



Cell suspension culture of *Rhizoma zedoariae* in a two-stage perfusion bioreactor system for β -elemene production

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Abstract

We presented a two-stage combined bioreactor system consisting of a stir-tank and an airlift column, and challenged with *Rhizoma zedoariae* cell suspensions for β -elemene production. Two-stage culture was initiated when the cell concentration in both vessels was maintained at an appropriate density. The cells were proliferated in stirred-tank with the maximal growth rate of 0.17 d^{-1} to present enough cells for β -elemene synthesis. In the airlift column, continuous cell separation from culture medium was achieved by using a cell retention device based on centrifugal and gravity settling when the system was performed in perfusion mode. The results indicated that additives can efficiently promote the accumulation of β -elemene in *R. zedoariae* cells. In addition, the β -elemene content showed higher levels in cell lines of overexpressing 3-hydroxy-3-methylglutaryl coenzyme-A reductase, Farnesyldi phosphate synthase, and *STO2C* genes.

Keywords β -Elemene · Bioreactor · Cell culture · *Rhizoma zedoariae* · Secondary metabolism

Introduction

β -Elemene is one of the sesquiterpenes used for the treatment of ovarian, breast, and lung cancers (Zhao et al. 2015). Presently, the raw material of β -elemene is mainly extracted from *Rhizoma zedoariae*, one of the Traditional Chinese Medicines, collected from farms (Zhou et al. 2016). Field cultivation is time-consuming and easily interfered by climate. Therefore, cell-suspension culture was considered as a promising way to produce this valuable pharmaceutical metabolite.

Much progress has been made in terpenoid synthesis (Fig. 1). Isopentenyl pyrophosphate (IPP) is a common precursor of terpenoid synthesis. 3-hydroxy-3-methylglutaryl coenzyme-A reductase (*HMGR*) and 1-Deoxy-D-xylulose 5-phosphate (*DXS*) are two rate-limiting enzymes of the mevalonic acid pathway (MVA) and mevalonate pathway (MEP) respectively for IPP biosynthesis (Liu et al. 2005; Aquil et al. 2009). Farnesyldiphosphate synthase (*FDS*) catalyzed the synthesis of

fluorinated polypropylene (FPP) to increase the metabolic flux towards sesquiterpenes (Ping et al. 2012). FPP can be converted into germacrene A with one-step synthesis, and germacrene A was further converted to β -elemene at high temperature without any biochemical catalysts (Leila et al. 2015).

Secondary metabolites are important defensive substances released by plants in response to adverse environmental stresses or attacking of pests and pathogens (Duan et al. 2013). Studies have shown that conditions suitable for plant cell proliferation often reduce the synthesis of secondary metabolites (Qi et al. 2014). Thus, it is a challenge to increase both biomass and target substance accumulation in plant cell suspensions in one bioreactor system.

Two-stage cultivation of plant cell refers to the culture procedures, which can be divided into two different stages according to the dyssynchrony of cell proliferation and secondary metabolites synthesis (Marisol et al. 2016). For a traditional two-stage run, cell proliferation and target substance synthesis usually proceed in different bioreactor systems, which increases the risk of contamination during cell transfer between bioreactors. Furthermore, it takes time for cells to adapt to a new growth environment.

When cell suspensions are cultivated in bioreactor, target metabolites released in the nutrient solution can be degraded or used to synthesize new compounds (Wang et al. 2010). Moreover, the synthesis of target substances can be disturbed

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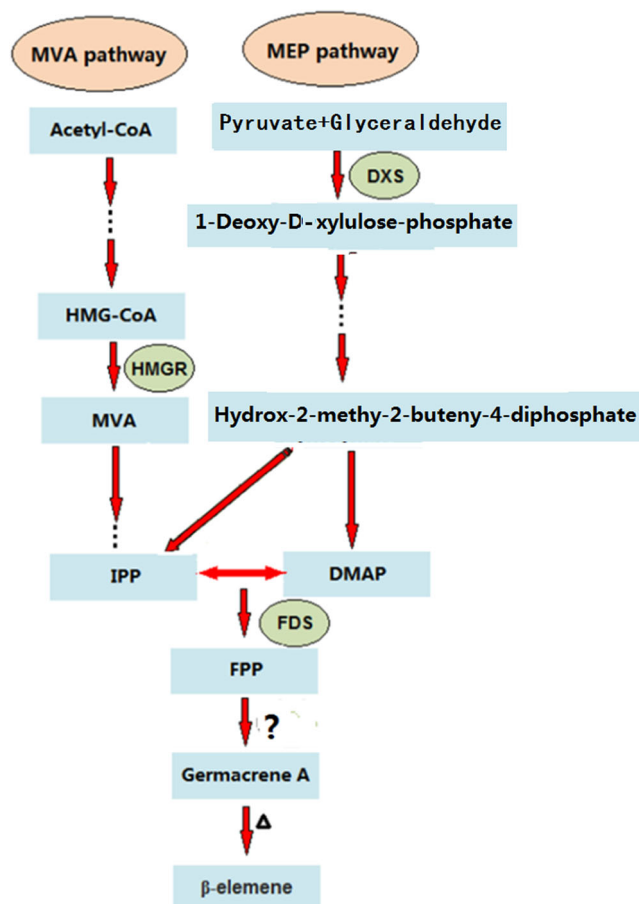


Figure 1. Biosynthetic pathway of β -elemene in plant.

due to feedback inhibition (Wang et al. 2015). Our early study (Wang and Qi 2009) also indicated that parts of *in vitro* plant cells were subjected to lethal browning due to oxidized phenolic compounds. Perfusion culture seems to have potential to decrease cell browning and feedback inhibition. Through constant medium replacement, the metabolites and other harmful substances are removed from the bioreactor, while additional nutrient materials are provided to cells.

For perfusion culture of plant suspension cells, continuous cell separation from the medium is also a challenge. *In situ* filtration has been applied to mammalian cell retention (De la Torre et al. 2010), but it has not been widely used in plant cells due to filter clogging at high cell densities. Gravitational sedimentation seems to be an alternative to retain cells in perfusion runs. But our studies (Wang and Qi, 2009; Wang et al. 2010) show that the cell retention efficiency based on the gravitational settling was less efficient at high cell density.

Retaining cells by centrifugal settling is likely to be effective especially for culture solutions with high viscosity (Christopher et al. 2009; Christopher et al. 2011); nevertheless, this is rarely used in plant cell culture. In this study, a combined bioreactor system consisting of a stir-tank and an airlift reaction column was used to conduct the two-stage perfusion

cultivation of *R. zedoariae* suspension cells in one bioreactor system. Cell proliferation and β -elemene accumulation was achieved simultaneously in one bioreactor. The main components of the cell retention devices were a spiral-type centrifugal settling pipe and a gravity settling pipe with which we accomplished continuous cell separation from culture medium. In addition, the factors influencing cell retention, mass transfer, β -elemene synthesis, and cell growth were also investigated.

Materials and methods

Cell culture and maintenance The tubers of *R. zedoariae* were sliced and then transferred to solid Murashige & Skoog medium to induce callus with 2 mg L^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.2 mg L^{-1} 6-benzylaminopurine (6-BA). The suspension cell lines were obtained when these calluses were transferred to liquid medium containing 28 g L^{-1} sucrose, 0.5 mg L^{-1} 6-BA, 1.0 mg L^{-1} naphthylacetic acid (NAA), 1.0 mg L^{-1} 2,4-D, and kept at a rotational speed of 110 g. The cell cultures were sub-cultured each 2 wk with shaking at 120 g. All cultures were kept at 25°C in the dark.

Bioreactor configuration and perfusion culture A schematic representation of the bioreactor system is shown in Fig. 2. This bioreactor system includes a stir-tank and airlift bioreactor with the total working volume of 2 L. The stirred tank and airlift column were connected by a glass pipe. The medium inlet in the airlift column was higher than liquid surface, which made the two reaction vessels relatively independent of each other. The stir-tank bioreactor with a working volume of 1 L was used in the first stage for cell growth and division. The

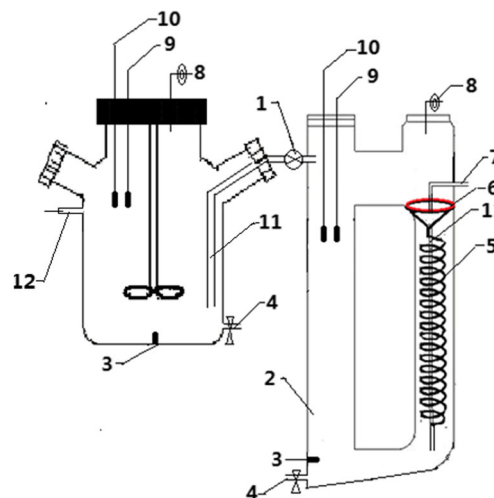


Figure 2. Schematic representation of the two-stage bioreactor system. 1, valve. 2, upstream zone. 3, air sparger. 4, sampling port. 5, spiral-type centrifugal settling pipe. 6, liquid inlet of downstream zone. 7, outlet of discarded medium. 8, air filter. 9, pH probe. 10, dissolved oxygen probe. 11, gravity settling pipe. 12, inlet of fresh medium.

connected pipe also served as a gravitational settling device for primary cells retention when the system was run in perfusion mode. The external loop air-lift reactor with a working volume of 1 L was used in the second stage for secondary metabolites accumulation. An important component of this system is the cell retention device that positioned in the air-lift reactor includes a spiral-type centrifugal settling pipe and a gravity settling pipe with the diameter of 2 cm, through which we achieved continuous cell separation from culture medium. A peristaltic liquid pump controlled the perfusion medium feed rate, and a microporous glass flake embedded in the pipe acted as a gas sparger. The liquid was rotated clockwise once gas was ventilated into the airlift reactor *via* the gas sparger located in the riser region. A microprocessor was used to monitor dissolved oxygen (DO) levels and pH.

Bioreactor cultivation was also conducted using the same culture media ingredients in Erlenmeyer flasks. Cell suspensions were transferred from shake flasks into the bioreactor and inoculated at an initial cell density of 5 g L⁻¹ dry weight. The perfusion rates were 5–20% (v/v) every day and the aeration rates were set at 0.1–0.5 air volume/culture volume/min (v/v/m). A batch mode was performed as well under the same conditions as those in the perfusion mode with no exchange of culture medium.

Determination of cell growth rate and cell retention efficiency

In order to remove the residual sugar and other impurities from cells, the cell samples from bioreactor were filtered on filter paper under vacuum and then washed in distilled water three times. The filtered cells were weighed in pre-weighted dishes to obtain their fresh weight (FW) and the dry weight (DW) after drying at 40°C for 3 d. The average cell growth rate was calculated as the formula as follows:

(Maximum dry cell weight-Initial dry cell weight) / (Initial dry cell weight × Δt)

where Δt refers to the cultivation period during which the maximum dry cell mass was obtained (Wang and Qi 2009). The cell retention efficiency referred to the cell loss rate that was extracted from the discarded medium.

Determination of the volumetric oxygen mass transfer coefficient (k_La)

The k_La was measured by the dynamic gassing-in and gassing-out method in distilled water (Wang et al. 2015) at different aeration and stirring rates. O₂ gas was degassed by feeding N₂ gas that fed at the same flow rate as air. The value of k_La was defined as follows:

$$\ln \frac{c^*}{c^*-c} = k_L a \cdot t$$

where c* is the oxygen solubility in the liquid (mg L⁻¹); and c is the oxygen concentration in the liquid (water) at time t (mg L⁻¹).

Determinating the content of β-elemene Dry cells were ground to fine powder in a mortar, and then the powder was filtered through a 40-mesh sieve. Ether was added to the filtered powder and extracted with sonication for 90 mins. HPLC analysis was performed on an Agilent1100 apparatus equipped with a chromatographic column of Kromasil C18 (4.6 mm × 250 mm × 5 μm). The temperature of column was 25°C and the mobile phase consisted of acetonitrile-water (65:35, v/v). The detection wavelength was 214 nm and the flow rate was 1.00 mL min⁻¹. The injection volume was 10 μl. β-Elemene were detected and quantified by comparison with authentic standard curves and retention times. The standard sample was 0.1 mg mL⁻¹ β-elemene solution (Sigma, Shanghai division). The regression equation of standard curves were β-elemene of Y = 2240.1 x + 21.907, r = 0.9999 respectively.

Quantitative PCR for HMGR, DXS, and FDS gene expression

Quantitative PCR (qPCR) was performed with a qPCR kit (TaKaRa, Shanghai, China) with SYBR Green PCR Master Mix. The results showed that methyl jasmonic acid induced β-elemene biosynthesis. Total RNA was extracted from common cells (control) and high β-elemene content cell lines (treated by jasmonic acid). The primer sequences for qPCR were showed in Table 1.

For quantitative PCR reaction, the total volume was 20 μL and the reaction was as follows: The cDNA was denatured at 94°C for 2 min followed by 45 cycles of amplification (15 s at 94°C; 15 s at 55°C; 30 s at 68°C) and actin was used as a reference gene. Fold increase/RQ was calculated using the formula RQ = 2^{-ΔΔCt}, where ΔCt = average Ct (gene of interest)–average Ct (Actin), and ΔΔCt = ΔCt – ΔCt (control of the experiment; cDNA of common cell suspensions). All experiments were designed with three biological replicates.

Cloning of HMGR, Germacrene A synthetase (GAS), and FDS genes

The complete sequences of *HMGR* and *FDS* genes were obtained in transcriptome database of *R. zedoariae*, and they were cloned by one-step RT-PCR kit (TaKaRa,

Table 1. The primer sequences for qPCR

Name of primers	Prime sequences
<i>HMGR</i> -Q-F	GTAAAGGGTGCGAACTTGGA-3'
<i>HMGR</i> -Q-R	AATCTTTGCTGGATCGGTTG-3'
<i>DXS</i> -Q-F	AATTCCTCATCACGGTGGAG-3'
<i>DXS</i> -Q-R	AATTCCTCATCACGGTGGAG-3';
<i>FDS</i> -Q-F	GGATCGAGAAGATGCTGGAC-3'
<i>FDS</i> -Q-R	CTAAACCAGCAAGGCTGACC-3'
<i>Actin</i> -Q-F-5'-	GAG TCATCTTCTCGCGTTTC-3
<i>Actin</i> -Q-R-5'-	CCACAAAGGTGTGATGGTTG-3'

Shanghai, China). Hyun and David (2012) characterized a terpene synthase named *ST02C* in ginger. The assay for *ST02C* with FPP as a substrate produced main substance of germacrene A (49.3%). There is only 49% homology between the *GAS* in *Artemisia annua* and *ST02C* in ginger. Though the whole sequences of *GAS* gene and *ST02C* gene were reported, we failed to clone them in *R. zedoariae* and cannot detect them in the two transcriptome database of *R. zedoariae* leaf and tuber. Hence, we cloned the *GAS* gene in *Artemisia annua* by RT-PCR and artificially synthesized the *ST02C* gene according to the amino acid sequences reported in the literature above. The primer sequences were showed in Table 2.

For each reaction, the total volume was 50 μL and the reaction was as follows: The cDNA was denatured at 94°C for 5 min followed by 35 cycles of amplification (1 min at 94°C; 1 min at 52–58°C; 1–1.5 min at 72°C) and the final incubation at 72°C was extended to 10 min.

Introducing *ST02C*, *HMGR*, *FDS*, and *GAS* genes into cells of *R. zedoariae* Four plasmids containing the cDNAs encoding *HMGR*, *FDS*, *GAS* as well as *ST02C* driven by CaMV35S promoter, were separately introduced into the *Agrobacterium* GV3101 strain. After infection with the *Agrobacterium* that contained target genes, the tuber explants were transferred to selective medium to obtain positive transformants of callus which was confirmed by PCR of target genes. The cells from positive callus were established as engineered cell strains named with different number.

Statistical analysis All the experiments were repeated three times. Mead SE are shown for biological triplicates. The statistical significance difference was analyzed by one sample t test and the errors were analyzed using the one-way analysis of variance (ANOVA) via SPSS11.5 software.

Results

Cell culture for increased biomass – Cell distribution in two reaction vessels at different cell concentration In order to accumulate sufficient cells to prepare for two-stage culture, the cells were inoculated into the reactor with valve 1 closed for biomass increase. The appropriate cultivation parameters for biomass increase were agitation rate 120 g and aeration rate 0.3 *vvm* in batch mode. When the cell concentration was allowed to rise to more than 20 g L^{-1} , the cell cultures in both culture vessels became very thick. A large portion of cells were deposited at bottom of the reactors, which were not conducive to mass transfer and cell growth. Therefore, we lowered the cell concentration to about 5 g L^{-1} , meanwhile, opened valve 1 to conducted perfusion run at various velocities of perfusion medium feeding and ventilating.

Table 2. The primer sequences for clone of *HMGR*, *FDS*, and *GAS* genes

Name of primers	Prime sequences
<i>HMGR</i> -f	ATGGCCATGGAACTCCGTCG TAG
<i>HMGR</i> -r	CAGGCCCTCCCGGTTGTCCGC
<i>FDS</i> -f	ATGGCGGAGGCGACGGCGAACG
<i>FDS</i> -r	TTACTTCTGCCTTTGTAGA
<i>GAS</i> -f	ATGGCAGCGGTTCAAGCTAATG
<i>GAS</i> -r	TTACACGGGTAGAGAACCCAC

The gravity settling pipe in the stirred tank was used to adjust the velocity of cell transferring from stirred tank to airlift column. At the initial stage, only a minority of cells transferred into the airlift reactor. But with cell density increasing, the gravity settling became less efficient and an increasing number of cells flowed into airlift reactor. Our early researches show that the cell sedimentation efficiency was positively related to diameter of gravity settling pipe and cell density (Wang and Qi 2009). In this study, restricted by the reactor geometric structure, the diameter of the gravity settling pipe was set as 2 cm. The distribution of cells in two reactors at different cell concentration was investigated. The results in Fig. 3, indicated that most of cells were retained in the stirred tank when the cell concentration was lower than about 15 g L^{-1} . But as cell density increased, obvious differences in cell density were detected between the two reaction vessels due to the cells transferring. Interestingly, the cell density in the stirred tank can be maintained at a relatively stable value of 18 g L^{-1} or so at the perfusion rate of 20%, and no obvious cell layer was observed at the bottom of the reactor. The cell concentration in the airlift column was also maintained at about 18 g L^{-1} by regular harvesting. Thus, the cell density in both reaction vessels was approximately similar and the two-stage cultivation mode was initiated.

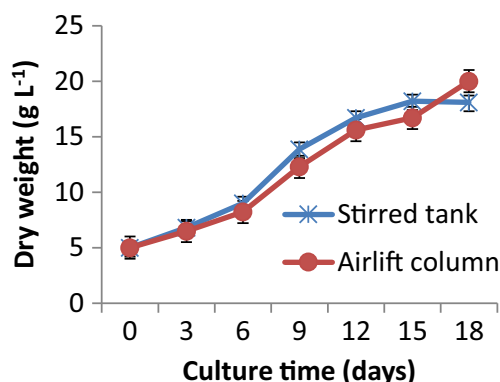


Figure 3. Cell distribution in two reaction vessels. Means \pm SE of triplicates are shown. For each compound columns with different letters are significantly different at $p < 0.05$ by ANOVA.

Table 3. Cell growth and retention under different culture conditions

No	Aeration Rate (vvm) A	Helix pitch (cm) B	Perfusion rate (%) C	Blank control	Growth rate (day ⁻¹)		Retention efficiency (mg L ⁻¹)
					Stirred-tank	Air-lift	
1	0.1	1	5	1	0.16 ± 0.02	0.08 ± 0.03	0.03 ± 0.01
2	0.1	2	10	2	0.16 ± 0.04	0.14 ± 0.03	0.06 ± 0.03
3	0.1	3	20	3	0.17 ± 0.06	0.13 ± 0.06	0.09 ± 0.02
4	<i>0.3</i>	<i>1</i>	<i>10</i>	<i>3</i>	<i>0.17 ± 0.03</i>	<i>0.15 ± 0.03</i>	<i>0.02 ± 0.03</i>
5	0.3	2	20	1	0.17 ± 0.02	0.16 ± 0.03	0.04 ± 0.07
6	0.3	3	5	2	0.16 ± 0.07	0.15 ± 0.02	0.08 ± 0.03
7	0.5	1	20	2	0.17 ± 0.02	0.12 ± 0.03	0.01 ± 0.03
8	0.5	2	5	3	0.16 ± 0.03	0.11 ± 0.02	0.04 ± 0.01
9	0.5	3	10	1	0.17 ± 0.03	0.15 ± 0.03	0.09 ± 0.04

The experiments were designed by using an orthogonal test of L₉ (3⁴). The data was collected within 15 d of perfusion cell culture with the inoculum density of 5 g L⁻¹ and the cells were not forced by additives or ultraviolet radiation

The conditions of No. 4 in italics are the most appropriate for cell growth and retention

Cell retention efficiency of the bioreactor system When perfusion mode was conducted, cell cultures were continuously separated from medium by settling device. The settling cells then mixed with the culture solution again that circulated clockwise in airlift column. Cell retention efficiency was determined by detecting the cell loss in discarded medium. Table 3 depicted the influence of perfusion rate, aeration rate and helix pitch of spiral tube on cell retention. The results suggested that the helix pitch of spiral pipe was a key factor for cell settling. The smaller was the helix pitch, the more efficient were cells separated from the medium. Ventilation

in the airlift column was another significant contributor to cell settling. Cell retention efficiency improved obviously with the increase of aeration rate, and only minority of the cells transferred to exhausted medium at aeration rate of 0.5 vvm. Table 3 also indicated that lower cell loss was detected when ventilation rate was 0.5 vvm and the helix pitch of spiral tube was 1 cm. The combination of high aeration rate and small helix pitch can improve the rotation speed of cell cultures in the spiral tube, which can be attributed to the increasing of centrifugal force. Nevertheless, excessive ventilating caused negative effect on cell growth and division in the airlift column (Table 3). Under the conditions of aeration rate 0.3 vvm, helix pitch 1 cm, and perfusion rate 10%, both cell retention and cell proliferation were kept at an appropriate levels of 0.16 ± 0.04 d⁻¹ (stirred-tank), 0.14 ± 0.03 d⁻¹ (air-lift), and 0.06 ± 0.03 mg L⁻¹ (Table 3). The cells escaping from centrifugal force were further retained by a gravity settling pipe connected to the base of helical pipe. In agreement with the previous experiments, high perfusion medium feed rate was a negative factor for cell settling (Wang and Qi 2009).

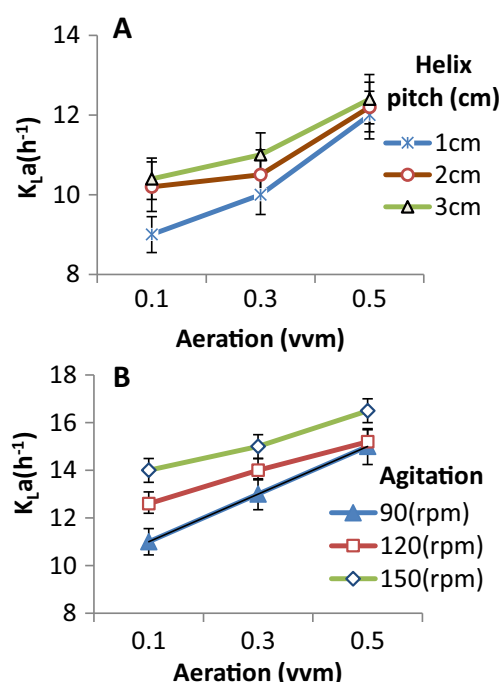


Figure 4. K_La of the bioreactor system. (A) Airlift column. (B) Stirred tank. Means ± SE of triplicates are shown. For each compound columns with different letters are significantly different at $p < 0.05$ by ANOVA.

Cell growth in the reactor system During the process of two-stage culture, the cells need to be proliferated continuously in the first stage to present enough cells for β-elemene synthesis in

Table 4. Influence of abiotic elicitation and precursor feeding on β-elemene accumulation

No.	Treatment	Cell status	β-Elemene (% dry wt.)
1	Control	Normal	0.142 ± 0.03
2	Salicylic acid	Brown	0.18 ± 0.03
3	Ethylene	Brown	0.21 ± 0.06
4	Methyl jasmonic acid	Normal	0.23 ± 0.02
5	FPP	Normal	0.23 ± 0.03
6	Ultraviolet irradiation	Normal	0.15 ± 0.02

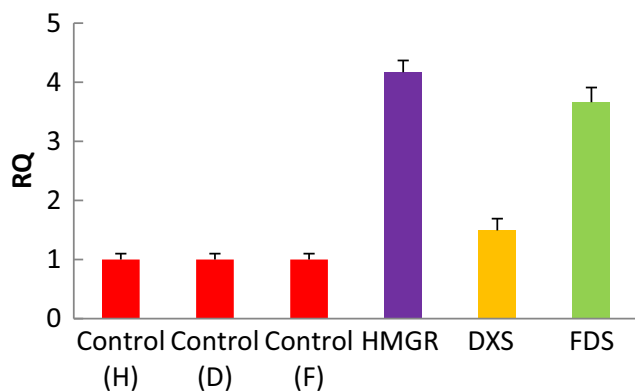


Figure 5. Expression patterns of *HMGR*, *DXS*, and *FDS* genes in suspension cells. Control (H), Control (D), and Control (F) represented the experiment control of *HMGR*, *DXS* and *FDS* gene respectively. Means \pm SE of triplicates are shown. For each compound, columns with different letters are significantly different at $p < 0.05$ by ANOVA.

the second stage. The cells tended to grow stably when the cell concentration was maintained to about 18 g L^{-1} in both vessels, and then the two-stage cultivation mode was performed with various factors of precursors, chemical additives and ultraviolet irradiation in airlift column. Before two-stage culture, the cell growth rate in the stirred tank was slightly higher than that in the airlift column (Table 3) due to different mass transfer efficiency between them (Fig. 4). However, when a two-stage run was started, the biomass of cells in airlift column did not increase because the additives and ultraviolet radiation made a relatively tough environment for cell growth and proliferation. In order to

optimize the culture conditions for two-stage culture, the influence of various operating parameters on cell growth was investigated. Table 3 indicated that all of the factors of perfusion rate, aeration rate, and helix pitch were relevant to cell growth. In the stirred tank, the perfusion medium feeding and aeration rates were important influences on cell growth though not prominent (data not shown). The optimum culture conditions was A2B1C2, under which the cell growth rate reached 0.17 d^{-1} and less cell loss was detected in discarded medium (Table 3). As mentioned above, the combination of high aeration rate and small helix pitch was not conducive to cell growth in airlift column due to the shear force generated by medium rotation. In addition, during the process of two-stage culture, more and more cells accumulated to the bottom of the airlift column for the reasons of cell sedimentation; hence, the redundant cells were continuously harvested and maintained the cell density at a relative stable value of approximately 18 g L^{-1} .

Cell culture for β -elemene synthesis – Abiotic elicitation and precursor feeding The anabolism of secondary metabolites in plants is influenced by various abiotic and biological stresses. It was reported that salicylic acid, ethylene, and methyl jasmonic acid could activate transcription factors in response to such stresses, which were involved in the synthesis of terpenes (Kai et al. 2004; Ana et al. 2014; Niels et al. 2015). Based on the previous studies, the following experimental strategy was designed to enhance the flux towards β -elemene synthesis in *R. zedoariae* cells. Salicylic acid,

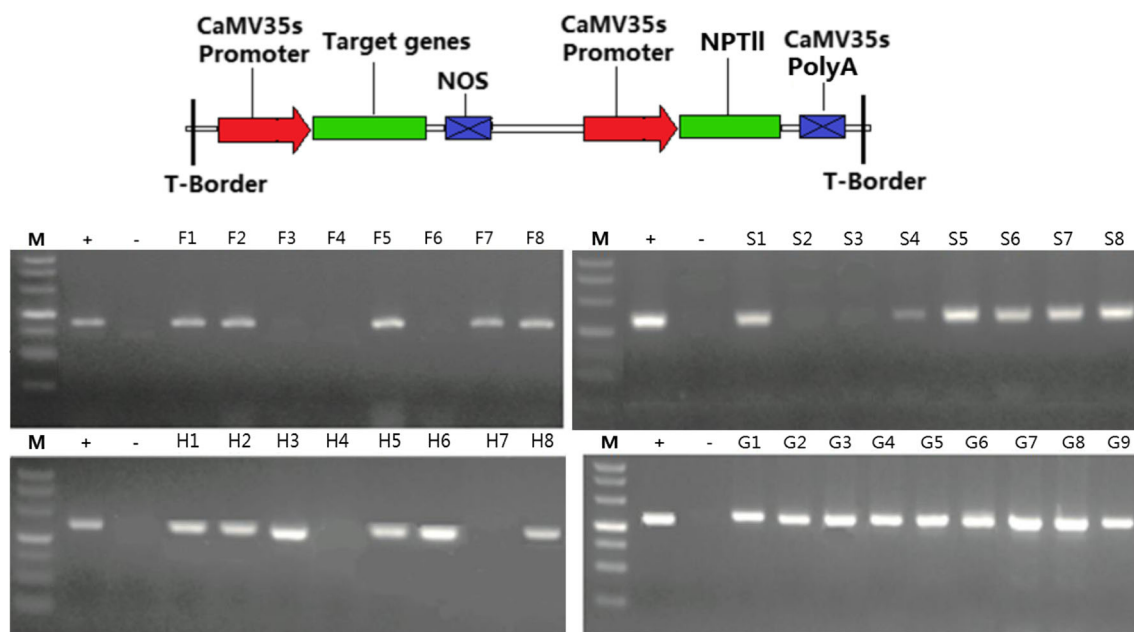


Figure 6. Vector construct and PCR analysis for the presence of four genes in cell lines of *R. zedoariae*. Lane M represents the DNA Marker DL400. Lane + represents positive control. Lane - represents negative control. Lane F1~F8 represent cell lines from transformation with pCambia2300-*FDS*. Lane S1~S8 represent cell lines from

transformation with pCambia2300-*ST02C*. Lane H1~H8 represent cell lines from transformation with pCambia2300-*HMGR*. Lane G1~G9 represent cell lines from transformation with pCambia2300-*GAS*.

Expression patterns of HMGR, DXS, and FDS genes in suspension cells *HMGR*, *DXS*, and *FDS* are important rate-limiting enzymes for terpene synthesis in plants. In this study, the expression patterns of *HMGR*, *DXS* and *FDS* genes in cell lines with higher β -elemene content were determined by PCR.

Figure 5 indicated that in cells of *R. zedoariae*, the IPPs used for β -elemene synthesis are mainly from MVA pathway because the expression level of *HMGR* gene was higher than that of *DXS* gene. As a key enzyme in the branch points of sesquiterpene synthetic pathway, *FDS* gene showed an active expression pattern. Accordingly,

HMGR and *FDS* should be two key points in controlling β -elemene metabolism.

Raising β -elemene content by overexpression of *HMGR*, *FDS*, and *ST02C* genes in cells of *R. zedoariae* The complete sequences of *HMGR* and *FDS* genes were obtained in a transcriptome database of *R. zedoariae*, and then confirmed by sequence alignment online at the URL of https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome. After cloning in *R. zedoariae*, the *HMGR* (Fig. 9) and *FDS* (Fig. 10) genes were transferred into plant expression

Figure 8. *GAS* gene in *Artemisia annual*.

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1 ATGGCAGCGGTTCAAGCTAATGTTACTGGTATCAAAGAAAAACACAAAACCTCCGCAGAG
1 M A A V Q A N V T G I K E N T K T S A E
61 CCAGTGC GGCCGCTGGCCAACCTTTCCTCCTTCAGTATGGGGTGATCGCTTCCTTTCATT
21 P V R P L A N F P P S V W G D R F L S F
121 TCTCTTGACAGATCGGAATTGGAGCGATATGCCATAGCTATGGAGAAGCCGAAGGAGGAT
41 S L D R S E L E R Y A I A M E K P K E D
181 TTGAGAAAAGTTGATCGTGGATCCGACCATGGATTCTAATGAGAAAAGCTAGGTTTGATTTAT
61 L R K L I V D P T M D S N E K L G L Y I
241 TCTGTGCATCGTCTCGGTTTGACATATATGTTCTTGCAAGAGATCGAAAAGCCAGCTTGAC
81 S V H R L G L T Y M F L Q E I E S Q L D
301 AAGCTTTTCAACAAGTTTAGCTTGAAGATTACGAAGAAGTTGATCTGTACACTATTTTCG
101 K L F N K F S L Q D Y E E V D L Y T I S
361 ATTAATTTCAAGTTTTCGACACCTTGGTTACAACTACCTTGTGATGTCTTTAAACAAA
121 I N F Q V F R H L G Y K L P C D V F N K
421 TTCAAGGACGTTAGCTCGGGTACTTTCAAGGCATCCATTAGTGTGATGTGAGGGGTATG
141 F K D V S S G T F K A S I M S D V R G M
481 TTGGCCCTATATGAATCTGCACAGTTGAGAATAAGAGGAGAAAAGATACTTGTAGAGGCC
161 L G L Y E S A Q L R I R G E K I L D E A
541 TCAGTTTTCACTGAAGCTAACTCAAGAGTGTAGTGAACACTCTGAAGGTGATCTTGCA
181 S V F T E A K L K S V V N T L E G D L A
601 CAACAAGTGACACAATCTTTAAGGAGACCATTCCATCAAGGGATGCCAATGGTAGAGGCA
201 Q Q V T Q S L R R P F H Q G M P M V E A
661 AGGCTCTATTCTCTAACTATGAAAAGAATGTTCCACTTATGACTCATTGCTAAGCTT
221 R L Y F S N Y E K E C S T Y D S L K L
721 GCAAAGCTTCACTTTAAGTATTGGAGCTACGACAAAAGAAGAACTGCGAATTTGTACCA
241 A K L H F K Y L E L R Q K E E L R I V P
781 AAGTGGTGAAGGACATGAGGTTTCACGAACTACTCCTTATATAAGGGATAGAGTACCA
261 K W W K D M R F H E T T P Y I R D R V P
841 GAGATTTACTTATGGATATTGGGATTATACTTTGAGCCTCGTTACTCCTTGGCAAGAAC
281 E I Y L W I L G L Y F E P R Y S L A R I
901 ATCGCAACAAAATAACGTTGTTTCTCGTGGTTTTAGATGACACATYATGATGCTTATGCT
301 I A T K I T L F L V L V L D T Y D A Y A
961 ACCATTGAAGAGATTGCGCCCTCCAACAGATGCGATAAGCAAGTGGGATATTAGTGTATG
321 T I E E I R P P T D A I S K W D I S A M
1021 GAGCAAATCCAGAGTATATTAGACCATTTTACAAAATCTTTTAGATGAATATGCTGAA
341 E Q I P E Y I R P F Y K I L L D E Y A E
1081 ATTGAGAAGAAAATGGCTAAAGAAGGGAGAGCAAATACTGTTATTGCTTCAAAAAGAGCG
361 I E K K M A K E G R A N T V I A S K E A
1141 TTCCAAGACATAGCTAGAGGTTACCTTGAAGAGGCTGAATGGACAAACAGTGGATATGTG
381 F O D I A R G Y L E E A E W T N S G Y V
1201 GCATCATTCCGGAGTATATGAAGAATGGATTAATCACTTCTGCCTATAATGTCATTTC
401 A S F P E Y M K N G L I T S A Y N V I S
1261 AAATCGGCTTTAGTTGGTATGGGIGAGATTGTTAGTGAAGATGCTTTGGCTTGGTATGAA
421 K S A L V G M G E I V S E D A L A W Y E
1321 AGTCATCCAAAGACTTTCGCAAGCTTCTGAGTTGATTTCAAGACTTCAAGATGATGTCATG
441 S H P K T L Q A S E L I S R L Q D D V M
1381 ACTTACCAGTTTGAACGTGAAAGAGGTCAATCTGCCACTGGCGTGTATGGGTATATTAAG
461 T Y Q F E R E R G Q S A T G V D A Y I K
1441 ACTTATGGTGTCTCAGAAAAGGAAGCTATTGACGGCTCAAGATCATGATTGAGAATGCA
481 T Y G V S E K E A I D A L K I M I E N A
1501 TGGAAAGATATAAACGAGGGATGTCTAAAGCCAAGACAAGTCTCAATGGATTTGCTTGCT
501 W K D I N E G C L K P R Q V S M D L L A
1561 CCAATTCCTAACCTTGAAGAATGATAGATGTGGTATACAGGTACGACGACGGGTTCACT
521 P I L N L A R M I D V V Y R Y D D G F T
1621 TTTCCAGGAAAGACTCTGAAAGAGTATATCAATCTTCTGTTTGGGGTCTCTACCCGTT
541 F P G K T L K E Y I N L L F V G S L P V
1681 TAA
561 *
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Figure 9. *HMGR* gene in *R. zedoariae*.

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1 ATGCCATGGAAGTCCGTCGTCAGGCTTCCTCCGAGCGTTGCTCTGCATCCAAGGGGCGC
1 M A M E L R R R L P P S V A S A S K G R
61 AAGCCTACCCCGGAGGCTCCTGTGCAGGCCCTCCGATGCCCTCCCGTCCCGATCCGCTAT
21 K P T P E A P V Q A S D A L P V P I R Y
121 ACTAACCTCCTCTTCTCCGCGCTCTTCGCGCCTCTCTGGTTTTCTTATGGCCGCGTGG
41 T N L L F S A L F A A S L V F L M R R W
181 CGCGAGAAAGTCCGTCCTTCTTACCTCTCCATGTCGTTGGCCAGCCGAGATCGTCGCC
61 R E K V R L S S P L H V V G P A E I V A
241 ATCGTCGGCCTTGTCCGCTCCCTCATCTACCTCCTCAACTTTTTGGGATCGCTTTCGTC
81 I V G L V A S L I Y L L N F F G I A F V
301 CAGTCCATCGTTTCTCGAACGACGAGGAGGACGATTTTCTACTCTCCGATCCACGGAG
101 Q S I V S S N D E E D D F L L S D S T E
361 CCCTCTGCCGCTCCCGCTCCGCTCCCATTCCTCCATCTCCCATCTTGGCGCTTGCCTGCT
121 P S A A P A S A P I P S P T L A P C S L
421 CTCTGCTCTGCTTCGGCCCTCGAGAAAATGCCAGATCTCACCGATGATGACGAGGAGATC
141 L C S A S A L E K M P D L T D D D E E I
481 ATCTCTCCGTTGTCCGCGGAAAATCCATCCTACGTTCTGGATCCAAAGTGGCGAT
161 I S S V V A G K I P S Y V L E S K L G D
541 TGCCGAAGAGCGGCCGGGATCCGGAAGAGCGCTGAGCGGACAAACGGGAGGGGCGCTG
181 C R R A A G I R K E A L R R T T G R G L
601 GTGGGCTTCCCTTGACGGATTTCGACTACGCTCCATTTGGGACAAATGTTGCGAACTC
201 V G L P L D G F D Y A S I L G Q C C E L
661 CCGATTGGGTACGTGAGCTGCCGTTGGGATCGCCGGGCCACTTCTGCTCAATGGCCGA
221 P I G Y V Q L L P V G I A G P L L L N G R
721 CAATACTATGTCCATGCGGACCCGAGGGTGGCTGTTGGGACAAATGTTGCGAACTC
241 Q Y Y V P M A T T E G C L V A S T N R G
781 TGTAAGCCATCTTGGAGTCTGGGGAGCCACCGCAATCTTAAAGGATGGTATGACG
261 C K A I L E S G G A T A I V L K D G M T
841 AGAGCTCCGGCAGTGAGGCTGCCGTCGGCAAGGGGTGCTCGGGAGCTCAAGGCTTTTTG
281 R A P A V R L P S A R G A A E L K A F L
901 GAGGATCCCAACAATCCGAGACGATTTCCCTCGTGTTCACAAATCAAGCAGATTTGCT
301 E D P N N S E T I S L V F N K S S R F A
961 AAGTTTCAGGGCATCAAAATGTGCACTGGCTGGGAGGAACCTTTATATGAGATTCAGGTGC
321 K F Q G I K C A L A G R N L Y M R F R C
1021 AGCACAGGAGATGCCATGGGTATGAATATGGTATCCAAGGGTGTGCAAAACGTTTTGGAC
341 S T G D A M G M N M V S K G V H M K V L D
1081 TATCTTCTGACAGATTTCCAGACATGGAAGTGAATGGTATTTCTGGTAATTATTGTTCA
361 Y L L T D F P D M E V I G I S G N Y C S
1141 GACAAGAACTGCTGCAGTTAATGGATTGAAGCCGGGGCAAATCTGTGGTCTGTGAA
381 D K K P A A V N W I E G R G A N S V V C E
1201 GCAATTATCAAGAGGAGGTGGTGAAGAAGACTCTCAAGATCAATGTACCAGCTCTAGTC
401 A I I K E E V V K K T L K I N V P A L V
1261 GAATCAACATGATTAAGAACCCTTGTGGGTCTGCAGTTGTGGAGCTCTCGGAGGTTTT
421 E L N M I K N L A G S A G V A N L D G G F
1321 AACGCTCATGCCAGCAATATTGTCTCTGCGGTATTCATTGCTACTGGCCAAAGATCCTGCT
441 N A H A S N I V S A V F I A T G Q D P A
1381 CAAAATGTTGAAAGTTCTCACTGCATCACCATGATGGAAGCTGTGAACGATGGAAGGAT
461 Q N V E S S H C I T M M E A V G A N L D K D
1441 CTCCATGATCTGTGACTATGCCATCCATTGAGGTAGGCACGGTGGTGGTGGGACTCAG
481 L H V S V T M P S I E V G T V G G G T Q
1501 TTGGCTTCTCAGGCTGCCTGTTTGGACCTTCTGGGCGTAAAGGGTGCGAANTGGACACC
501 L A S Q A A C L D L L G V K G A N L D T
1561 CCTGGGGCAAATGCCAGGCTCCTGGCCACCATTGTGGCTGGTGTCTTCTAGCTGGAGAA
521 P G A N A R L L A T I V A G A V L A G E
1621 CTTTCTTCTCTCTGCCCCTTGTGCGGGCCAGCTTGTGAAGAGCCACATGAAATACAA
541 L S L L S A L A A G Q L V K S H M K Y N
1681 CGATCCAGCAAAGATTTCAACAAAGTTGCCCCCTAA
561 R S S K D F N K V A P *

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vectors respectively used for transformation of *R. zedoariae*. *ST02C* gene and *GAS* gene and could catalyze FPP converted to germacrene A in different plants (Figs. 6 and 7). We failed to clone either of these genes in *R. zedoariae*. Therefore, we cloned *GAS* gene from *Artemisia annua* (Fig. 8) and synthesized the *ST02C* gene (Fig. 7). The plant expression vectors containing *ST02C* gene or *GAS* gene were constructed as well for transformation of *R. zedoariae*. As shown in Fig. 6, a total of 26 positive lines were obtained (Figs. 9 and 10).

The β -elemene content in transgenic cell lines of *R. zedoariae* is shown in Fig. 11. Cells overexpressing *HMGR* can efficiently promote accumulation of β -elemene in cell lines from H2 to H5 with the range of 0.15–0.17% (*w/v*), which was higher than the control (0.11% (*w/v*)). This implied that the upstream approach of β -elemene biosynthesis was based more on the MVA pathway in *R. zedoariae*. The levels of β -elemene in FDS-transformed lines also showed higher content of β -elemene ranging from 0.14% (*w/v*) to 0.18% (*w/v*) compared to

Figure 10. *FDS* gene in *R. zedoariae*.

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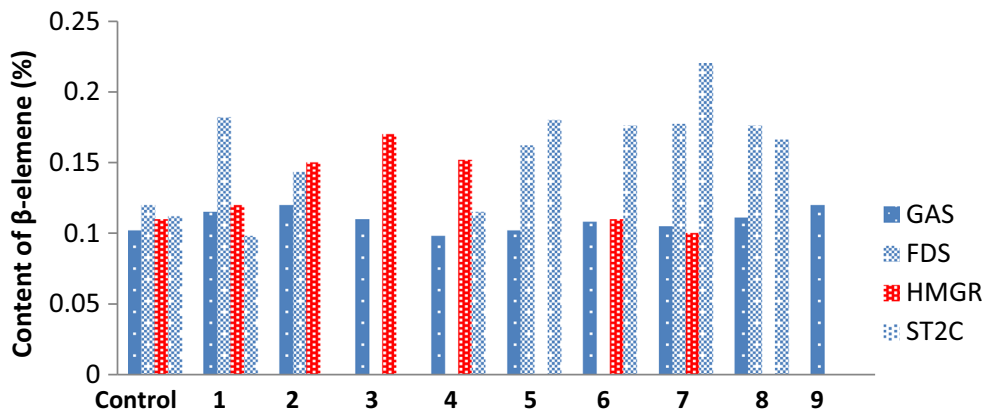
1 ATGGCGGAGGCGACGGCGAACGGAGCGGCGAGCGGTGCGCAGCAGGTGGGGATCGGAAGGCG
1 M A E A T A N G A A A V A A G G D R K A
61 GCCTTCCTCCAGATCTATGTTCAACTTAAGGAGGATCTGCTGCGGGATCCCGCCTTTGAC
21 A F L Q I Y V Q L K E D L L R D P A F D
121 TACACCGACGAGTCCCGCGAATGGATCGAGAAGATGCTGGACTACAACGTACCTGGAGGC
41 Y T D E S R E W I E K M L D Y N V P G G
181 AAGTTGAACCGAGGAATATCTGTATAGATAGCTACAAGTTGCTTAAACAAGGAAACCAA
61 K L N R G I S V I D S Y K L L K Q G N Q
241 CTCACGTGACACTGAGTTCTTTCTTGGTGGTTCCTTGGATGGTGCATCGAATGGCTTCAA
81 L T D T E F F L G G V L G W C I E W L Q
301 GCATATTTCTAGTCCCTTGATGATATTATGGATAACTCAGTCACCAGGCGTGGTCAGCCT
101 A Y F L V L D D I M D N S V T R R G Q P
361 TGCTGGTTTAGGGTTCCTAAGGTTGGTCTTATTGCTACAATGATGGAATCTTAATGCGC
121 C W F R V P K V G L I A T N D G I L M R
421 AATCACATTCCCTCGAATGCTTAAAAAGTATTTAAGGGAAAGCCTTACTATGTTGATCTC
141 N H I P R M L K K Y F K G K P Y Y V D L
481 CTCGATCTGTTTAAACGAGGTTGAGTTTCAGACAGCTTCAGGACAGTTGTTGGATCTCATT
161 L D L F N E V E F Q T A S G Q L L A D L I
541 ACAACCCATGAGGGAGAACAGAACCCTATCAAATAACAATCACTGTTTACAATCGCATT
181 T T H E G E Q N L S K Y N I T V Y N R I
601 GTGCAATACAAAACCTGCTTATTATTCCTTCTATCTCCAGTAGCATGCGCATTGCTATTG
201 V Q Y K T A Y Y S F Y L P V A C A L L L
661 GCAGGCAAAAATCTGGACAACCTTCTCGAAGTGAAGACATTCTTATTCAAATGGGAACA
221 A G K N L D N F L E V K D I L I Q M G T
721 TATTTCCAGATACAGGATGATTACCTGGATTGCTTCGGTCATCCTGATQTAATTGGTAAG
241 Y F Q I Q D D Y L D C F G H P D V I G K
781 ATTGGAACGTATATTAGGACTTCAAGTGCCTTTGGCTTGTAGTGAAGCCCTAGGTCTT
261 I G T D I Q D F K C S W L V V Q A L G L
841 GCTAATGAGAGCCCACTGAAGATTTTGTGCGGACAACATGCGGAAAACGTATCCAGCATGT
281 A N E S Q L K I L S D N Y G K T D P A C
901 GTTGA AAAAGTGA AACGCCTTACA AAGATCTTA AACTGGAGAAATATTGCTGAGTAT
301 V E K V K R L Y K D L N L E K L F A E Y
961 GAGCGGACTAGCTACGGAAAACCTGATATCTTCTATTGAAGCCCAGCCAGGCAAGAAGTG
321 E R T S Y G K L I S S I E A Q P G K E V
1021 CAGCATGTGTGAAGTCTTTCTCGAAAAGATCTACAAAAGGCAGAAGTAA
341 Q H V L K S F L E K I Y K R Q K *
    
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the control level of 0.12% (w/v). The highest β -elemene content of 0.22% (w/v) was detected in *ST02C*-transformed lines (S5) when compared to the control lines (0.11 (w/v)). Whether the *ST02C* gene was present in *R. zedoariae* or another gene was involved in the β -elemene biosynthesis needs to be confirmed through further analyses. Most of transgenic lines showed higher levels of β -elemene, reflecting that transgenic technology is a promising way to improve β -elemene accumulation in *R. zedoariae* cells.

Discussion

Application of the two-stage culture system Two-stage cultivation is a common strategy used for plant cell culture. For traditional two-stage run, both cell proliferation and target substance synthesis proceed in different bioreactor systems. Traditional two-stage culture suffered from the drawbacks of slow adaptation of cells to the new growth environment and an increased risk of contamination during cell transfer between bioreactors. In this study, a combined system consisting

Figure 11. β -Elemene content in transgenic cell lines of *R. zedoariae*. 1~9 represent the transgenic cell lines of *G1~G9*, *F1~F8*, *H1~H8*, and *S1~S8* respectively. Control represents non-transgenic cell lines.



of stir-tank and airlift bioreactors was used to conduct a two-stage perfusion cultivation of *R. zedoariae* suspension cells. The application of this apparatus can accomplish two-stage culture in one bioreactor system. The continuous cell culture was also achieved *via* a cell settling device composed by a spiral-type centrifugal settling pipe and a gravity settling pie that built into the air-lift column.

In the two-stage culture system, the stress of additives and ultraviolet radiation made the airlift column a tough environment for cell growth. The medium in the airlift column did not flow back to the stirred tank due to the medium inlet in airlift column was above the liquid surface. Therefore, the two reaction vessels were relatively independent each other though they were connected by a glass pipe. Cells in the stirred tank grew well and were continuously presented to the airlift column for β -elemene synthesis. In the airlift column, β -elemene content in cells was increased by additives and genetic transformation; meanwhile, normal growth of cells was maintained. Both biomass and secondary metabolites production were achieved simultaneously in one bioreactor system. This bioreactor culture system is stable, convenient, and inexpensive, with potential industrial applications. We plan further studies on scale-up and volume increase of this bioreactor system in future.

Preliminary study on the metabolic pathway of β -elemene in *R. zedoariae*

Progress has been made on the upstream pathway of terpene synthesis in plants. Advances in understanding the downstream pathway are urgently needed, but have not yet been achieved in most plants. Two independent pathways of IPP biosynthesis, the MVA and MEP pathways, have been found in higher plants. In some plants, the precursors of sesquiterpenes and triterpenes are largely from the contribution of MVP pathway, whereas the MEP pathway is involved to the synthesis of diterpenes as well as tetraterpenes (Liu et al. 2005). *HMGR* and *DXS* are the key rate-limiting enzymes of the MVA and MEP biosynthetic pathway respectively (Aquil et al. 2009). These enzymes often serve as important regulatory sites in the upstream pathway for terpene synthesis. In the present study, the *HMGR* gene was actively expressed in cells with high β -elemene content. In addition, cells overexpressing *HMGR* can more efficiently promote accumulation of β -elemene compared to the control line, implying that the upstream approach of β -elemene biosynthesis are based more on the MVA pathway in *R. zedoariae*. *FDS* was considered as a key enzyme in the branch points of sesquiterpene synthetic pathway in plants (Ping et al. 2012). The present study also indicated that it is essential to increase the metabolic flux towards sesquiterpenes *via* overexpression of *FDS* gene in *R. zedoariae*. *ST02C* is a key enzyme for β -elemene biosynthesis in ginger. In this study, the highest β -elemene content of 0.22% (*w/v*) was detected in *ST02C*-transformed lines, but we failed to obtain the nucleotide sequence of this gene from *R. zedoariae*. Whether these genes are absent in genome of *R. zedoariae* or a new pathway is

present in *R. zedoariae* for β -elemene synthesis needs to be confirmed. There is only 49% homology between the *GAS* in *Artemisia annua* and *ST02C* in ginger, so it can also be speculated that another gene, similar in function to the *ST02C* or *GAS*, may be present in *R. zedoariae*. Further studies will be conducted to characterize more candidate genes in *rhizoma zedoaria* to obtain fine transgenic cell lines with higher β -elemene content.

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