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Cloning and Characterization of *δ*-Guaiene Synthase Genes Encoding a Sesquiterpene Cyclase from *Aquilaria microcarpa* Cell Cultures

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Abstract

Three cDNA clones encoding *δ*-guaiene synthase, a sesquiterpene cyclase, were isolated from tissue cultures of *Aquilaria microcarpa*, and data mining analysis of the orthologous genes suggested that 10 and 9 amino acid residues of N- and C-terminal ends of the translated products of these clones remained undefined. The recombinant enzyme proteins, to which the putative missing N- and C-terminal amino acid sequences (MSSAKLGSAS and ALLRHAIEI, respectively) were ligated, exhibited the catalytic activities of sesquiterpene biosynthesis. Among these three *δ*-guaiene synthases, two isoforms were capable of liberating *α*-guaiene, *δ*-guaiene, *β*-elemene plus *α*-humulene as a minor product, while remaining one isoenzyme generated *α*-, *δ*-guaiene and *β*-elemene but not *α*-humulene. Although the enzyme protein solely lacking in the N-terminal 10 amino acid residues was capable of synthesizing the sesquiterpenoids, the protein without 9 amino acids at C-terminal did not exhibit the catalytic activity. These results suggest that two types of *δ*-guaiene synthase; *α*-, *δ*-guaiene, *β*-elemene-producing type, and *α*-, *δ*-guaiene, *β*-elemene plus *α*-humulene-producing type; concomitantly occur in *A. microcarpa* cell cultures, and several amino acid residues at C-terminal of the synthase protein are essential to exhibit the catalytic activities as the sesquiterpene cyclase.

Keywords

Methyl Jasmonate, Yeast Extract, Sesquiterpene, *δ*-Guaiene Synthase, *Aquilaria microcarpa*

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1. Introduction

It has been widely known [1] that *Aquilaria*, and *Gyrinops* species sometimes form dark resinous heartwood, called agarwood, in the trunk of the plants in response to mechanical wounding or microbial infection. Agarwood produces a variety of sesquiterpene compounds with aroma, and, therefore, it has been used as the scent, perfume and traditional medicines [1]. These highly valuable tissues are not formed under healthy conditions, and artificial transformation of these trees to agarwood has been very difficult. Okudera and Ito, however, reported [2] that biosynthetic activities of several sesquiterpenoids, such as δ-guaiene, α-guaiene and α-humulene, are induced in cell cultures of *A. crassna* by the treatment with methyl jasmonate or yeast extract. Guaianolide sesquiterpene compounds are thought to be synthesized via two cyclization reactions [3]: i) C1-to-C10 cyclization generating germacrene-like macrocyclic intermediate; and ii) C2-to-C6 cyclization forming guaiane-skeleton (**Figure 1**). A part of the macrocyclic intermediate is converted to α-humulene or germacrene A without the second cyclization. It has been also demonstrated [4] [5] that β-elemene is an artificially formed compound by the pyrolysis of germecrene A. Recently, several cDNA clones encoding δ-guaiene synthase, a sesquiterpene cyclase, have been isolated from *A. crassna* and *A. sinensis* [3] [6]. Catalytic specificities of the translated proteins of these genes have been extensively studied, and it has been shown that the enzymes liberate multiple sesquiterpene compounds, such as δ-guaiene, α-guaiene, β-elemene and α-humulene, from farnesyl diphosphate as the substrate. However, only very little is known about the molecular basis of the enzymatic reaction of the synthase generating multiple products.

We have recently shown [5] [7] that treatment of cell cultures of *A. microcarpa* with methyl jasmonate or yeast extract results in the marked transcriptional activation of δ-guaiene synthase gene, and a cDNA clone (*GS*-1, GenBank accession No. KF800046) encoding the enzyme protein has been isolated [5]. It has been also demonstrated that *GS*-1 translate catalyzes the formation of δ-guaiene, α-guaiene and β-elemene, but not α-humulene [5]. In the present experiments, we have isolated three additional clones of the synthase gene although several amino acid residues at both N- and C-terminal ends of the translated proteins remain undefined. In order to understand the inducible biosynthetic activities of *A. microcarpa* liberating multiple sesquiterpene compounds, we have ligated putative missing amino acid sequences to N- and C-terminals of the recombinant proteins, and examined the catalytic properties of these 'deficiencies supplied' δ-guaiene synthases.

2. Materials and Methods

2.1. Cloning of δ-Guaiene Synthase Genes from *A. microcarpa* Cell Culture

Seeds of *A. microcarpa* were generous gift from Professor Shigetoshi Kadota, Institute of Natural Medicine, University of Toyama, and they were germinated and grown in a greenhouse at Experimental Station for Medi-

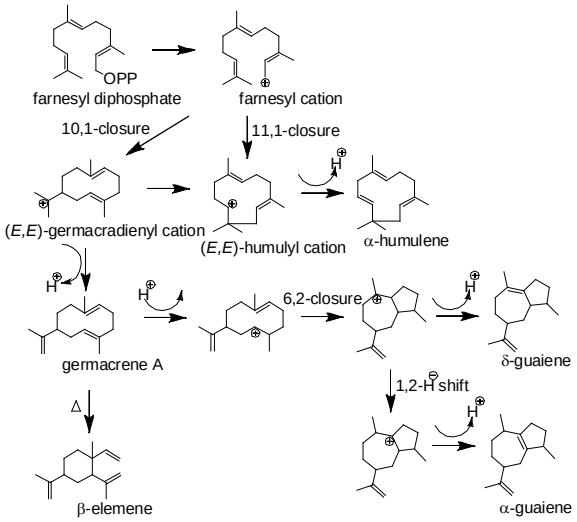

Figure 1. Predicted biosynthetic pathway of sesquiterpene compounds of *Aquilaria* plants.

cinal Plant Research of our university. Sterilized young leaf tissues of the plant were placed on Murashige and Skoog's agar medium [8] supplemented with 3% (w/v) sucrose and 3 μM of 2,4-dichlorophenoxyacetic acid, and callus formation was observed in the leaf veins after 4 weeks. The dedifferentiated cells were then incubated on the medium in the presence of 3 μM of 2,4-dichlorophenoxyacetic acid and 3 μM of N^6-benzyladenine at 26˚C under darkness. After establishment of the callus culture, the cells were transferred onto a fresh medium every 3 weeks as described previously in detail [9] [10]. Total RNA was isolated from yeast extract-treated cultured cells (approximately 100 mg) with RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) [5] [9], and cDNA templates were generated by reverse transcription reaction using Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). The cDNA fragments presumably encoding the translatable region of *δ*-guaiene synthase were isolated by PCR amplification employing the primer pair designated from the nucleotide sequence of the homologous gene previously isolated [5] (5'-ATG TCT TCG GCA AAA CTA GGT TCT GCC TCC-3' as the forward and 5'-TCA GAT TTC AAT AGC ATG ACG CAA CAA GGC-3' as the reverse primer). The amplified cDNAs were subcloned into the pGEM-T Easy vector (Promega, Fitchburg, WI, USA), and the nucleotide sequences were determined on both strands using the dye-terminator method on a PRISM 3130 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA).

2.2. Preparation and Purification of Recombinant *δ*-Guaiene Synthase Proteins

The modified *δ*-guaiene synthase genes, *GS*-2, 3 and 4, in which undefined sequences of the translatable regions were ligated, were amplified using a common primer pair (5'-ATG TCT TCG GCA AAA CTA GGT TCT GCC TCC-3' as the forward and 5'-TCA GAT TTC AAT AGC ATG ACG CAA CAA GGC-3' as the reverse primer, 1644 mer as the product). Amplification of *GS*-2*ΔNΔC*, the undefined regions-omitted gene, was performed with 5'-GAA GAT GTT AGC CGC CGA GAT GCC-3' as the common forward primer and 5'-TCA GGC AAT TCT ATC CTT GGT CAC-3' as the gene specific reverse primer employing pGEM-T Easy vector harboring *GS*-2 as the template. *GS*-3*ΔNΔC* and *GS*-4*ΔNΔC* were similarly amplified by the combination of the common forward primer and the gene specific reverse primer (5'-TCA GGC AAT TCT ACC CTT GGT CAC-3' for *GS*-3*ΔNΔC*, and 5'-TCA GGC AAT TCT ATC TTT GGT CAC-3' for *GS*-4*ΔNΔC*, respectively). *GS*-4*ΔN* and *GS*-4*ΔC* were also amplified by the appropriate combinations of the common primer and the specific primers (5'-GAA GAT GTT AGC CGC CGA GAT GCC-3' and 5'-TCA GAT TTC AAT AGC ATG ACG CAA CAA GGC-3' for *GS*-4*ΔN* while 5'-ATG TCT TCG GCA AAA CTA GGT TCT GCC TCC-3' and 5'-TCA GGC AAT TCT ATC TTT GGT CAC-3' for *GS*-4*ΔC*) using *GS*-4 subcloned into pGEM-T Easy vector as the template.

Over expression of *δ*-guaiene synthase genes in *Escherichia coli* was performed using the *E. coli* Expression System with Gateway Technology (Invitrogen, Waltham, MA, USA). Desired regions of the synthase genes amplified by PCR were successively subcloned into pENTR and pDEST15 (Invitrogen, Waltham, MA, USA), and then, *E. coli* BL21 cells were transformed with the constructed expression vectors. The cell cultures were grown in LB medium overnight at 25˚C, and, after transfer into the fresh medium, 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. The cultures were further incubated at 25˚C for 5 h. *δ*-Guaiene synthase proteins were recovered as the glutathione-S-transferase (GST) tagged form, and were purified by an affinity chromatography on a Glutathione-Sepharose 4B column (GE Healthcare Life Sciences, Little Chalfont, UK) according to the instruction manual. Protein concentrations were determined by the method of Bradford [11], and, if necessary, the purified enzyme solutions were appropriately concentrated by Amicon Ultra-15 (Merck Millipore, Billerica, MA, USA). The samples were, then, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10% gel) according to the method of Laemmli [12], and the separated proteins were stained with Coomassie brilliant blue.

2.3. Catalytic Activities of Recombinant *δ*-Guaiene Synthase Proteins

In order to examine sesquiterpene biosynthetic activities of the recombinant *δ*-guaiene synthases, the purified enzyme proteins were incubated with farnesyl diphosphate as the substrate, and the reaction products were analyzed by GC-MS. The assay mixtures (200 μl) contained 50 mM Tris-HCl (pH 7.0), 20 mM $MgCl_2$, 10 mM dithiothreitol, 46 μM farnesyl diphosphate (Sigma-Aldrich, St. Louis, MO, USA), 10% (v/v) glycerol, purified GST-*δ*-guaiene synthase proteins (approximately 10 μg) and limonene (0.2 μg, nacalai tesque, Kyoto, Japan) as

an internal standard. In a control experiment, the enzyme proteins were heat denatured (95˚C, 10 min) prior to the assay. The mixtures were incubated at 25˚C for 4 h, and the reaction products were extracted using headspace solid phase microextraction assembly with a 100 µm polydimethylsiloxane fiber (Supelco, Bellefonte, PA, USA). The fiber was exposed to the sample headspace for 30 min, and, after adsorption of the reaction products, sesquiterpene compounds were analyzed by GC-MS (Shimadzu, GCMS-QP5000; Agilent, J&W DB-1ms 0.32 mm × 30 m column) in the splitless mode. The injection port and the interface temperature were adjusted at 250˚C, and the mass range was set from m/z 40 to 400. The carrier flow (He) was at 1.4 ml/min. The oven temperature was started at 50˚C for 2 min and was increased to 170˚C at the rate of 4˚C/min, then raised to 300˚C at 30˚C/min. The reaction products were separated under the above conditions to calculate retention indices, and the identification of the compounds was based on the comparison of reported retention indices [13] [14] and mass spectra with those in the NIST02 and Wiley 7 MS databases.

3. Results

3.1. *δ*-Guaiene Synthase Genes of *A. microcarpa* Cell Culture

It has been demonstrated [3] [6] that sesuqiterpene biosynthetic genes encoding *δ*-guaiene synthase in *Aquilaria* plants are usually organized as the multigene family. To our knowledge, seven orthologues of the synthase genes have been isolated from *Aquilaria* plants (*A. crassna*, *A. sinensis* and *A. microcarpa*), and the translated products of these genes are found to be highly conserved. All of the synthase proteins consist of 547 amino acid residues and the sequences also show very high homology (98% - 99% identity) [3] [5] [6]. In order to isolate *δ*-guaiene synthase genes other than *GS*-1 from *A. microcarpa* cell cultures, we employed the primer pair corresponding to the N- and C-terminal ends of the translatable region of *GS*-1 (30 nucleotides each, including stop codon for C-terminal). Three new clones presumably encoding *δ*-guaiene synthase were isolated (*GS*-2*ΔNΔC*, *GS*-3*ΔNΔC* and *GS*-4*ΔNΔC*; GenBank accession No. KT283579, KT283580 and KT283581, respectively), and the putative amino acid sequences of the translated products showed high homology with *GS*-1 (**Figure 2**). Two specific motifs, the double arginine residue (RRX8W) at the N-terminal end and the aspartate rich motif (DDXXD) in the reaction center of the protein [15] [16], were observed in these three clones, and replacement of amino acid residues toward *GS*-1 was found at only three positions in *GS*-2, four in *GS*-3 and eight in *GS*-4, respectively (**Figure 2**).

Since nucleotide sequences corresponding to N- and C-terminal ends of *GS*-1 were employed as primers for PCR amplification, 10 and 9 amino acid residues of the terminals remained to be defined. A number of attempts were made to elucidate the undefined translatable and untranslatable regions of these three *δ*-guaiene synthase genes, and the rapid amplification of cDNA ends method have been systematically performed to obtain the DNA fragments. However, these experiments have not succeeded possibly because only very few nucleotides are replaced in the newly isolated three clones.

3.2. Preparation and Purification of Recombinant *δ*-Guaiene Synthase Proteins

Among seven *δ*-guaiene synthases reported from *Aquilaria* [3] [5] [6], six enzyme proteins share the common N-terminal sequence MSSAKLGSAS while remaining one is MSSAKLGSTS (**Table 1**). On the other hand, C-terminal sequence of all of the seven proteins is identical (ALLRHAIEI). Therefore, we ligated the most probable "missing" amino acid sequences to N- and C-terminal ends, and catalytic properties of these deficiencies-supplied enzymes (*GS*-2, *GS*-3 and *GS*-4) were examined together with the terminals-lacking translates of *GS*-2*ΔNΔC*, *GS*-3*ΔNΔC* and *GS*-4*ΔNΔC*. The recombinant enzymes were obtained as GST-tagged proteins, and were purified by an affinity chromatography on a Glutathione-Sepharose 4B column. In SDS-PAGE analysis (**Figure 3**), the protein bands corresponding to the position of GST-fused *GS*-2, 3 and 4 in IPTG-treated cell extracts (approximately 91.5 kDa) were shown to be sufficiently purified. The recombinant enzymes lacking in the terminal structures were also prepared as GST-tagged forms and purified in a similar manner.

3.3. Catalytic Activities of Recombinant *δ*-Guaiene Synthase Proteins

The recombinant *δ*-guaiene synthases of *A. microcarpa* were incubated with farnesyl diphosphate, and the reaction products were analyzed by GC-MS. We have previously reported [5] that translate of *GS*-1 liberates *δ*-guaiene, *α*-guaiene and germacrene A as the reaction products. We have also demonstrated [5] that germa-

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GS-1          MSSAKLGSASEDVSRRDANYHPTVWGDFFLTHSSNFLENNHSILEKHEELKQEVRNLLVVETSDLPSKIQLTDKIIRLGV 80
GS-2ΔNΔC      ----------EDVSRRDADYHPTVWGDFFLTHSSNFLENNHSILEKHEELKQEVRNLLVVETSDLPSKIQLTDKIIRLGV 70
GS-3ΔNΔC      ----------EDVSRRDANYHPTVWGDFFLTHSSNFLENNDNILEKHEELKQEVTNLLVVETSDLPSKIQLTDEIIRLGV 70
GS-4ΔNΔC      ----------EDVSRRDANYHPTVWGDFFLTHSSNFLENNDNILEKHEELKQEVRNLLVVETSDLPSKIQLTDKIIRLGV 70

GS-1          GYHFEMEIKAQLEKLHDHQLHLNFDLLTTSVWFRLLRGHGFSISSDVFKRFKNTKGEFETEDARTLWCLYEATHLRVDGE 160
GS-2ΔNΔC      GYHFEMEIKAQLEKLHDHQLHLNFDLLTTSVWFRLLRGHGFSISSDVFKRFKNTKGEFETEDARTSWCLYEATHLRVDGE 150
GS-3ΔNΔC      GYHFEMEIKAQLEKLHDHQLHLNFDLLTTSVWFRLLRGHGFSISSDVFKRFKNTKGEFETEDARTLWCLYEATHLRVDGE 150
GS-4ΔNΔC      GYHFEMEIKAQLEKLHDHQLHLNFDLLTTSVWFRLLRGHGFSISSDVFKRFKNTKGEFETEDARTLWCLYEATHLRVDGE 150

GS-1          DILEEAIQFSRKKLEALLPELSFPLNECVRDALHIPYHRNVQRLAARQYIPQYDAELTKIESLSLFAKIDFNMLQALHQS 240
GS-2ΔNΔC      DILEEAIQFSRKKLEALLPELSFPLNECVRDALHIPYHRNVQRLAARQYISQYDAELTKIESLSLFAKIDFNMLQALHQS 230
GS-3ΔNΔC      DILEEAIQFSRKKLEALLPELSFPLNECVRDALHIPYHRNVQRLAARQYIPQYDAELTKIESLSLFAKIDFNMLQALHQS 230
GS-4ΔNΔC      DILEEAIQFSRKKLEALLPELSFPLNECVRDALHIPYHRNVQRLAARQYIPQYDAELTKIESLSLFAKIDFNMLQALHQS 230

GS-1          ELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNRIIGITSLIDDTYDVYGTLEEVTLFTEAVER 320
GS-2ΔNΔC      ELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNRIIGITSLIDDTYDVYGTLEEVTLFTEAVER 310
GS-3ΔNΔC      ELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNRIIGITSLIDDTYDVYGTLEEVTLFTEAVER 310
GS-4ΔNΔC      ELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNRIIGITSLIDDTYDVYGTLEEVTLFTKAVER 310

GS-1          WDIEAVKDIPKYMQVIYTGMLGIFEDFKDNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIIS 400
GS-2ΔNΔC      WDIEAVKDIPKYMQVIYTGMLGIFEDFKDNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIIS 390
GS-3ΔNΔC      WDIEAVKDIPKYMQVIYTGMLGIFEDFKDNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIIS 390
GS-4ΔNΔC      WDIEAVQDIPKYMQVIYTGMLGIFEDFKDNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIIS 350

GS-1          GGYKMFIILMLIGRAEFELKETLDWASTIPEMVKASSLIARYIDDLQTYKAEEERGETVSAVRCYMREYGVSEEEACKKM 480
GS-2ΔNΔC      GGYKMFIILMLIGRAEFELKETLDWASTIPEMVKASSLIARYIDDLQTYKAEEERGETVSAVRCYMREYGVSEEEACKKM 470
GS-3ΔNΔC      GGYKMFIILMLIGRAEFELKETLDWASTIPEMVKASSLIARYIDDLQTYKAEEERGETVSAVRCYMREYGVSEEEACKKM 470
GS-4ΔNΔC      GGYKMFIILMLIGRGEFELKETLDWASTIPEMVKASSLIARYIDDLQTYKAEEKRGETVSAVRCYMREYGVSEEEACKKM 470

GS-1          REMIEIEWKRLNKTTLEADEISSSVVIPSLNFTRVLEVMYDKGDGYSDSQGVTKDRIAALLRHAIEI 547
GS-2ΔNΔC      REMIEIEWKRLNKTTLEADEISSSVVIPSLNFTRVLEVMYDKGDGYSDSQGVTKDRIA--------- 528
GS-3ΔNΔC      REMIEIEWKRLNKTTLEADEISSSVVIPSLNFTRVLEVMYDKGDGYSDSQGVTKGRIA--------- 528
GS-4ΔNΔC      REMIEIEWKKLNKTTLEANEISSSVVIPSLNFTRVLEVMYDKGDGYSDSQGVTKDRIA--------- 528
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Figure 2. Alignment of amino acid sequence of *δ*-guaiene synthases from *A. microcarpa*. Non-conserved amino acids were shaded, and GenBank accession numbers are as follows; *GS*-1, KF800046; *GS*-2*ΔNΔC*, KT283579; *GS*-3*ΔNΔC*, KT283580; *GS*-4*ΔNΔC*, KT283581.

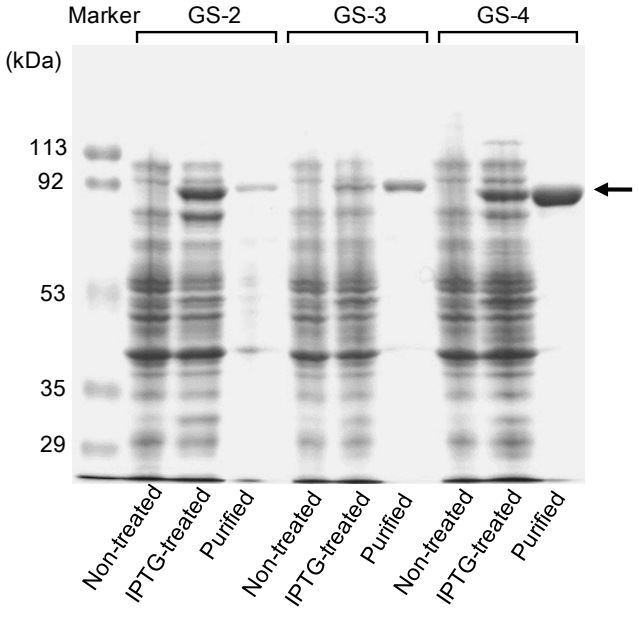

Figure 3. SDS-PAGE analysis of production and purification of *δ*-guaiene synthase proteins. *GS*-2, *GS*-3 and *GS*-4 were overexpressed in *E. coli* cells, and the translated products were obtained as GST-fusion proteins. The crude extracts prepared from non-treated control, IPTG-treated cells and the purified proteins were analyzed by SDS-PAGE, and the expected position of GST-fused *δ*-guaiene synthase (approximately 91.5 kDa) was indicated by an arrow.

Table 1. Amino acid sequences of N- and C-terminal ends of *δ*-guaiene synthases isolated from *Aquilaria* plants.

N-terminal end		
A. microcarpa GS-1	MSSAKLGSAS	10
A. sinensis-1	MSSAKLGSAS	10
A. sinensis-2	MSSAKLGSAS	10
A. sinensis-3	MSSAKLGSAS	10
A. crassna-1	MSSAKLGSAS	10
A. crassna-2	MSSAKLGSAS	10
A. crassna-3	MSSAKLGSAS	10
C-terminal end		
A. microcarpa GS-1	ALLRHAIEI	547
A. sinensis-1	ALLRHAIEI	547
A. sinensis-2	ALLRHAIEI	547
A. sinensis-3	ALLRHAIEI	547
A. crassna-1	ALLRHAIEI	547
A. crassna-2	ALLRHAIEI	547
A. crassna-3	ALLRHAIEI	547

GenBank accession numbers are as follows; *A. sinensis*-1, JQ712682; *A. sinensis*-2, JQ712683; *A. sinensis*-3, JQ712684; *A. crassna*-1, GU083697; *A. crassna*-2, GU083698; *A. crassna*-3, GU083699.

crene A is easily converted to *β*-elemene by pyrolysis, and, therefore, under the usual GC-MS conditions, together with *α*- and *δ*-guaiene, *β*-elemene was observed as the apparent product of the enzymatic reaction instead of germacrene A. As shown in **Figure 4(a)**, *GS*-2 produced at least three sesquiterpene compounds, and peak numbers 1, 2 and 4 were assigned to be *β*-elemene, *α*-guaiene and *δ*-guaiene by the retention indices and mass spectra (**Figure 5**) in the data base, respectively [13] [14]. In sharp contrast, detectable amount of the reaction products was not observed when the assay was carried out with the terminals sequences-omitted form, *GS*-2*ΔNΔC* (**Figure 4(a)**). Heat denatured enzyme proteins also did not show the catalytic activity (data not shown). *GS*-3 was also capable of producing *β*-elemene, *α*-guaiene and *δ*-guaiene as the major products, however, unlike in *GS*-1 [5] and *GS*-2, this protein was found to liberate *α*-humulene (peak 3) as an additional minor product (**Figure 4(b)** and **Figure 5**). As well as *GS*-2*ΔNΔC*, *GS*-3*ΔNΔC* did not exhibit the sesuqiterpene biosynthetic activity. *GS*-4 showed the catalytic specificity essentially similar to that of *GS*-3, and this enzyme produced *β*-elemene, *α*-guaiene, *δ*-guaiene and low amount of *α*-humulene (**Figure 4(c)** and **Figure 5**). As was in other *δ*-guaiene synthases, *GS*-4*ΔNΔC* was shown to be an inactive protein. In **Figure 4(a)**, it appeared that a minor peak could be observed at the retention time similar to *α*-humulene (peak 3), and, therefore, we carefully examine the possible formation of this sesquiterpene compound in *GS*-2-catalyzed reaction. However, based on the mass spectra analysis, it was revealed that the apparent minor peak was due to the formation of unknown compound but not *α*-humulene.

In order to elucidate whether or not both N- and C-terminal structures of *δ*-guaiene synthase are essential to exhibit the catalytic activity, 10 amino acid residues at N-terminal or 9 amino acids at C-terminal of the enzyme were selectively omitted, and the catalytic activities of these proteins were examined. In this set of the experiments, *GS*-4 which showed the highest recovery in the protein purification processes was selected, and N-terminal-lacking and C-terminal-lacking enzyme proteins (*GS*-4*Δ*N and *GS*-4*Δ*C) was prepared as the GST-fused form. As well as *GS*-4, N-terminal lacking enzyme *GS*-4*Δ*N exhibited the catalytic activity, if somewhat reduced, and the peaks of the sesquiterpene compounds were clearly detected (**Figure 6**). In sharp contrast, *GS*-4*Δ*C apparently did not show the enzymatic activity, and no peak of the reaction product was observed as far as tested. These results strongly suggest that 9 amino acid residues at C-terminal of *δ*-guaiene synthase proteins should be an essential structure to function as the sesquiterpene cyclase.

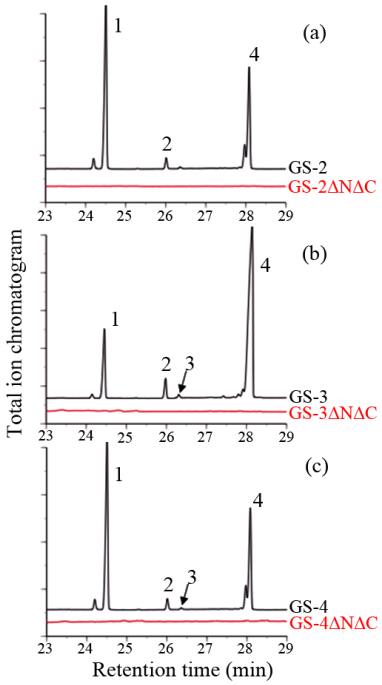

Figure 4. Total ion chromatograms of the reaction products formed by the incubation of recombinant GST-*δ*-guaiene synthase proteins with farnesyl diphosphate. (a) *GS*-2 and *GS*-2*ΔNΔC*; (b) *GS*-3 and *GS*-3*ΔNΔC*; (c) *GS*-4 and *GS*-4*ΔNΔC*.

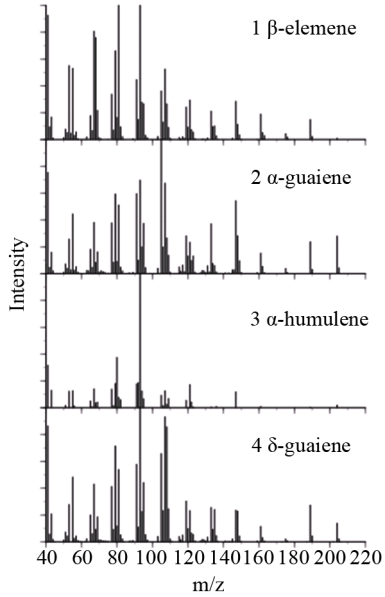

Figure 5. Mass spectra of the compounds corresponding to the peaks shown in **Figure 4**.

4. Discussion

As shown in **Figure 1**, *α*- and *δ*-guaiene are thought to be synthesized from germacrene A as an intermediate. On the other hand, *α*-humulene biosynthesis shares the common pathway with guaienes-forming processes only in the early stage. Kumeta and Itoh [3] isolated three *δ*-guaiene synthase genes from *A. crassna*, and showed that the translated proteins of all of these genes generate *δ*-guaiene, *α*-guaiene plus *α*-humulene as the minor product.

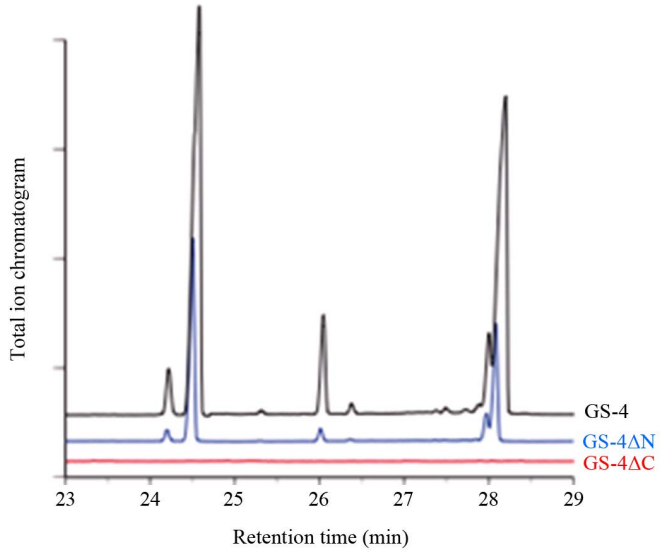

Figure 6. Total ion chromatograms of the reaction products liberated by recombinant *δ*-guaiene synthases; *GS*-4, *GS*-4*Δ*N and *GS*-4*Δ*C.

In contrast, Xu *et al*. have recently reported [6] that three homologous genes of *δ*-guaiene synthase were isolated from *A. sinensis*, and the translated proteins liberate *δ*-guaiene, *α*-guaiene and *β*-elemene but not *α*-humulene. Therefore, it is assumed that two classes of *δ*-guaiene synthase would occur in *Aquilaria* plants; *δ*-guaiene, *α*-guaiene and *β*-elemene producing group (*A. sinensis*), and *δ*-guaiene, *α*-guaiene and *α*-humulene producing group (*A. crassna*). We have previously reported [5] that *GS*-1, the translated product of a *δ*-guaiene synthase gene isolated from *A. microcarpa* cell culture, is capable of producing *δ*-guaiene, *α*-guaiene and *β*-elemene, however, the formation of *α*-humulene by *GS*-1 was not observed. In the present experiments, we have isolated three additional cDNA clones encoding the partial structures of *δ*-guaiene synthase (*GS*-2*ΔNΔC*, *GS*-3*ΔNΔC* and *GS*-4*ΔNΔC*) from *A. microcarpa* (**Figure 2**). We have also shown that the modified proteins, in which probable missing amino acid residues at N- and C-terminals were supplied (*GS*-2, *GS*-3 and *GS*-4), showed unique catalytic properties (**Figure 4** and **Figure 5**). The reaction products of *GS*-2 were *α*-guaiene, *δ*-guaiene and *β*-elemene, and liberation of *α*-humulene was not observed. Therefore, catalytic specificity of *GS*-2 was found to be similar to that of *GS*-1 isolated from *A. microcarpa* [5] and three *δ*-guaiene synthases from *A. sinensis* [6]. In contrast, *GS*-3 and *GS*-4 produced *α*- and *δ*-guaiene, *β*-elemene plus *α*-humulene, and, therefore, characteristics of the enzyme reaction of *GS*-3 and *GS*-4 should resemble the synthases from *A. crassna* [3]. These observations imply that two classes of *δ*-guaiene synthase, *α*-humulene producing and non-producing types, would concomitantly occur in *A. microcarpa* cells. In order to clarify the molecular bases of these unique specificities of *δ*-guaiene synthases in *Aquilaria* plants, preparation and characterization of several mutant proteins of the synthase are in progress in our laboratory in which the non-conserved amino acids presented in **Figure 2** are appropriately replaced.

Although recombinant *GS*-2, 3 and 4 proteins showed the biosynthetic activity of the sesquiterpene compounds, N- and C-terminals-deleted proteins, *GS*-2*ΔNΔC*, *GS*-3*ΔNΔC* and *GS*-4*ΔNΔC*, did not catalyze the terpenoids production (**Figure 4**). However, *GS*-4*Δ*N, deleted solely N-terminal end, clearly showed the enzymatic activity (**Figure 6**) while *GS*-4*Δ*C without C-terminal was found to lose the catalytic function. These observations strongly suggest that several amino acid residues at C-terminal end of *GS*-4 protein are essential to exhibit the terpene cyclase activity. We are attempting to construct the 3D model of the enzyme protein employing the suitable related protein(s) as the template for the elucidation of the biochemical functions of C-terminal amino acid sequence of *δ*-guaiene synthase.

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