Enzymatic $^{13}$C Labeling and Multidimensional NMR Analysis of Miltiradiene Synthesized by Bifunctional Diterpene Cyclase in Selaginella moellendorffii

Yoshinori Sugai, Yohei Ueno, Ken-ichiro Hayashi, Shingo Oogami, Tomonobu Toyomasu, Sadamu Matsumoto, Masahiro Natsume, Hiroshi Nozaki, and Hiroshi Kawaide

From the Institute of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan, Faculty of Science, Okayama University of Science, Okayama, Okayama 700-0005, Japan, and Faculty of Agriculture, Yamagata University, Tsuruoka, Yamagata 997-8555, Japan, and Tsukuba Botanical Garden, National Museum of Nature and Science, Tsukuba, Ibaraki 305-0005, Japan

Diterpenes show diverse chemical structures and various physiological roles. The diversity of diterpene is primarily established by diterpene cyclases that catalyze a cyclization reaction to form the carbon skeleton of cyclic diterpene. Diterpene cyclases are divided into two types, monofunctional and bifunctional cyclases. Bifunctional diterpene cyclases (BDTCs) are involved in hormone and defense compound biosyntheses in bryophytes and gymnosperms, respectively. The BDTCs catalyze the successive two-step type-B (protonation-initiated cyclization) and type-A (ionization-initiated cyclization) reactions of geranylgeranyl diphosphate (GGDP). We found that the genome of a model lycophyte, Selaginella moellendorffii, contains six BDTC genes with the majority being uncharacterized. The cDNA from $S$. moellendorffii encoding a BDTC-like enzyme, miltiradiene synthase (SmMDS), was cloned. The recombinant SmMDS converted GGDP to a diterpene hydrocarbon product with a molecular mass of 272 Da. Mutation in the type-B active motif of SmMDS abolished the cyclase activity, whereas (+)-copalyl diphosphate, the reaction intermediate from the conversion of GGDP to the hydrocarbon product, rescued the cyclase activity of the mutant to form a diterpene hydrocarbon. Another mutant lacking type-A activity accumulated copalyl diphosphate as the reaction intermediate. When the diterpene hydrocarbon was enzymatically synthesized from [U-$^{13}$C$_5$]mevalonate, all carbons were labeled with $^{13}$C stable isotope (>99%). The fully $^{13}$C-labeled product was subjected to $^{13}$C-$^{13}$C COSY NMR spectroscopic analyses. The direct carbon-carbon connectivities observed in the multidimensional NMR spectra demonstrated that the hydrocarbon product by SmMDS is miltiradiene, a putative biosynthetic precursor of tanshinone identified from the Chinese medicinal herb Salvia miltiorrhiza. Hence, SmMDS functions as a bifunctional miltiradiene synthase in $S$. moellendorffii. In this study, we demonstrate that one-dimensional and multidimensional $^{13}$C NMR analyses of completely $^{13}$C-labeled compound are powerful methods for biosynthetic studies.

Terpenoids are the largest class of natural products and function as primary and secondary metabolites including hormones, cell constituents, fragrances, and medicinal substances. Terpene cyclases are one of the committed enzymes in terpenoid biosynthesis that are involved in formation of carbon skeletons. In plants, several diterpene cyclases play crucial roles in hormone synthesis (1) and induce the production of defense compounds against pathogens (2). Cyclic diterpenes are derived from geranylgeranyl diphosphate (GGDP; $C_{20}$) through specific cyclases localized in plastids. Among them, the structure and function of ent-kaurene synthases are well characterized. ent-Kaurene is a tetracyclic hydrocarbon that is a biosynthetic intermediate phytosterol of gibberellins. It is formed from GGDP via ent-copalyl diphosphate (ent-CDP) by combination of the monofunctional ent-copalyl diphosphate synthase (CPS) and ent-kaurene synthase (KS) in flowering plants (3). By comparison, these steps are catalyzed by a single peptide bifunctional CPS/KS in moss (Physcomitrella patens) and liverwort (Jungermannia subulata) (4, 5). These non-vascular land plants have only a bifunctional ent-kaurene synthase. Fungal ent-kaurene synthases are also bifunctional, although the hydrocarbon product by SmMDS is miltiradiene, a putative biosynthetic precursor of tanshinone identified from the Chinese medicinal herb Salvia miltiorrhiza. Hence, SmMDS functions as a bifunctional miltiradiene synthase in $S$. moellendorffii. In this study, we demonstrate that one-dimensional and multidimensional $^{13}$C NMR analyses of completely $^{13}$C-labeled compound are powerful methods for biosynthetic studies.

* This work was supported in part by the NMR facility of RIKEN Yokohama Institute (non-proprietary use) and Research Fellowship for Young Scientists 227977 (to Y. S.) and Grant-in-aid for Scientific Research C 20510210 (to H. N.) from the Japan Society for Promotion of Science.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank(TM)/EBI Data Bank with accession number(s) AB668998.

1 To whom correspondence should be addressed. Tel.: 81-42367-5903; Fax: 81-42367-5698; E-mail: hkawaide@cc.tuat.ac.jp.

2 The abbreviations used are: GGDP, geranylgeranyl diphosphate; ADS, abietadiene synthase; BDTC, bifunctional diterpene cyclase; CDP, copalyl diphosphate; CPS, ent-copalyl diphosphate synthase; KS, ent-kaurene synthase; MDS, miltiradiene synthase; HSQC, heteronuclear single quantum coherence; INADEQUATE, incredible natural abundance double quantum coherence; RACE, rapid amplification of cDNA ends; rSmMDS, recombinant $\Delta N$98SmMDS fused with a His$_6$ tag.
their amino acid sequences are not significantly similar to the plant bifunctional CPS/KS (6, 7).

Conversion from GGDP into ent-CDP is initiated by protonation of a C=C double bond in the GGDP (type-B cyclization), and synthesis of ent-kaurene from ent-CDP is initiated by carbocation formation caused by release of a diphosphate group (type-A cyclization) (1). The type-B and type-A cyclizations require an aspartate-rich DXD motif near the N terminus and a DD motif near the C terminus, respectively. The monofunctional CPS and KS have the DXD and DD motifs, respectively, whereas the bifunctional CPS/KS from moss, liverwort, and fungi has both motifs (4, 5, 8). These characteristics are common among bifunctional diterpene cyclases (BDTCs) involved in labdane-related diterpene biosyntheses (3). For instance, the two motifs are also conserved in bifunctional abietadiene-related cyclases (9). The abietadiene and its related hydrocarbons are converted from GGDP via (+)-CDP and are responsible for the biosynthesis of resin acids in gymnosperms (10).

Notably, recent x-ray studies of the two monofunctional diterpene cyclases taxadiene synthase (type-A cyclase) and Arabidopsis CPS (type-B cyclase) revealed that they consist of three α-helical domains (α, β, and γ) (11, 12). The overall structure of taxadiene synthase and CPS are significantly similar, although the cyclization process they catalyze is quite different. Additionally, the mutation of a single amino acid in type-A cyclase affects the cyclization steps to change the product profile (5, 13). Therefore, it would be difficult to predict the structures of the cyclized products from GGDP on the basis of enzyme sequences. The functional analysis of an unidentified diterpene cyclase is a challenging task due to the numerous varieties of terpenoid structures, particularly if the phytochemical constituents are not known from the target plant species.

For confirmation of the identification of a novel compound, comparison with an authentic sample is desirable. However, the mass spectra of diterpene hydrocarbons are so similar that structural determination of compounds using this means is difficult. Spectroscopic analysis such as NMR gives crucial information about structures, although it requires a relatively large amount of sample for precise analysis. Diterpene hydrocarbons often have quaternary carbon atoms, which prevent proton detection by NMR analyses. Although INADEQUATE, a type of homonuclear 13C-13C NMR spectroscopy, can directly determine carbon-carbon connectivities, this method suffers from poor sensitivity and the low natural abundance of the 13C isotope (1.1%) (14). To overcome the faults, we established a full 13C labeling technique by using a one-pot terpenoid synthesis system (enzyme mixture) containing recombinant enzymes responsible for terpenoid biosyntheses. In our previous work, fully 13C-labeled gibberellins were synthesized from [U-13C6]mevalonate via ent-kaurene. In that report, one-dimensional NMR analysis of fully 13C-labeled ent-kaurene showed high sensitivity and carbon-carbon coupling (15). If such a fully 13C-labeled compound is analyzed by homonuclear multidimensional 13C NMR experiments, these spectra showing carbon-carbon connectivities would allow unambiguous determination of the chemical structures of various terpenoids with high sensitivity.

The lycophyte Selaginella moellendorffii is a model organism that has great interest as a plant system with a unique evolutionary placement. The complete genome of S. moellendorffii was sequenced, and almost all the genes were recently annotated (16). A homology search using a genomic database suggested that S. moellendorffii has at least 13 genes encoding labdane-related monofunctional diterpene cyclases or BDTCs (16, 17). The gymnosperms also have monofunctional diterpene cyclases for gibberellin biosynthesis and BDTCs for resin acid biosynthesis. However, resin acids and defense-related diterpenes have not been isolated and identified from ferns and lycophytes. The homologs of monofunctional CPS and KS in S. moellendorffii should be responsible for ent-kaurene synthesis leading to gibberellins (18). In contrast, little is known about the function and physiological roles of BDTCs in S. moellendorffii.

In this study, we adapted enzymatic total synthesis, 13C labeling, and multidimensional NMR techniques for the functional analysis of a BDTC of S. moellendorffii. Notably, we successfully synthesized a uniformly 13C-labeled product by a BDTC homolog (SmMDS) in S. moellendorffii in vitro and performed multidimensional NMR experiments to elucidate the structure of the product, miltiradiene. Miltiradiene was first isolated from a Chinese medicinal plant, Salvia miltiorrhiza, and was presumed to be an intermediate of tanshinone, a pharmaceutical secondary metabolite (19).

**EXPERIMENTAL PROCEDURES**

**Plant Material and Chemicals—**S. moellendorffii was from the Tsukuba Botanical Garden of the National Museum of Nature and Science. [U-13C6]Mevalonate was prepared by fermentation of Saccharomyces fibuligera. Recombinant enzymes for the labeled GGDP production were produced in Escherichia coli and purified with nickel-nitrilotriacetic acid resin (Qiagen, Valencia, CA) according to the manufacturer’s protocol. An enzyme mixture for GGDP synthesis was prepared by the methods described previously (15).

**Cloning for Full-length cDNA of SmMDS—**Total RNA was extracted from the aerial part of S. moellendorffii (300 mg wet weight) by using an RNAeasy® plant minikit (Qiagen) according to the manufacturer’s protocol. The poly(A)+ RNA was purified, and the cDNA was synthesized by reverse transcriptase with oligo(dT)18 primers (GE Healthcare) as described in a previous report (4). A gene-specific primer set (sense, 5′-TGCTC-GTCCGGAGGTGAG-3′; antisense, 5′-CACTTTTGCTCA-ATGTTGTCG-3′) was used for amplification of the target cDNA fragment by PCR. The temperatures for annealing and extension were 65 °C (30 s) and 72 °C (2.7 min), respectively. The 5′- and 3′-end sequences of the cDNA were determined using a GeneRacer kit (Invitrogen) according to the manufacturer’s protocols. PCR for the 5′-rapid amplification of cDNA ends (RACE) and 3′-RACE was performed using gene-specific primers as follows: 5′-AACGCTGTGGAGCAGCCGCAATCCG-3′ (antisense primer for primary PCR), 5′-TGCTTGTCAGCAGGATGACG-3′ (antisense primer for secondary PCR in 5′-RACE), 5′-CAAGGCCGCTGCTCA-ATGGTTGTCG-3′ (sense primer for primary PCR), and 5′-AAATCCATTCGCGC-
Bifunctional Mitiradiene Synthase in S. moellendorfii

TCCACGAC-3’ (sense primer for secondary PCR in 3’-RACE).

Heterologous Expression of SmMDS Gene and Recombinant Protein Production—The open reading frame cDNA encoding SmMDS lacking the predicted transit peptide sequence (Δ98) was amplified by PCR (Phusion Hi-Fi DNA polymerase, Finnzyme, Vantaa, Finland) using the following primer set: Δ98 sense primer 5’-ATGAGAGAACCTGCCGTGCTC-3’ (ATG for the start codon is underlined) and SmMDS anti-sense primer 5’-TCACAAAGTTCTATAAGTTTCTAAACA- AAAC-3’. The amplified cDNA fragment was treated with T4 polynucleotide kinase (Toyobo, Shiga, Japan) and successively introduced into the SmaI site in the pQE30 expression vector (Qiagen). This construct (pQE-SmMDS) was used for transformation of E. coli JM109. The transformant was incubated in 800 ml of 2× YT medium (1.6% tryptone, 1.0% yeast extract, and 1.0% NaCl adjusted pH 7.5 with NaOH) containing 100 μg/ml of ampicillin at 37 °C. When the A600 value reached 0.6, isopropyl 1-thio-β-d-galactopyranoside (1 mM) was added, and the cells were incubated at 18 °C for 24 h. The recombinant SmMDS protein was extracted from the cells as described previously (15) and purified with nickel-nitrilotriacetic acid-agarose resin (Qiagen).

Site-directed Mutagenesis of SmMDS—The two aspartate residues, Asp611 and Asp612 in the 611DDLMD motif and Asp391 and Asp392 in the 389DIDD motif, were substituted by glycine to abolish the type-A cyclase activity and type-B activity of SmMDS, respectively. According to the procedures described previously (15) and purified with nickel-nitrilotriacetic acid-agarose resin (Qiagen).

Enzymatic Synthesis and Purification of 13C-Labeled Hydrocarbon Product—The recombinant SmMDS protein (24 μg) was used for reaction with GGDG in the presence of 5 mEq magnesium ion. After L.5-h incubation at 28 °C, the product was extracted with cyclohexane and analyzed by GC-MS. For synthesis of the fully 13C-labeled product, SmMDS protein was mixed with GGDP synthetic enzyme mixture (15). The mixture contained six enzymes (1.3 mg of mevalonate kinase, 1.3 mg of phosphomevalonate kinase, 1.3 mg of diphosphomevalonate decarboxylase, 1.3 mg of isopentenyl diphosphate isomerase, 3.2 mg of GGDP synthase, and 11.5 mg of SmMDS), 5 mM MgCl₂, 10 mM ATP, and 7.5 mg of [U-13C₆]mevalonate as the substrate in 32 ml of reaction buffer. After the enzyme reaction at 28 °C for 12 h, the product was extracted three times with cyclohexane and concentrated under a gentle stream of dry N₂ gas. To purify the enzyme product, the extract was loaded onto a column packed with silica gel (Bond Elut SI, 500 mg/3 ml; Agilent Technologies, Santa Clara, CA) and eluted with cyclohexane. The flow-through fraction was further purified by HPLC (Silver Column KANTO (5-μm particle, 4.6-mm inner diameter × 250 mm; Kanto Chemical, Tokyo, Japan)) using cyclohexane as the mobile phase at 1 ml min⁻¹ (column temperature, 40 °C).

Spectroscopic Analysis—The 13C-labeled and non-labeled hydrocarbon products were analyzed by GC-MS and 13C NMR. GC-MS spectral data were measured with JMS-Bu25 (JEOL, Tokyo, Japan). The capillary column DB-5 (0.25-mm inner diameter × 15 m, 0.25-μm film thickness; J&W Scientific, Folsom, CA) was used with GC-MS conditions as described previously (15). The NMR analysis was performed at the NMR facility at RIKEN Yokohama Institute. The NMR sample was dissolved in cyclohexene-d₆ (99%) and aliquoted into the NMR tube (Shigemi microsample tube, 5-mm inner diameter; Shigemi, Tokyo, Japan). The solvent signals were used as the reference. NMR spectra were obtained at 300 K and a 1H reference frequency of 700 MHz on an Avance III spectrometer (Bruker, Karlsruhe, Germany). To clarify the resonances derived from quaternary carbon atoms, we acquired the one-dimensional 13C spectrum with an isotope-filtered module to purge the 1H-attached 13C resonances (20). Two-dimensional heteronuclear single quantum coherence (HSQC) experiments were recorded with a total of 1,024 and 256 complex points in the F2 and F1 dimensions, respectively. To discriminate the multiplicity of the attached proton and carbon atoms, edited HSQC and constant time HSQC as well as HSQC were measured for the SmMDS product (21, 22). Two-dimensional homonuclear correlation spectroscopy (13C-13C COSY) experiments were recorded using the pulse sequence reported previously in the literature (23) that describes the pulse data with spectral widths of 22,935.779 Hz (F2) and 22,884.779 Hz (F1) and with complex data points of 2,048 (F2) and 512 (F1). The three-dimensional HCCH COSY experiment was recorded by using the pulse scheme according to a report by Bax et al. (24). The spectral width was 10,504.202 Hz along the 1H (F3) dimension, 10,561.783 Hz along the 13C (F2) dimension, and 10,500.499 Hz along the 1H (F1) dimension. The number of complex data points was 1,024 (1H) × 64 (13C) × 128 (1H). The (H)CCH COSY was recorded using the pulse sequence reported previously (25). In addition, the CCH COSY experiments, which start with the evolution of the 13C magnetization, were performed by the modification of the (H)CCH COSY pulse sequence. The spectral widths were 10,504.202 Hz along the 1H (F3) dimension, 7,041.189 Hz along the 13C (F2) dimension, and 7,041.189 Hz along the 13C (F1) dimension. The number of complex data points were 1,024 (1H) × 128 (13C) × 128 (13C). All NMR data were processed using the programs TOPSPIN 3.0 (Bruker) and NMRpipe. Analyses of the processed data were performed using programs TOPSPIN 3.0 and NMRView (26) with KUJIRA (27).

RESULTS

Molecular Cloning and Characterization of SmMDS cDNA—A homology search suggested that S. moellendorfii has six putative BDTC genes possessing both DXDD and DDXXD motifs in their deduced amino acid sequences. Among them, we cloned an expressed cDNA encoding a putative BDTC from...
the aerial part by RT-PCR and named it SmMDS. The full-length sequence of the 2743-bp cDNA was determined by 5'- and 3'-RACE. Its open reading frame is 2604 bp encoding 867 amino acids (DDBJ accession number AB668998). This deduced amino acid sequence has a putative transit peptide and 3' UTR sequence of the 2743-bp cDNA was determined by 5'-RACE (H11032) and named it SmMDS. The full-length cDNA was amplified by RT-PCR from the aerial part by RT-PCR and sequenced. The open reading frame was determined by 5'- and 3'-RACE. Its open reading frame is 2604 bp encoding 867 amino acids (DDBJ accession number AB668998). This deduced amino acid sequence has a putative transit peptide and 3' UTR sequence of the 2743-bp cDNA was determined by 5'-RACE (H11032) and named it SmMDS. The full-length cDNA was amplified by RT-PCR from the aerial part and sequenced.

**Enzyme Activity of Recombinant SmMDS and Uniform 13C Labeling Experiments**—The recombinant SmMDS protein was expressed using a His6 tag (rSmMDS) was used for the conversion assay with a His6 tag (rSmMDS) was used for the conversion assay (100%), and 105 (60%). The GC-MS analysis on the 13C-labeled product showed good agreement with that of the theoretically 13C-labeled product (Fig. 1B) by comparison of natural abundance (Fig. 1A) (f<sub>r</sub> = 6.73 min; m/z 292 (M<sup>+</sup>, 54%), 276 (26%), 246 (12%), 158 (60%), 144 (100%), and 113 (79%)). The yield of 13C-labeled product was ~1 mg (26%; seven steps) from 7.5 mg of mevalonate estimated by GC total ion intensity.

**Determination of Copalyl Diphosphate Intermediate**—The two aspartate-rich motifs, 399DD and 611DDL, that are required for the type-B and type-A cyclization reactions, respectively, suggested that SmMDS catalyzes the successive cyclization of GGDP via a CDP intermediate. It was shown that a loss-of-function mutation in the DDXD motif in bifunctional ent-kaurene synthases from fungi and mosses resulted in the accumulation of an intermediate ent-CDP from GGDP (5). In the conversion assay of rSmMDS described above, the CDP intermediate and residual GGDP substrate were not detected. Hence, to abolish the type-A activity and consequently detect the CDP intermediate from the conversion of GGDP to hydrocarbon, both Asp<sup>611</sup> and Asp<sup>612</sup> residues were substituted by glycine (D611G/D612G rSmMDS). A recombinant D611G/D612G rSmMDS was prepared by an E. coli expression system and used for the enzyme assay. GC-MS analysis was carried out after dephosphorylation by alkaline phosphatase treatment to detect the alcohol derivative of the CDP intermediate. A single peak at 8 min was detected from incubation of GGDP and D611G/D612G rSmMDS, and its retention time and mass spectrum were identical to those of authentic ent-copalol (mass spectra of the peak in Fig. 2A, m/z 290 (M<sup>+</sup>, 5%), 275 (29%), 272 (12%), 257 (26%), 177 (21%), 137 (100%), and 82 (72%); mass spectra of the peak in Fig. 2B, m/z 290 (M<sup>+</sup>, 5%), 275 (21%), 272 (13%), 257 (23%), 177 (21%), 137 (98%), and 82 (100%). Authentic ent-copalol was obtained by dephosphorylation of the ent-CDP product from GGDP by recombinant Arabidopsis CPS (AtCPS). These data suggested that D611G/D612G rSmMDS might convert GGDP to ent-CDP.

However, we could not conclude that D611G/D612G rSmMDS was converting GGDP to ent-CDP because the retention time and mass spectrum of ent-copalol were identical to those of (+)-copalol in our GC-MS system. To determine the stereostructure of the CDP intermediate and to study the substrate specificity of the type-A domain of SmMDS, we carried out a conversion assay using three stereoisomers of CDP, (−)-CDP, (+)-CDP, and syn-CDP, as substrates. (+)-CDP and syn-CDP were obtained by GGDP conversion experiments using recombinant Phomopsis amygdali diterpene cyclase2 (PaDC2) and rice CPS4 (OsCPS4), respectively (30, 31). To remove the type-B activity of SmMDS, we prepared another mutant, D391G/D392G rSmMDS, in which both Asp<sup>391</sup> and Asp<sup>392</sup> residues were substituted by glycine. When the D391G/D392G rSmMDS mutant was incubated with ent-CDP, syn-CDP, or (+)-CDP, only (+)-CDP was converted to the diterpene hydrocarbon of which the GC-MS data were identical to those of the product obtained using wild type rSmMDS (Fig. 2...
From these results, we concluded that SmMDS converted a diterpene hydrocarbon via (−)-CDP.

SmMDS Is Bifunctional Miltiradiene Synthase—The mass spectrum of the rSmMDS product showed a molecular ion at m/z 272 corresponding to a diterpene hydrocarbon with five degrees of unsaturation. This was supported by 20 carbon signals in the quantitative inverse gated decoupling NMR spectrum of the 13C-labeled product (Table 1). The rSmMDS final product is cyclized from the (−)-CDP intermediate, suggesting that the stereostructure of the A/B ring junction would be retained from (−)-CDP. The 13C-13C COSY, which allows analysis of the carbon-carbon connectivity, showed cross-peaks from C8 to C17, establishing the connection of the C ring (C8–C14) and isopropyl group (C13–C17) as shown in Fig. 3. Additionally, the 13C-13C COSY correlations of C7/C8 and C9/C10 indicated the junction of the B ring and a C ring (Fig. 3A). However, the 13C-13C COSY cross-peaks from C4, C6, and C10 were unclear because these chemical shifts were close to those of C19, C2, and C1, respectively (Table 1). HCCH COSY correlations showed the connection of C1–C3 and C5–C7 that could not be determined by 13C-13C COSY (Fig. 3, D and E). To identify the correlation between quaternary carbon atoms (C4 and C10) and the vicinal protons, we performed (H)CCH COSY experiments as well as CCH COSY experiments. By comparison of the two spectra, we could clarify the correlation with quaternary carbon atoms, resulting in confirmation of the A/B ring structure corresponding to the A/B ring of (−)-CDP (Fig. 3F). The rSmMDS final product is cyclized from the (−)-CDP intermediate, suggesting that the stereostructure of the A/B ring junction would be the same configurations as that of (−)-CDP. Finally, the spectroscopic data of the product agreed with the published data of miltiradiene (19). These results demonstrated that SmMDS was a bifunctional miltiradiene synthase catalyzing the cyclization reaction of GGDP via (−)-CDP to afford miltiradiene as a final product (Fig. 4).

### TABLE 1

| Carbon number | Chemical shift* | Coupling pattern* |
|---------------|-----------------|-------------------|
| 1             | 37.80 ppm       | t                 |
| 2             | 19.80 ppm       | t                 |
| 3             | 42.77 ppm       | t                 |
| 4             | 33.94 ppm       | quintet           |
| 5             | 52.47 ppm       | q                 |
| 6             | 19.76 ppm       | t                 |
| 7             | 32.66 ppm       | t                 |
| 8             | 124.28 ppm      | dt                |
| 9             | 135.82 ppm      | dt                |
| 10            | 37.91 ppm       | quintet           |
| 11            | 26.18 ppm       | t                 |
| 12            | 117.14 ppm      | dd                |
| 13            | 139.98 ppm      | dt                |
| 14            | 33.84 ppm       | t                 |
| 15            | 35.22 ppm       | q                 |
| 16            | 21.65 ppm       | d                 |
| 17            | 21.50 ppm       | d                 |
| 18            | 22.02 ppm       | d                 |
| 19            | 33.64 ppm       | d                 |
| 20            | 19.90 ppm       | d                 |

* C6D12 was used as reference (26.4 ppm).
* Coupling patterns were directly observed in one-dimensional 13C NMR. quintet; q, quartet; d, doublet; t, triplet; dt, double triplet; dd, double doublet.

**FIGURE 2.** Determination of intermediate during cyclization reaction of SmMDS from GGDP to hydrocarbon product. The intermediate from the GGDP cyclization reaction was determined by functional analysis of SmMDS and the site-directed mutant proteins. A, AtCPS is a type-B cyclase containing a DIDD motif that catalyzes the cyclization reaction of GGDP to ent-CDP. The product of ent-CDP was determined by GC-MS in its dephosphorylated form, ent-copalol. The retention time was 8.00 min after injection. B, the mutant protein of SmMDS, D611G/D612G, lacked type-A activity because the DDLMD motif in SmMDS was modified. This mutant accumulated a possible intermediate at 8.00 min after injection at the same retention time as ent-copalol. C, recombinant SmMDS wild type produced diterpene hydrocarbon as the final product. D, the mutant protein D391G/D392G produced no diterpene product from GGDP because it lacked type-B activity due to the modification of the DIDD motif. Co-incubation of the D391G/D392G mutant and (−)-CDP produced the same diterpene product as that of SmMDS wild type.
DISCUSSION

It was previously known that plant BDTCs are responsible for ent-kaurene biosynthesis in bryophytes and for resin acid biosynthesis (such as abietadiene/levopimaradiene synthases) in gymnosperms. In this study, we identified a cDNA, SmMDS, encoding a bifunctional miltiradiene synthase via (+)-CDP. It was recently reported that another BDTC from *S. moellendorffii* catalyzes a two-step reaction from GGDP to produce labda-7,13E-diene-15-ol (32). Miltiradiene, an abietane-type diterpene, was first identified as the reaction product of a

FIGURE 3. Two-dimensional and three-dimensional NMR experiments revealed structure of SmMDS diterpene product as miltiradiene. A–C represent the three expanded regions of the two-dimensional $^{13}$C–$^{13}$C COSY spectra. A, low field (F2) × high field (F1) region; B, low field (F2) × low field (F1) region; C, high field (F2) × high field (F1) region. Because the chemical shifts of C2 and C6 are very close, the cross-peak of C7-C6 correlation observed by $^{13}$C–$^{13}$C COSY was further confirmed by HCCH COSY. D and E represent the two-dimensional constant time HSQC spectrum and three-dimensional HCCH COSY spectrum, respectively. D, constant time HSQC spectrum of the sample. In this spectrum, resonances in the low field region are decoupled by selective inversion pulse during $t_1$ evolution time. Negative peaks are boxed. E, F1/F3 slice of HCCH COSY spectrum. The plane corresponds to F2 equal to 36.6 ppm in which the C7 carbon resonates. F summarizes the correlations obtained from the present NMR study for the chemical shift assignment of miltiradiene. The bold lines indicate correlations confirmed by $^{13}$C–$^{13}$C COSY, the solid arrows indicate correlations determined by HCCH COSY, and the dashed arrows indicate the correlations confirmed by the comparison between (H)CCH COSY and CCH COSY experiments.
monofunctional type-A diterpene cyclase from *S. miltiorrhiza*, a traditional medicinal plant in China (19). Miltiradiene is also a putative biosynthetic intermediate of an abietane-type secondary metabolite, tan shinone, that has a variety of pharmaceutical activities. Although abietane-type diterpenes are biosynthesized by BDTCs in gymnosperms and SmMDS in *S. moellendorffii*, miltiradiene synthesis in *S. miltiorrhiza* involves two distinct monofunctional cyclases (SmiCPS and SmiKSL). Monofunctional diterpene cyclases responsible for secondary metabolite biosynthesis are also involved in the biosynthesis of steviosides in *Stevia rebaudiana* (33). In this case, these monofunctional cyclases are CPS and KS, and no bifunctional CPS/KS is used for production of steviosides. In our earlier work, we discovered that bryophytes such as *P. patens* and *J. subulata* use a bifunctional CPS/KS for *ent*-kaurene biosynthesis (4, 5). In fact, it is not known whether lycophytes and ferns produce defense-related diterpenes against pathogens and pests, although the diterpenes antheridiogens were identified from ferns (34). In contrast to non-vascular land plants, both the vascular plants lycophytes and gymnosperms may use BDTCs for the biosynthesis of secondary metabolites, whereas monofunctional CPS-like and KS-like genes found in the genome suggest that they may use monofunctional CPS and KS for *ent*-kaurene biosynthesis (17, 35). Although we could not identify miltiradiene from *S. moellendorffii*, further analyses are required for the study of secondary metabolites in lycophytes.

Recently, a spruce levopimaradiene/abietadiene synthase was found to initially produce a thermally unstable allylic alcohol (13-hydroxy-8(14)-abietene) after cyclization and carboxylation quenching reactions. Then the dehydration of the alcohol product occurs to form diterpene hydrocarbon compounds such as levopimaradiene, abietadiene, neoabietadiene, and palustradiene (36). AgADS is also known to be a multiprotein diterpene cyclase that produces levopimaradiene, abietadiene, and neoabietadiene (37). The product made enzymatically by SmMDS was carefully analyzed according to the enzyme assay procedure for AgADS, and the results indicated that SmMDS produced miltiradiene as the single product. The amino acid sequence of SmMDS is similar to that of AgADS (45% identity) whereas the *ent*-kaurene synthase from several plant species contains Gly (supplemental Fig. S1 and Ref. 38). Phylogenetic tree analysis shows that SmMDS is closer to the gymnosperm-specific group than SmiKSL (supplemental Fig. S2). This indicates that the BDTCs in the lycophyte-specific group may be ancestors of gymnosperm-specific BDTCs involved in the biosyntheses of a variety of secondary metabolites. Moreover, SmiKSL may not originate from SmMDS and instead may be independently derived from monofunctional type-A cyclases.

Our functional assay was based on an enzymatic total synthesis system that provides not only *in vitro* synthesis of terpenoids from acetate or mevalonate but also stable labeling of all carbon atoms. We previously successfully synthesized *in vitro* ent-kaurene, taxadiene, and amorphaadiene by combining the appropriate enzymes in a mixture (15). In this study, rSmMDS was added into the [13C]GGDP synthetic mixture to synthesize 13C-labeled miltiradiene. This fully 13C-labeled miltiradiene was analyzed by one-dimensional, two-dimensional, and three-dimensional NMR measurements. From the one-dimensional NMR experiments, inverse gated decoupling analysis was carried out to reveal peak numbers estimated from peak area. In addition, two-dimensional 13C-13C COSY spectra, not INADEQUATE, revealed the planar structure of miltiradiene. Both 13C-13C COSY and INADEQUATE can measure carbon–carbon connectivities of the carbon skeleton of organic molecules. However, the INADEQUATE pulse sequence was developed for compounds with a relatively low natural abundance level around 1–20% (14). Hence, 13C-13C COSY experiments could be utilized for 13C isotope-labeled (>99%) compounds that were enzymatically synthesized from [U-13C6]mevalonate. Thus, 13C-13C COSY and isotope labeling allow us to analyze relatively low quantities of sample synthesized by using enzyme mixtures. Both 13C-13C COSY and three-dimensional NMR experiments provided unequivocal assignment of all 13C NMR signals. From the literature values of the 13C NMR assignment for miltiradiene obtained from SmiKSL, δc 33.58 and δc 25.65 (in CDCl3) were assigned to C11 and C14, respectively (19). In our direct carbon–carbon connective analysis, C11 and C14 were noted at δc 26.18 and δc 33.84 (in C6D12), respectively. In addition, although it was reported that the signal derived from C19 was δc 14.34 (in CDCl3), the correct signal was observed at δc 33.64 (in C6D12). Such mistaken assignments of some signals were clearly corrected by the results of the direct connectivity obtained from the multidimensional NMR experiments using the 13C-labeled product. In contrast to proton detection NMR experiments, a 13C-13C COSY experiment can provide unambiguous structural information about carbons that are not attached to protons. Thus, completely 13C-labeled terpenoids synthesized by using enzyme mixtures combined with multidimensional NMR experiments can easily lead to precise struc-
Bifunctional Miltiradiene Synthase in S. moellendorffii

REFERENCES

1. MacMillan, J., and Beale, M. H. (1999) Comprehensive Natural Product Chemistry, pp. 217–243, Elsevier Science Ltd., Oxford
2. Trapp, S., and Croteau, R. (2001) Annu. Rev. Plant Physiol. Plant Mol. Biol. 52, 689–724
3. Toyomasu, T., and Sassa, T. (2010) Comprehensive Natural Product II Chemistry and Biology, pp. 643–672, Elsevier, Oxford
4. Hayashi, K., Kawaide, H., Notomi, M., Sakigi, Y., Matsu, A., and Nozaki, H. (2006) FEMS Lett. 580, 6175–6181
5. Kawaide, H., Hayashi, K., Kawanabe, R., Sakigi, Y., Matsu, A., and Natsume, M., and Nozaki, H. (2011) FEMS J. 278, 123–133
6. Kawaide, H., Imai, R., Sassa, T., and Kamiya, Y. (1997) J. Biol. Chem. 272, 21706–21712
7. Toyomasu, T., Kawaide, H., Ishizaki, A., Shinoda, S., Otsuka, M., Mitsuhashi, W., and Sassa, T. (2000) Biosci. Biotechnol. Biochem. 64, 660–664
8. Kawaide, H. (2006) Biosci. Biotechnol. Biochem. 70, 583–590
9. Vogel, B. S., Wildung, M. R., Vogel, G., and Croteau, R. (1996) J. Biol. Chem. 271, 23262–23268
10. Martin, D. M., Fäldt, J., and Bohlmann, J. (2004) Plant Physiol. 135, 1908–1927
11. Köksal, M., Iin, Y., Coates, R. M., Croteau, R., and Christianson, D. W. (2011) Nature 469, 116–120
12. Köksal, M., Hu, H., Coates, R. M., Peters, R. J., and Christianson, D. W. (2011) Nat. Chem. Biol. 7, 431–433
13. Wilderman, P. R., and Peters, R. J. (2007) J. Am. Chem. Soc. 129, 15736–15737
14. Claridge, T. D. W. (2009) High-Resolution NMR Techniques in Organic Chemistry, 2nd Ed., pp. 189–232, Elsevier, Oxford
15. Sugai, Y., Miyazaki, S., Mukai, S., Yumoto, I., Natsume, M., and Kawaide, H. (2011) Biosci. Biotechnol. Biochem. 75, 128–135
16. Banks, J. A., Nishiyama, T., Hasebe, M., Bowman, J. L., Gribskov, M., dePamphilis, C., Albert, V. A., Aono, N., Aoyama, T., Ambrose, B. A., Ashton, N. W., Axtell, M. J., Barker, E., Barker, M. S., Benetten, J. L., Bonawitz, N. D., Chapple, C., Cheng, C., Correa, L. G., Dacre, M., DeBarry, J., Dreyer, I., Elias, M., Enstrom, E. M., Estelle, M., Feng, L., Finet, C., Floyd, S. K., Frommer, W. B., Fujita, T., Gramzow, L., Gutenson, M., Harholt, J., Hattori, M., Heyl, A., Hirai, T., Hiwatashi, Y., Ishikawa, M., Iwata, M., Karol, K. G., Koehler, B., Kolukisaoglu, U., Kubo, M., Kurata, T., Lalonde, S., Li, K., Li, Y., Litt, A., Lyons, E., Manning, G., Maruyama, T., Michael, T. P., Mikami, K., Miyazaki, S., Morinaga, S., Murata, T., Mueller-Roeber, B., Nelson, D. R., Ohara, M., Oguri, Y., Olimstead, R. G., Onodera, N., Petersen, B. L., Pils, B., Prigge, M., Rensing, S. A., Riaño-Pachón, D. M., Roberts, A. W., Sato, Y., Scheller, H. V., Schulz, B., Schulz, C., Shakirov, E. V., Shibagaki, N., Shinohara, N., Shippen, D. E., Serensen, L., Sotooka, R., Sugimoto, N., Suga, M., Sumikawa, N., Tanurdzic, M., Theissen, G., Ulsvikov, P., Wakazuki, S., Weng, J. K., Willats, W. W., Wipf, D., Wolf, P. B., Yang, L., Zimmer, A. D., Zhu, Q., Mitros, T., Hellsten, U., Loquè, D., Ottillar, R., Salamov, A., Schmutz, J., Shapiro, H., Lindquist, E., Lucas, S., Rokhsar, D., and Grigoriev, I. V. (2011) Science 332, 960–963
17. Chen, F., Tholl, D., Bohlmann, J., and Pichersky, E. (2011) Plant J. 66, 212–229
18. Hirano, K., Nakajima, M., Asano, K., Nishiyama, T., Sakakibara, H., Kojima, M., Katoh, E., Xiang, H., Tanahashi, T., Hasebe, M., Banks, J. A., Ashikari, M., Kitano, H., Ueguchi-Tanaka, M., and Matsuoka, M. (2007) Plant Cell 19, 3058–3068
19. Gao, W., Hillwig, M. L., Huang, L., Cui, G., Wang, X., Kong, J., Yang, B., and Peters, R. J. (2009) Org. Lett. 11, 5170–5173
20. Breeze, A. L. (2000) Prog. Nucl. Magn. Reson. Spectrosc. 36, 323–372
21. Willker, W., Leibfritz, D., Kerssebaum, R., and Bormel, W. (1993) Magn. Reson. Chem. 31, 287–292
22. Vuister, G. W., and Bax, A. (1992) J. Magn. Reson. 98, 428–435
23. Nagayama, K., Kumar, A., Wüthrich, K., and Ernst, R. R. (1980) J. Magn. Reson. 40, 321–334
24. Bax, A., Cleare, G. M., and Gronenborn, A. M. (1990) J. Magn. Reson. 88, 425–431
25. Gehring, K., and Ekiel, I. (1998) J. Magn. Reson. 135, 185–193
26. Johnson, B. A. (2004) Methods Mol. Biol. 278, 313–352
27. Kobayashi, N., Iwashara, J., Koshiba, S., Tomizawa, T., Tochio, N., Güntert, P., Kigawa, T., and Yokoyama, S. (2007) J. Biomol. NMR 39, 31–52
28. Sun, T. P., and Kamiya, Y. (1994) Plant Cell 6, 1509–1518
29. Yamaguchi, S., Sun, T., Kawaide, H., and Kamiya, Y. (1998) Plant Physiol. 116, 1271–1278
30. Otomo, K., Kenmoku, H., Oikawa, H., König, W. A., Toshima, H., Mitsuhashi, W., Yamanaka, S., Sassa, T., and Toyomasu, T. (2004) Plant J. 39, 886–893
31. Toyomasu, T., Niida, R., Kenmoku, H., Kanno, Y., Miura, S., Nakano, C., Shinno, Y., Mitsuhashi, W., Toshima, H., Oikawa, H., Hoshino, T., Dairi, T., Kato, N., and Sassa, T. (2008) Biosci. Biotechnol. Biochem. 72, 1038–1047
32. Mafu, S., Hillwig, M. L., and Peters, R. J. (2011) ChemBioChem 12, 1984–1987
33. Richman, A. S., Gijzen, M., Starratt, A. N., Yang, Z., and Brandle, J. E. (1999) Plant J. 19, 411–421
34. Yamane, H., Satoh, Y., Nohara, K., Nakayama, M., Murofushi, N., Takanashi, N., Takeno, K., Furuya, M., Furber, M., and Mander, L. N. (1988) Tetrahedron Lett. 29, 3959–3962
35. Keeling, C. I., Dullat, H. K., Uyen, M., Ralph, S. G., Jancisik, S., and Bohlmann, J. (2010) Plant Physiol. 152, 1197–1208
36. Keeling, C. I., Madliao, L. L., Zerbe, P., Dullat, H. K., and Bohlmann, J. (2011) J. Biol. Chem. 286, 21145–21153
37. Peters, R. J., Flory, J. E., Jetter, R., Ravn, M. M., Lee, H. J., Coates, R. M., and Croteau, R. B. (2000) Biochemistry 39, 15592–15602
38. Zhou, K., and Peters, R. J. (2009) Phytochemistry 70, 366–369

Acknowledgments—We are grateful to Drs. Yutaka Muto (RIKEN Systems and Structural Biology Center, Yokohama, Kanagawa, Japan) and Hajime Sato (Bruker Biospin, Yokohama, Kanagawa, Japan) for help with multidimensional NMR measurements.