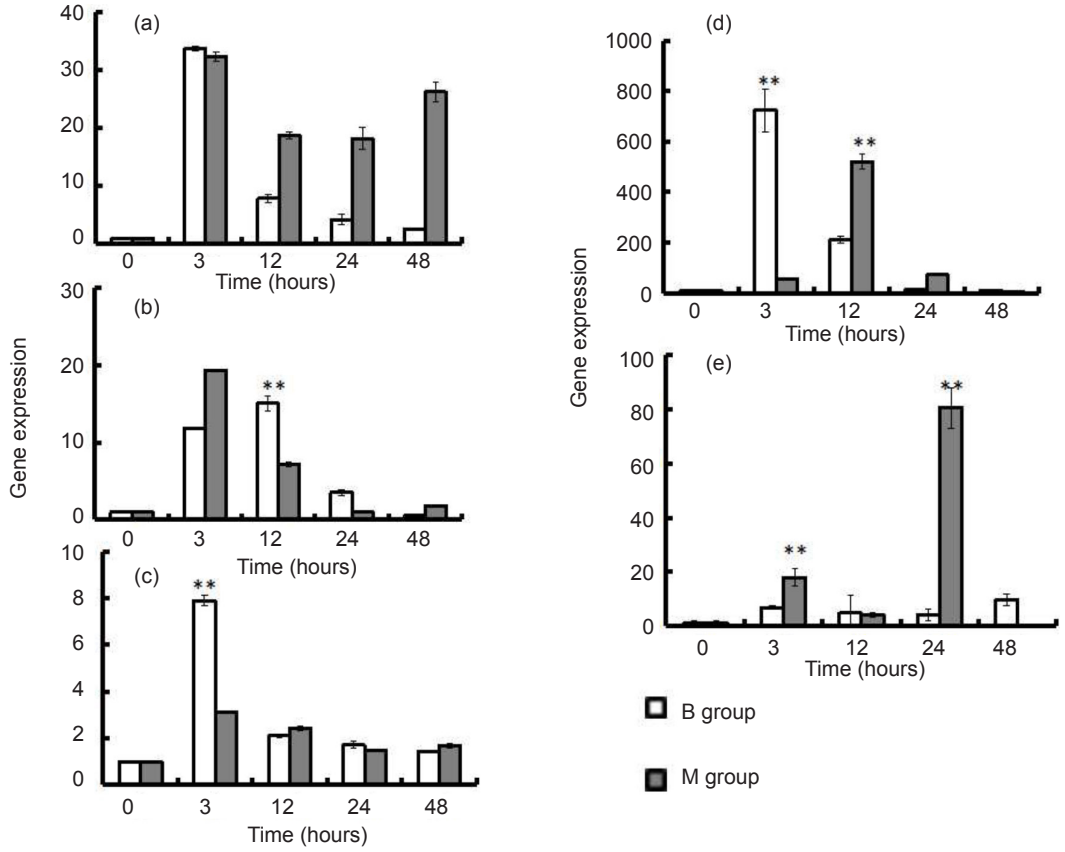

Figure 3 Gene expressions of sesquiterpenes synthetase in Agar-Bit (B) and mechanical (M) groups at different times compared with histone for (a) HMGR, (b) FPS, (c) DXS1, (d) ASS1 and (e) TPS; values are means ± standard deviations (n = 3),** = $p < 0.01$

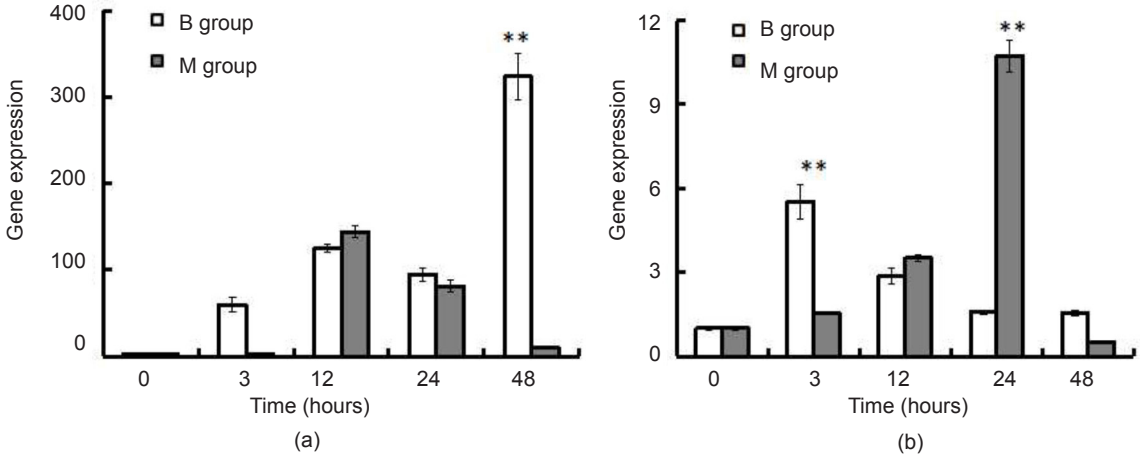

Figure 4 Gene expressions of flavoniod synthetases in Agar-Bit (B) and mechanical (M) groups at different times compared with histone for (a) CHS1 and (b) CHI1; values are means ± standard deviations (n = 3), ** = $p < 0.01$

sinensis the expression of ASS1 was significantly upregulated approximately 800 times in response to mechanical wounding and 1000 times in response to methyl jasmonate treatment (Xu et al. 2013). In the stem of *A. sinensis* ASS1 is more sensitive when stimulations were carried out in 3-year-old trees. The results demonstrated that inducible expression of ASS was responsible for the formation of agarwood sesquiterpenes in *A. sinensis*. For TPS the expression in Agar-bit group showed no significant intensity. However, in the mechanical group, TPS expressed almost

80 times more than control at 24 hours after mechanical stimulation. In the mechanical group CHS1 was expressed to the maximum at 48 hours and the expressive level was twice that of the Agar-Bit group (highest at 12 hours). Expression of CHI1 in the mechanical group was high at 3 hours but still lower than the value at 24 hours for the Agar-Bit group. In general the genes involved expressed differently in degree and time after different stimulations. Most genes except for TPS were strongly upregulated by biophysical treatment 48 hours after stimulation, especially DXS1, ASS1 and CHS1 which expressed quicker and at higher levels compared with the mechanical group. Different treatments induced different expressions of sesquiterpenes and flavonoids synthesis genes. When *A. sinensis* trees were treated by burning nails method, physical and low/high temperature treatments showed different gene expressions after 2 hours (Gao et al. 2013). Expression levels in this study was within 48 hours. Unlike other artificial methods, Agar-Bit can stimulate the main synthesis of compounds at gene levels.

Essential oil content

Average moisture contents of all samples from Agar-Bit, mechanical and natural groups were 4.86, 5.75 and 6.35% respectively (Figure 5). The yield of essential oils obtained after diethyl ether extract of Agar-Bit, mechanical and natural groups are shown in Figure 4. Agarwood from Agar-Bit group contained more essential oil than the natural group by cold-maceration, indicating higher oil productivity. Many essential oil extraction methods have been applied in agarwood production including steam distillation and solvent extracts (Chen et al. 2011). Diethyl ether was a common solvent in agarwood essential oil extraction (Mei et al. 2013) especially for further analysis by GC-MS. The solvent can extract main sesquiterpenes and chromones of agarwood and essential oils content obtained can be a simple parameter for agarwood quality control. Essential oils content of Agar-Bit group was similar to that reported by Liao et al. (2016) who also used diethyl ether extraction but was much higher than that obtained by Liu et al. (2013) using Agar-Wit steam distillation.

Total chromone analysis

Chromone only occurs in the resin-filled reaction wood and this is unique to agarwood (Chen et al. 2012). Compared with sesquiterpenes in agarwood, chromone was easier to identify. Colorimetric method in this study can determine the total content of chromone which is simple and stable for the quality control of agarwood. Total chromone contents of Agar-Bit were much higher than those of mechanical group, but similar to natural group. The content of chromone in mechanical group is almost the same as in healthy white wood (Figure 4) . Based on total chromone content, samples from Agar-Bit were more qualified than those from mechanical group.

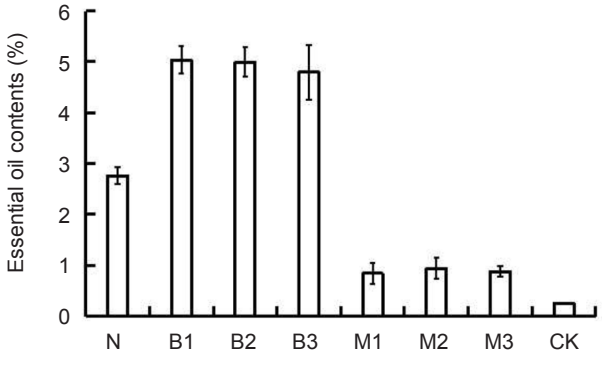

Figure 5 Essential oil contents in *A. sinensis* trees; B1–3 = different trees by Agar-Bit and M1–3 = different trees by mechanically stimulating method, N = natural, CK = control; values are means ± standard deviations (n = 3)

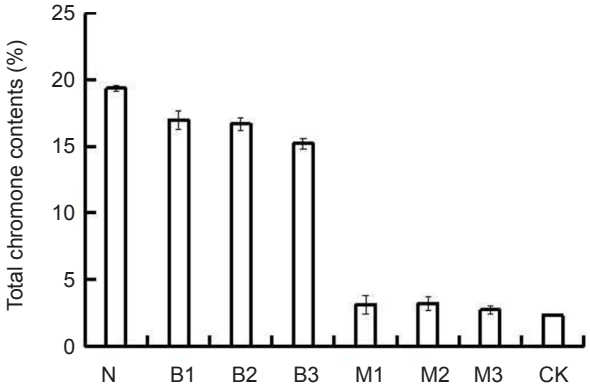

Figure 6 Total chromone contents in *A. sinensis* trees; B1–3 = different trees by Agar-Bit, M1–3 = different trees by mechanically stimulating method, N = natural, CK = control; values are means ± standard deviations

CONCLUSIONS

In this study gene expressions of key synthetases in pathway of sesquiterpenes and chalcone-related enzymes that varied at different times and levels were determined after both biophysical and mechanical stimulation of *A. sinensis* trees. The genetic activation or inhibition of these synthetases was analysed by comparison of the two treated groups. Agar-Bit group showed an apparent response to the stimulation as some genes expressed intensely and quickly. In addition the results of chemical comparisons of essential oil and total chromones contents showed that Agar-Bit had superiority in producing agarwood and characteristic compounds could be detected 6 months after stimulation. This study has proven that, from genetical and chemical aspects, Agar-Bit is a novel option for production of agarwood.

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