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Efficient Production of δ-Guaiene, an Aroma Sesquiterpene Compound Accumulated in Agarwood, by Mevalonate Pathway-Engineered *Escherichia coli* Cells

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Abstract

Mevalonate pathway for isoprenoid biosynthesis was constructed in *Escherichia coli* cells by the transformation with a gene cluster isolated from *Streptomyces* sp., and farnesyl diphosphate synthase and δ-guaiene synthase genes were coexpressed in this strain. This transformant was capable of liberating an appreciable amount of δ-guaiene, an aroma sesquiterpene compound accumulated in agarwood, and its concentration was elevated to more than 30 μg/ml culture by the incubation with mevalonolactone as an isoprene precursor in a nutrient-enriched Terrific broth. Coexpression of type 1 isopentenyl diphosphate isomerase plus acetoacetyl-CoA ligase genes also enhanced δ-guaiene production, and the concentration of the compound was approximately 38 - 42 μg/ml culture in the presence of mevalonolactone or lithium acetoacetate. These results clearly indicate that mevalonate pathway-engineered *E. coli* cells showed an appreciable δ-guaiene producing activity in the enriched medium in the presence of appropriate isoprene precursors.

Keywords

Engineered *Escherichia coli*, δ-Guaiene Production, Isoprenoids, Mevalonate Pathway, Secondary Metabolism, Sesquiterpene

1. Introduction

It is well known that *Aquilaria* plants very occasionally form dark resinous heartwood

called agarwood upon mechanical wounding or microbial infection [1]. These tissues produce a variety of sesquiterpene compounds with aroma such as *δ*-guaiene, *α*-guaiene and *α*-humulene, and, therefore, they have been used as a scent, perfume and traditional medicines. It has been assumed [2] that guaianolide sesquiterpenes are synthesized via two steps of cyclization reactions catalyzed by *δ*-guaiene synthase (GS) with farnesyl diphosphate (FPP) as the substrate (**Figure 1**). Although commercial value of agarwood is very high, this material is not formed under normal environmental conditions [1], and the artificial transformation of *Aquilaria* plants to agarwood has been quite difficult.

We showed previously [3] [4] that treatment of cell cultures of *A. microcarpa* with either a plant hormone methyl jasmonate or yeast extract resulted in a marked tran-

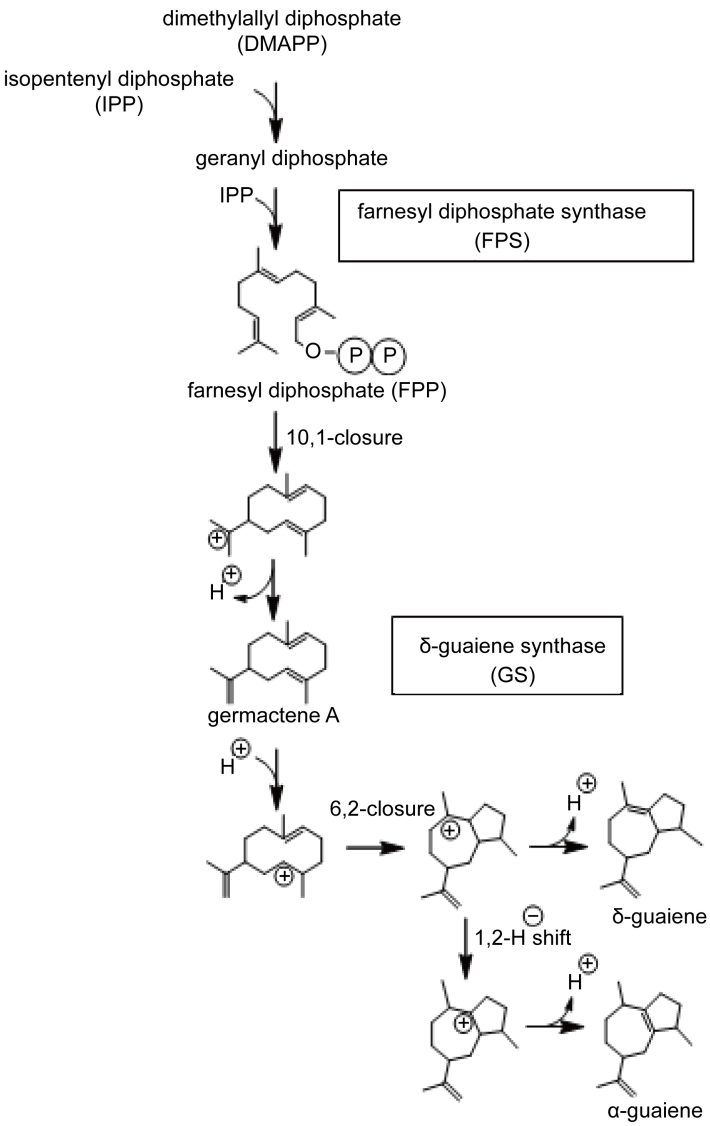

Figure 1. Predicted biosynthetic pathway of guaiene-type sesquiterpene compounds of *Aquilaria* plants.

scriptional activation of GS, and this enabled us to isolate several homologous *GS* genes. To our knowledge, *δ*-guaiene cannot be purchased from commercial venders, and, therefore, we attempted to construct the production system of this compound employing bacterial cells. A *GS* gene, together with a farnesyl diphosphate synthase gene (*FPS*) isolated from *A. microcarpa* [5], was inserted into pRSFDuet-1, and *Escherichia coli* cells transformed with this expression vector were found to generate *δ*-guaiene at the concentration of approximately 0.6 μg/ml culture in a nutrient-enriched Terrific broth [6].

In *E. coli*, isoprenoids are biosynthesized not through mevalonate (MVA) but through non-mevalonate pathway [7]. However, it was reported [8] [9] [10] [11] that *E. coli* transformed with MVA pathway-related genes obtained from heterogeneous sources showed appreciable terpenoid-producing activities by coexpression with appropriate biosynthetic enzyme genes. Recently, Harada *et al*. [12] constructed MVA pathway-engineered *E. coli* cells by the transformation with a gene cluster (**Figure 2**) isolated from *Streptomyces* sp. [13] [14], and demonstrated [15] that efficient carotenoid production was achieved in this strain by overexpression of several genes involved in the biosynthetic processes of the tetraterpene pigments. In the present experiments, we have attempted to construct the efficient producing system of *δ*-guaiene, which is biosynthesized in *Aquilaria* plants only under very limited condition, employing MVA pathway-engineered *E. coli* cells.

2. Materials and Methods

2.1. Construction of Expression Vectors

The translatable regions of *FPS* and *GS* isolated from cultured cells of *A. microcarpa* [3] [5] were amplified by PCR, and subcloned into pRSFDuet-1 (Novagen) according to the method described previously in detail [6]. This expression vector was designated as pRSF-*FPS*/*GS*. A gene cluster encoding six MVA pathway enzymes, 3-hydroxy-3-methylglutaryl CoA (HMGCoA) synthase, HMG-CoA reductase, MVA kinase, phosphomevalonate (PMVA) kinase, diphosphomevalonate (DPMVA) decarboxylase and type 2 isopentenyl diphosphate (IPP) isomerase (**Figure 2**), isolated from *Streptomyces* sp. strain CL190 [13] [14] was PCR-amplified, subcloned into pACYC184, and designated pAC-*Mev* (**Figure 3**). The successive insertion of type 1 IPP isomerase gene isolated from *Saccharomyces cerevisiae* (*Scidi*) and acetoacetate-CoA ligase gene from *Rattusnorvegicus* (*Aacl*) into pAC-*Mev* was performed as described previously in detail [12], and the constructed vectors were designated pAC-*Mev*/*Scidi* and pAC-*Mev*/*Scidi*/*Aacl*, respectively (**Figure 2** and **Figure 3**).

2.2. Transformation and Cultivation of *E. coli* Cells

Transformation and cultivation of *E. coli* was performed according to the method described previously [6] with several modifications. *E. coli* BL21 cells were transformed with four expression vectors, pRSF-*FPS*/*GS*, pAC-*Mev*, pAC-*Mev*/*Scidi* and pAC-*Mev*/*Scidi*/*Aacl*, and the transformants were grown in LB medium overnight at 37˚C. The

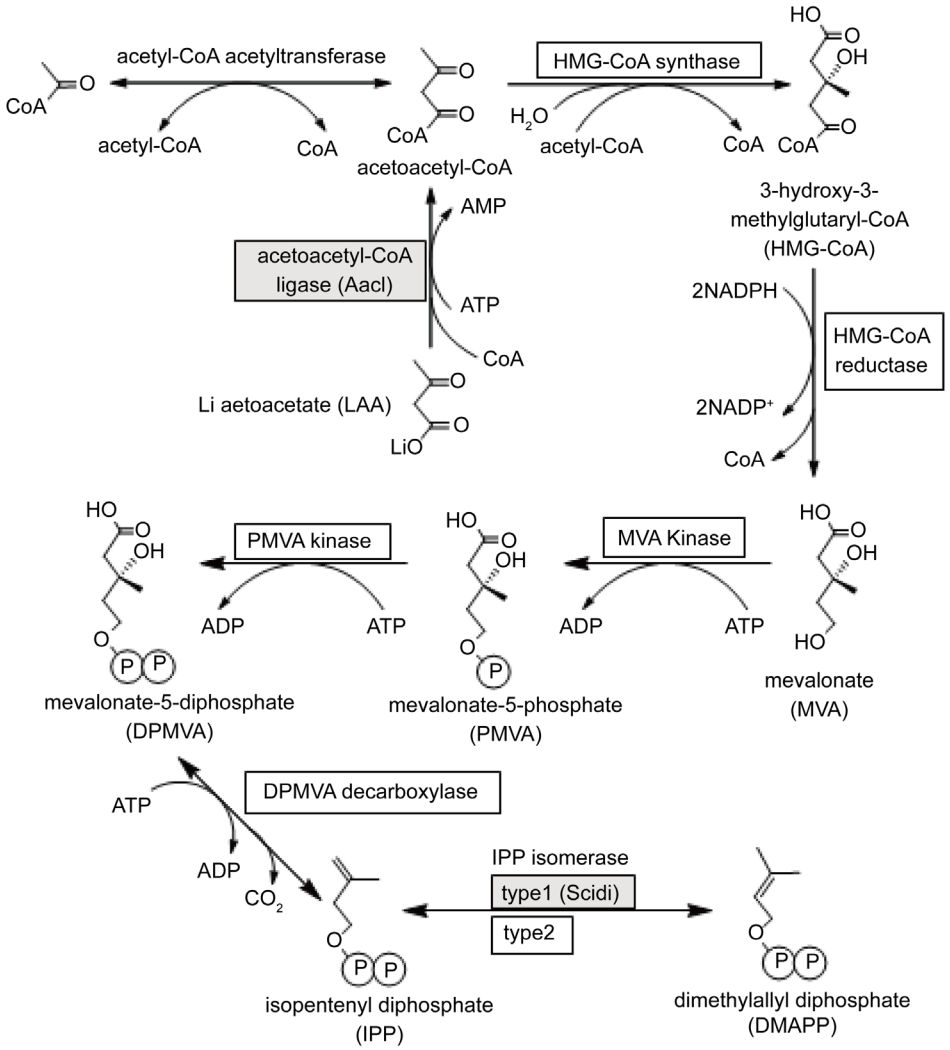

Figure 2. Enzymes and intermediates involved in MVA pathway. Six enzymes involved in the *Streptomyces* MVA gene cluster, 3-hydroxy-3-methylglutaryl CoA (HMGCoA) synthase, HMG-CoA reductase, MVA kinase, phosphomevalonate (PMVA) kinase and diphosphomevalonate (DPMVA) decarboxylase, and isopentenyl diphosphate (IPP) isomerase type 2, were presented in open squares.Additional two enzymes, type 1 isopentenyl isomerase isolated from *S. cerevisiae* (Scidi) andacetoacetate-CoA ligase from*R. norvegicus* (Aacl), were presented in the shaded squares.

cultures were transferred into fresh LB medium, and isopropyl β-D-1-thiogalactopyranoside (IPTG, final concentration 0.1 mM) was added to the cultures at an optical density of 0.4 - 0.5 at 600 nm. In parallel experiments, overnight culture of *E. coli* was transferred into a nutrient-enriched Terrific broth instead of LB medium, and, then, IPTG was added in a similar manner. Immediately after the addition of IPTG, 100 µl-aliquots were transferred into screw-capped 4 ml vials, and 0.2 µg limonene (NacalaiTesque, 1 µl ethanol solution) was added to the cultures as an internal standard. If necessary, 100 µg mevalonolactone (MVL, Tokyo Kasei, 1 µl-aliquot) and/or 100 µg lithium acetoacetate (LAA, Sigma-Aldrich, 1 µl-aliquot) were also added to the 100 µl *E.*

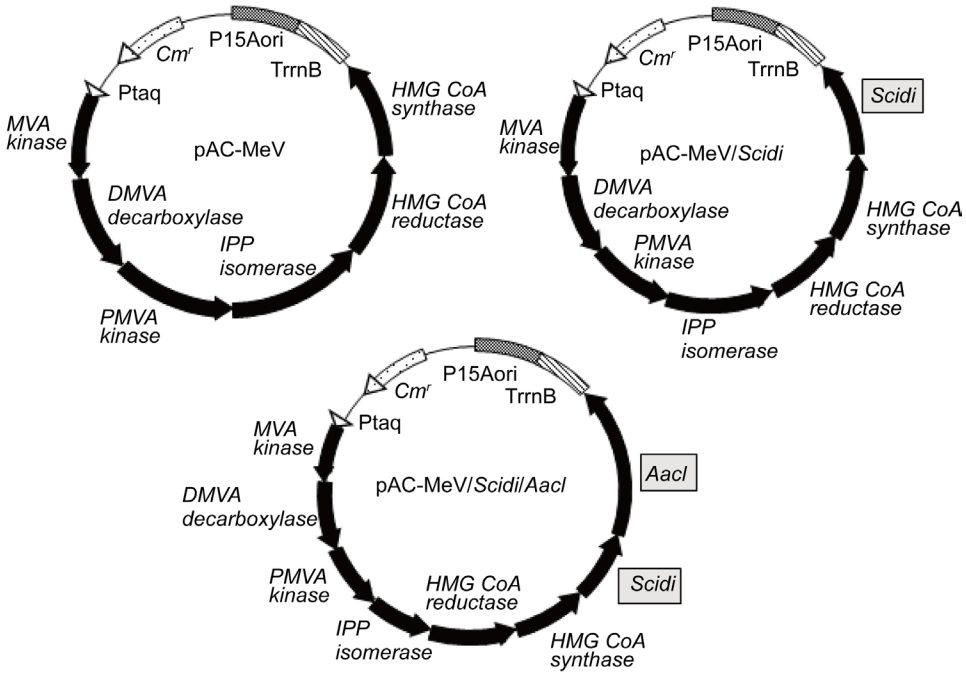

Figure 3. Schematic presentation of the vectors for MVA pathway-engineering, pAC-*Mev*, pAC-*Mev*/*Scidi*, pAC-*Mev*/*Scidi*/*Acal*.

coli cultures, and the mixtures were further incubated at 25˚C overnight.

2.3. GC-MS Analysis of *δ*-Guaiene

The sesquiterpene compounds accumulated in the head space of the cultures were extracted with a solid phase microextraction assembly for 30 min at room temperature (Supelco, 100 μm polydimethylsiloxane fiber), and identification of the products was performed by GC-MS analysis (Shimadzu, GCMS-QP2010 Ultra; Agilent, CAJ&W DB-5MS 0.32 mm × 30 m column) according to the method described previously in detail [6]. The reaction products were separated to calculate retention indices, and the identification of the compounds was based on the comparison of reported retention indices and mass spectra with those in databases as described previously [3] [16] [17].

2.4. Quantitative Determination of *δ*-Guaiene

Determination of *δ*-guaiene concentration was carried out by GC analysis (GL Sciences, GC-353; Supelco, SPB-1 0.32 mm × 30 m column; equipped with flame ionization detector and Hitachi D-2500 Chromatointegrator). The column pressure was at 260 kPa (carrier N2), and the injection port and the interface temperature were adjusted at 250˚C. The oven temperature was started at 50˚C for 2 min and was increased to 130˚C at the rate of 4˚C/min, then raised to 250˚C at 30˚C/min and kept for 10 min. The reaction products extracted with the microextraction assembly were injected in the splitless mode, and were separated under the conditions described above. *δ*-Guaiene concentration was determined by a calibration curve plotted the ratio of the peak areas of authen-

tic sample at various concentrations to the internal standard [6].

3. Results

3.1. Effect of Overexpression of MVA Pathway Genes on δ-Guaiene Production

We have previously reported [3] that sesquiterpene compounds produced by the pRSF-*FPS*/*GS*-transformed *E. coli* cells were detected neither in medium nor cells, but were recovered only from the head space of the cultures. In the total ion chromatogram of GC-MS analysis (**Figure 4(a)**), two peaks, together with the internal standard limonene, were found to be released from the transformed *E. coli* cultures, and they were identified as *α*-guaiene and *δ*-guaiene by the retention indices and mass spectra (**Figure 4(b)**) in the data base, respectively [3] [16] [17]. *E. coli* cells transformed with solely pRSF-*FPS*/*GS* liberated low concentration of *δ*-guaiene when they were cultivated in LB medium (0.4 μg/ml culture). However, the sesquiterpene-producing activity was significantly enhanced by the incubation of the transformant in a nutrient-enriched Terrific broth, and it increased to the level of 1.8 μg *δ*-guaiene/ml culture (**Figure 5**). An appreciable increase in sesquiterpene-producing activity was observed by the transformation of *E. coli* with pRSF-*FPS*/*GS* plus pAC-*Mev*, and the sesquiterpene contents were elevated to 1.0 μg/ml culture in LB medium and 4.8 μg/ml culture in Terrific broth, respectively. Since MVA pathway was expected to be constructed in the pAC-*Mev*-transformed *E.coli* cells, we examined the possible effect of the addition of an isoprene precursor, MVL, on the sesquiterpene production in this transformant. As shown in

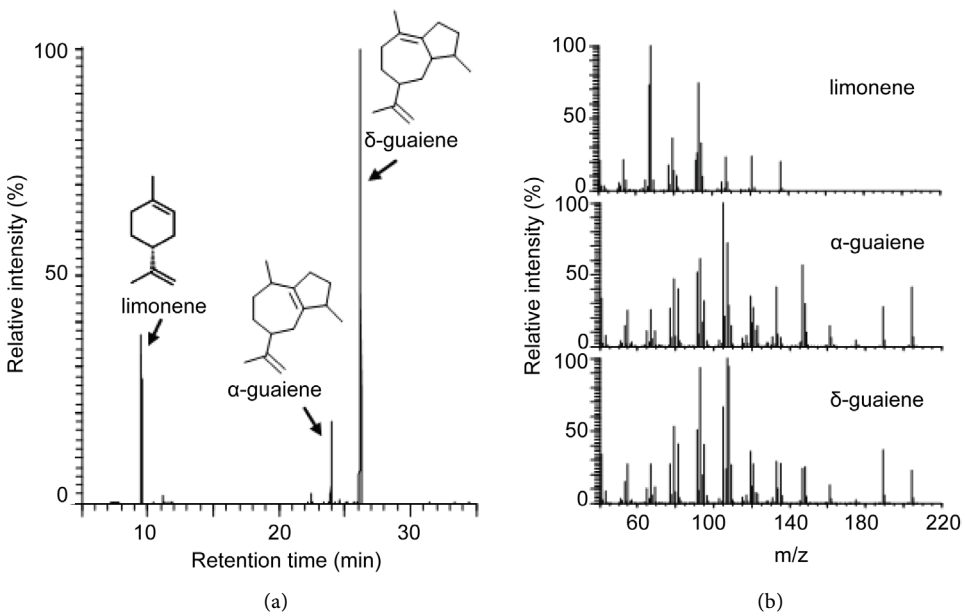

Figure 4. GC-MS analysis of the products liberated from the transformed *E. coli* cells. A, Total ion chromatogram of the products accumulated in the head space of the transformed *E. coli* cell cultures. Limonene was added as the internal standard immediately after the addition of IPTG. B, Mass spectra of limonene, *α*-guaiene and *δ*-guaiene.

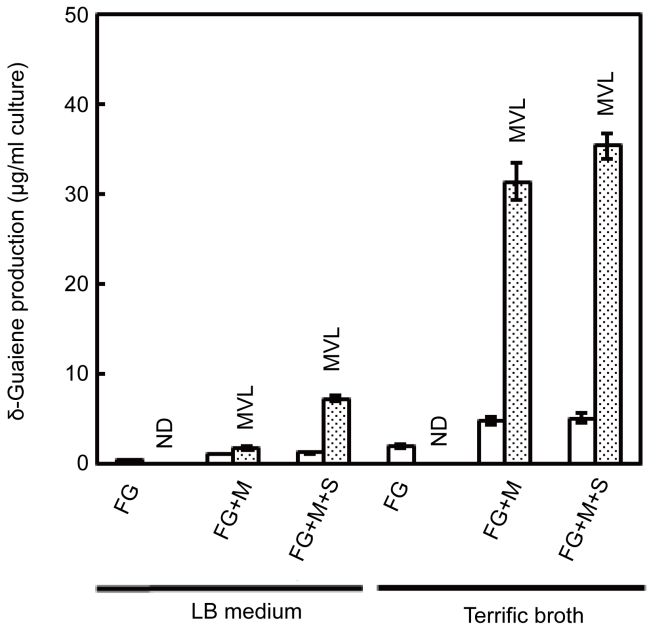

Figure 5. *δ*-Guaiene production in MVA pathway-engineered *E.coli*. *E. coli* cells transformed with *FPS*, *GS* plus MVA pathway genes from *Streptomyces* sp. were incubated in the absence (open columns) or presence (dotted columns) of MVL, and *δ*-guaiene contents in the head space of the cultures were determined by GC. Data are presented as means and standard deviations obtained from three replicate experiments (ND, not determined for MVL addition). FG, transformed with pRSF-*FPS*/*GS*; FG + M, transformed with pRSF-*FPS*/*GS* plus pAC-*Mev*; FG + M + S, transformed with pRSF-*FPS*/*GS* plus pAC-*Mev*/*Scidi*.

Figure 5, *δ*-guaiene accumulation in pAC-*Mev*-transformed cells grown in LB medium appeared to be slightly enhanced by the addition of MVL, and approximately 1.6-fold increase in the productivity was observed. In contrast, pAC-*Mev*-transformant cultivated in Terrific broth showed a marked elevation of the biosynthetic activity upon the coincubation with MVL, and the concentration of *δ*-guaiene was found to be 31.4 μg/ml culture.

3.2. Effect of Overexpression of *Scidi* Gene on *δ*-Guaiene Production

Harada *et al*. reported [12] that FPP content was estimated to increase several times when type 1 IPP isomerase gene derived from *S. cerevisiae* (*Scidi*) was introduced and overexpressed in *E. coli*, together with type 2 IPP isomerase (Figure 2). Therefore, *E. coli* cells were transformed with pRSF-*FPS*/*GS* plus pAC-*Mev*/*Scidi* (Figure 3), and the sesquiterpene-producing activity of the transformant was examined in the absence and presence of MVL (Figure 5). *E. coli* overexpressing *FPS*, *GS*, six MVA cluster genes plus *Scidi* liberated 1.2 and 7.2 μg *δ*-guaiene/ml culture in the absence and presence of MVL, respectively, when the cells were grown in LB medium. On the other hand, pAC-*Mev*/*Scidi*-transformed cells in Terrific broth generated the compound at the concentration of 5.0 and 35.3 μg/ml culture in the absence and presence of MVL.

3.3. Effect of Overexpression of *Aacl* Gene on δ-Guaiene Production

It was reported [15] that an efficient biosynthetic system of isoprenoids from acetoacetate could be established by the transformation of *E. coli* by a rat acetoacetate-CoA ligase gene (*Aacl*), together with the MVA pathway gene cluster, although *Aacl* translate is an important enzyme of butanoate metabolism rather than isoprenoid pathway (Figure 2 and Figure 3). In this case, it was also demonstrated that, LAA could be added to the culture medium as a convenient substrate of Aacl to form MVA (Figure 2). Therefore, we prepared *E. coli* cells transformed with pRSF-*FPS*/*GS* plus pAC-*Mev*/*Scidi*/*Aacl*, and examined the δ-guaiene productivity of the cells overexpressing these genes. As shown in Figure 6, the transformed *E. coli* in LB medium liberated 1.6 μg δ-guaiene/ ml culture, and the content increased to 12.0 and 17.3 μg/ml by the addition of MVL and LAA to the cultures, respectively. On the other hand, the transformed cells cultivated in Terrific broth generated 5.8 μg/ml culture of δ-guaiene in the absence of the precursors, while the concentration appreciably increased to 37.9 and 42.3 μg/ml culture by the addition of MVL and LAA.

We expected the possibility that some synergic effect of MVL and LAA on the sesquiterpene production might be observed upon the coincubation of these two precursors with the transformed cells. Therefore, MVL and LAA were concomitantly added to the transformant cultures, and δ-guaiene concentrations were determined (Figure 6). However, no enhancement of δ-guaiene production was observed in the transformed cells even in the presence of the two isoprene precursors, and the contents of the com-

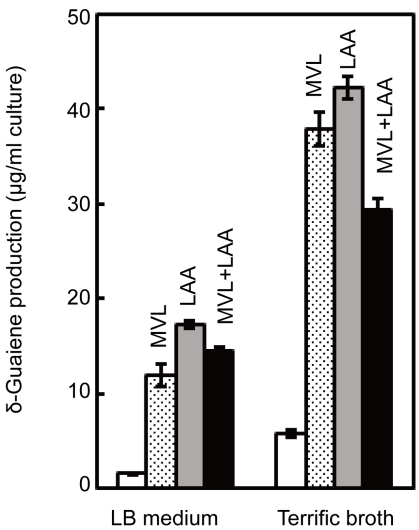

Figure 6. δ-Guaiene production by transformed *E. coli* cells utilizing acetoacetate as the isoprene precursor. *E. coli* cells transformed with pRSF-*FPS*/*GS* plus pAC-*Mev*/*Scidi*/*Aacl* were incubated in the absence and presence of MVL and/or LAA. δ-Guaiene contents in the cultures incubated in the absence of precursors (open columns), in the presence of MVL (dotted columns), LAA (shaded columns), and MVL plus LAA (closed columns) were determined by GC, and data are presented as means and standard deviations obtained from three replicate experiments.

pound decreased to 14.5 and 29.3 μg δ-guaiene/ml culture in LB medium and in Terrific broth, respectively. In repeated experiments, notable activation of δ-guaiene biosynthesis was not observed by the coincubation of the transformed cells with these two precursors, and the concentrations of the compound were almost similar or rather decreased though the values were varied in some extent.

4. Discussion

We showed previously [6] that δ-guaiene biosynthesis was markedly activated when *GS* and *FPS* were coexpressed in *E. coli* cells (**Figure 1**), and the content of the compound was approximately 1 - 2 μg/ml culture even in the nutrient-enriched Terrific broth. In the present study, we have demonstrated that *FPS*- and *GS*-overexpressing *E. coli* cells transformed with a *Streptomyces* MVA pathway gene cluster exhibited an appreciable biosynthetic activity of δ-guaiene, especially in the nutrient-enriched Terrific broth (**Figure 5**). Accumulation of the compound was increased to 31.4 μg/ml culture when the transformed cells were incubated in the presence of MVL. Therefore, 17.4-fold increase in δ-guaiene-producing activity was observed when MVA pathway-engineered *E. coli* cells were incubated in the enriched medium with the precursor supplement. Similarly, the sesquiterpene contents increased to 35.3 and 42.3 μg/ml culture in pAC-*Mev*/*Scidi*- and pAC-*Mev*/*Scidi*/*Aacl*-transformants in the presence of MVL and LAA (**Figure 5** and **Figure 6**), and the productivity was elevated to 19.6- and 23.5-fold levels, respectively, compared with a control *E. coli* strain expressing solely *FPS* plus *GS*. These results clearly indicate that the introduction of MVA pathway in *E.coli* is a powerful tool to construct δ-guaiene-producing system in the bacterial cells, as well as some other terpenoid compounds [8] [9] [10] [11].

Although addition of MVL or LAA to MVA pathway-engineered *E. coli* cell culture resulted in the marked enhancement of δ-guaiene biosynthesis, no synergic or somewhat inhibitory effect was observed when pAC-*Mev*/*Scidi*/*Aacl*-transformant was incubated in the presence of both of the precursors (**Figure 6**). At present, no direct evidence is available to explain this apparent discrepancy, however, it might be possible that intracellular concentration of the isoprene units and/or their precursors might be almost saturated in pAC-*Mev*/*Scidi*/*Aacl*-transformed *E. coli* culture supplemented with both MVL and LAA, and certain inhibitory mechanism might function under these "overdose" conditions.

Further improvement of *E. coli* cell culture system as the δ-guaiene-producing apparatus is in progress in our laboratory.

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References

[1] MNg, L.T., Chang, Y.S. and Kadir, A.A. (1997) A Review on Agar (Gaharu) Producing

Aquilaria Species. *Journal of Tropical Forest Products*, **2**, 272-285.

[2] Kumeta, Y. and Ito, M. (2010) Characterization of δ-Guaiene Synthases from Cultured Cells of *Aquilaria* Responsible for the Formation of the Sesquiterpenes in Agarwood. *Plant Physiology*, **154**, 1998-2007. http://dx.doi.org/10.1104/pp.110.161828

[3] Kurosaki, F., Hirohashi, S., Katoh, T., Taura, F. and Lee, J.B. (2015) Cloning and Characterization of δ-Guaiene Synthase Genes Encoding a Sesquiterpene Cyclase from *Aquilariamicrocarpa* Cell Cultures. *American Journal of Plant Sciences*, **6**, 2603-2611. http://dx.doi.org/10.4236/ajps.2015.616263

[4] Kurosaki, F. and Taura, F. (2015) Transcriptional Activation of Sesquiterpene Biosynthetic Enzyme δ-Guaiene Synthase Gene in Cell Cultures of *Aquilariamicrocarpa* Overexpressing *cam1* and *rac2* Encoding Calmodulin and RacGTPase. *Plant Gene*, **2**, 25-28. http://dx.doi.org/10.1016/j.plgene.2015.03.004

[5] Kenmotsu, Y., Ogita, S., Katoh, Y., Yamamura, Y., Takao, Y., Tatsuo, Y., Fujino, H., Kadota, S. and Kurosaki, F. (2011) Methyl Jasmonate-Induced Enhancement of Expression Activity of *Am-FaPS*-1, a Putative Farnesyl Diphosphate Synthase Gene from *Aquilariamicrocarpa*. *Journal of Natural Medicines*, **65**, 194-197. http://dx.doi.org/10.1007/s11418-010-0451-4

[6] Kato, T., Lee, J.B., Taura, F. and Kurosaki, F. (2016) Enhanced Production of δ-Guaiene, a Bicyclic Sesquiterpene Accumulated in Agarwood, by Coexpression of δ-Guaiene Synthase and Farnesyl Diphosphate Synthase Genes in *Escherichia coli*. *Natural Product Communications*, **11**, 1221-1224.

[7] Rohmer, M., Knani, M., Simonin, P., Sutter, B. and Sahm, H. (1993) Isoprenoid Biosynthesis in Bacteria: A Novel Pathway for Early Steps Leading to Isopentenyl Diphosphate. *Biochemical Journal*, **295**, 517-524. http://dx.doi.org/10.1042/bj2950517

[8] Martin, V.J., Pitera, D.J., Withers, S.T., Newman, J.D. and Keasling, J.D. (2003) Engineering a Mevalonate Pathway in *Escherichia coli* for Production of Terpenoids. *Nature Biotechnology*, **21**, 796-802. http://dx.doi.org/10.1038/nbt833

[9] Vadali, R.V., Fu, Y., Bennett, G.N. and San, K.Y. (2005) Enhanced Lycopene Productivity by Manipulation of Carbon Fow to Isopentenyl Diphosphate in *Escherichia coli*. *Biotechnology Progress*, **21**, 1558-1561. http://dx.doi.org/10.1021/bp050124l

[10] Yoon, S.H., Lee, Y.M., Kim, J.E., Lee, S.H., Lee, J.H., Kim, J.Y., Jung, K.H., Shin, Y.C., Keasling, J.D. and Kim, S.W. (2006) Enhanced Lycopene Production in *Escherichia coli* Engineered to Synthesize Isopentenyl Diphosphate and DimethylallylDiphosphate from Mevalonate. *Biotechnology and Bioengineering*, **94**, 1025-1032. http://dx.doi.org/10.1002/bit.20912

[11] Chang, M.C.Y. and Keasling, J.D. (2006) Production of Isoprenoid Pharmaceuticals by Engineered Microbes. *Nature Chemical Biology*, **2**, 674-681. http://dx.doi.org/10.1038/nchembio836

[12] Harada, H., Yu, F., Okamoto, S., Kuzuyama, T., Utsumi, R. and Misawa, N. (2009) Efficient Synthesis of Functional Isoprenoids from Acetoacetate through Metabolic Pathway-Engineered *Escherichia coli*. *Applied Microbiology and Biotechnology*, **81**, 915-925. http://dx.doi.org/10.1007/s00253-008-1724-7

[13] Takagi, M., Kuzuyama, T., Takahashi, S. and Seto, H. (2000) A Gene Cluster for the Mevalonate Pathway from *Streptomyces* sp. Strain CL190. *Journal of Bacteriology*, **182**, 4153-4157. http://dx.doi.org/10.1128/JB.182.15.4153-4157.2000

[14] Kaneda, K., Kuzuyama, T., Takagi, M., Hayakawa, Y. and Seto, H. (2001) An Unusual Isopentenyl Diphosphate Isomerase Found in the Mevalonate Pathway Gene Cluster from

Streptomyces sp. Strain CL190. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 932-937. http://dx.doi.org/10.1073/pnas.98.3.932

[15] Harada, H. and Misawa, N. (2009) Novel Approaches and Achievements in Biosynthesis of Functional Isoprenoids in *Escherichia coli*. *Applied Microbiology and Biotechnology*, **84**, 1021-1031. http://dx.doi.org/10.1007/s00253-009-2166-6

[16] Babushok, V.I., Linstrom, P.J. and Zenkevich, I.G. (2011) Retention Indices for Frequently Reported Compounds of Plant Essential Oils. *Journal of Physical and Chemical Reference Data*, **40**, 043101. http://dx.doi.org/10.1063/1.3653552

[17] Bos, R., Woerdenbag, H.J., Hendriks, H., Smit, H.F., Wikstroem, H.V. and Scheffer, J.J. (1997) Composition of the Essential Oil from Roots and Rhizomes of *Valerianawallichii* DC. *Flavour and Fragrance Journal*, **12**, 123-131. http://dx.doi.org/10.1002/(SICI)1099-1026(199703)12:2<123::AID-FFJ613>3.0.CO;2-4