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Note

Cloning of a Full-length cDNA Encoding *ent*-Kaurene Synthase from *Gibberella fujikuroi*: Functional Analysis of a Bifunctional Diterpene Cyclase

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We report here the nucleotide sequence of a full-length cDNA encoding *ent*-kaurene synthase that was isolated by a reverse-transcription polymerase chain reaction from *Gibberella fujikuroi* (Gcps/ks). This cDNA encodes 952 amino acid residues with a relative molecular mass of 107 kDa. The sequence similarity between Gcps/ks and *ent*-kaurene synthase of the gibberellin A_1-producing fungus, *Phaeosphaeria* sp. L487, is very high, suggesting that Gcps/ks is also a bifunctional diterpene cyclase. Its recombinant protein expressed in *Escherichia coli* converted geranylgeranyl diphosphate to copalyl diphosphate and *ent*-kaurene.

Key words: diterpene cyclase; *ent*-kaurene synthase; *Gibberella fujikuroi*; gibberellin biosynthesis

Gibberella fujikuroi is a rice pathogenic fungus producing large quantities of gibberellins (GAs), diterpenoid phytohormones, which regulate various aspects of plant development. Although such fungi as *Phaeosphaeria* sp. L487[1,2] also produce GAs, *G. fujikuroi* was historically paramount in the discovery of GAs; GAs were first isolated from its culture filtrate by Kurosawa[3] and crystallized by Yabuta and Sumiki.[4] This fungus has been used to study gibberellin biosynthetic enzymes because of its ability to produce large quantities of GAs. *ent*-Kaurene is an important hydrocarbon precursor of GAs,[5,6] and is formed by the two-step cyclization of geranylgeranyl diphosphate (GGDP) via copalyl diphosphate (CDP) (Fig. 1). Fall and West[7] have partially purified an *ent*-kaurene synthase from *Fusarium moniliforme*, an anamorph of *G. fujikuroi*, which had an estimated relative molecular mass of 430 to 490 kDa. A cDNA encoding *ent*-kaurene synthase has recently been isolated from *Phaeosphaeria*. This is designated as Pcps/ks in our paper, and its recombinant protein expressed in *E. coli* converted GGDP to *ent*-kaurene.[8] This showed the fungal *ent*-kaurene synthase to be a bifunctional enzyme, although this two-step cyclization in plants is catalyzed by two enzymes, CDP synthase and *ent*-kaurene synthase.[9] In this note, we report the isolation of a full-length cDNA encoding *ent*-kaurene synthase from *G. fujikuroi* and the biochemical function of the translational product as a bifunctional enzyme similar to Pcps/ks.

mRNA was extracted and purified from 3-day-cultured mycelia which were growing and producing GAs logarithmically. About an 870-bp band was amplified by the reverse-transcription polymerase chain reaction (RT-PCR) with the primers de-

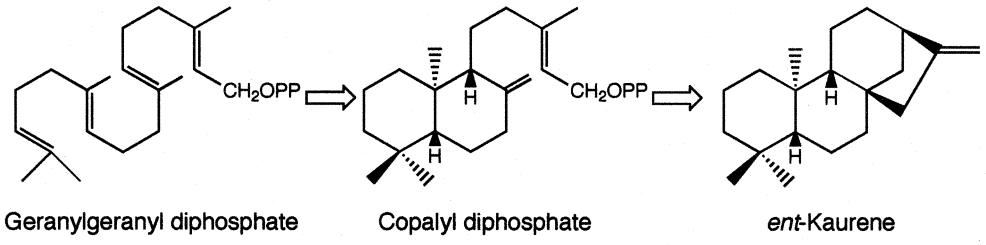

Fig. 1. Conversion Steps from Geranylgeranyl Diphosphate to *ent*-Kaurene.

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Abbreviations: CDP, copalyl diphosphate; GA, gibberellin; GGDP, geranylgeranyl diphosphate; RACE, rapid amplification of cDNA ends; RT-PCR, reverse-transcription polymerase chain reaction

scribed by Kawaide *et al.*,[8] and a sequence analysis showed that this band contained one fragment which is highly homologous to a partial sequence of Pcps/ks cDNA. The nucleotide sequence of the full-length cDNA was determined by 5′-rapid amplification of the cDNA ends (5′-RACE) and 3′-RACE by using gene-specific primers. This contained the predicted 2856-bp open reading frame, encoding a product of 952 amino acids, which was named Gcps/ks. Homology searches indicate that the derived amino acid sequences of Gcps/ks have a high level of similarity to other diterpene cyclases, especailly to Pcps/ks (45% identity). Figure 2 shows the alignment of Gcps/ks with Pcps/ks. Independently, Tudzynski *et al.*[10] isolated and published a genomic clone (accession No. Y15013) and a tentative cDNA sequence from *G. fujikuroi*. However, their deduced amino acid sequence is different from that of our Gcps/ks. Significant differences are that Met[1]-Pro[2] and Leu[383] in our sequence are replaced by Met[1]-Ser[8] and Pro[389]-Gly[390] in their sequence. A comparison of the sequence of our cDNA with that of their genomic DNA (Fig. 3) suggests that G[800]-G[848] in their genomic sequence is the first intron and that G[1690]-G[1738], which they deduced as the first intron, is in fact the second intron. We further suggest that the 5′ end of our cDNA is C[723] in their genomic sequence, and that the start codon in our cDNA is lo-

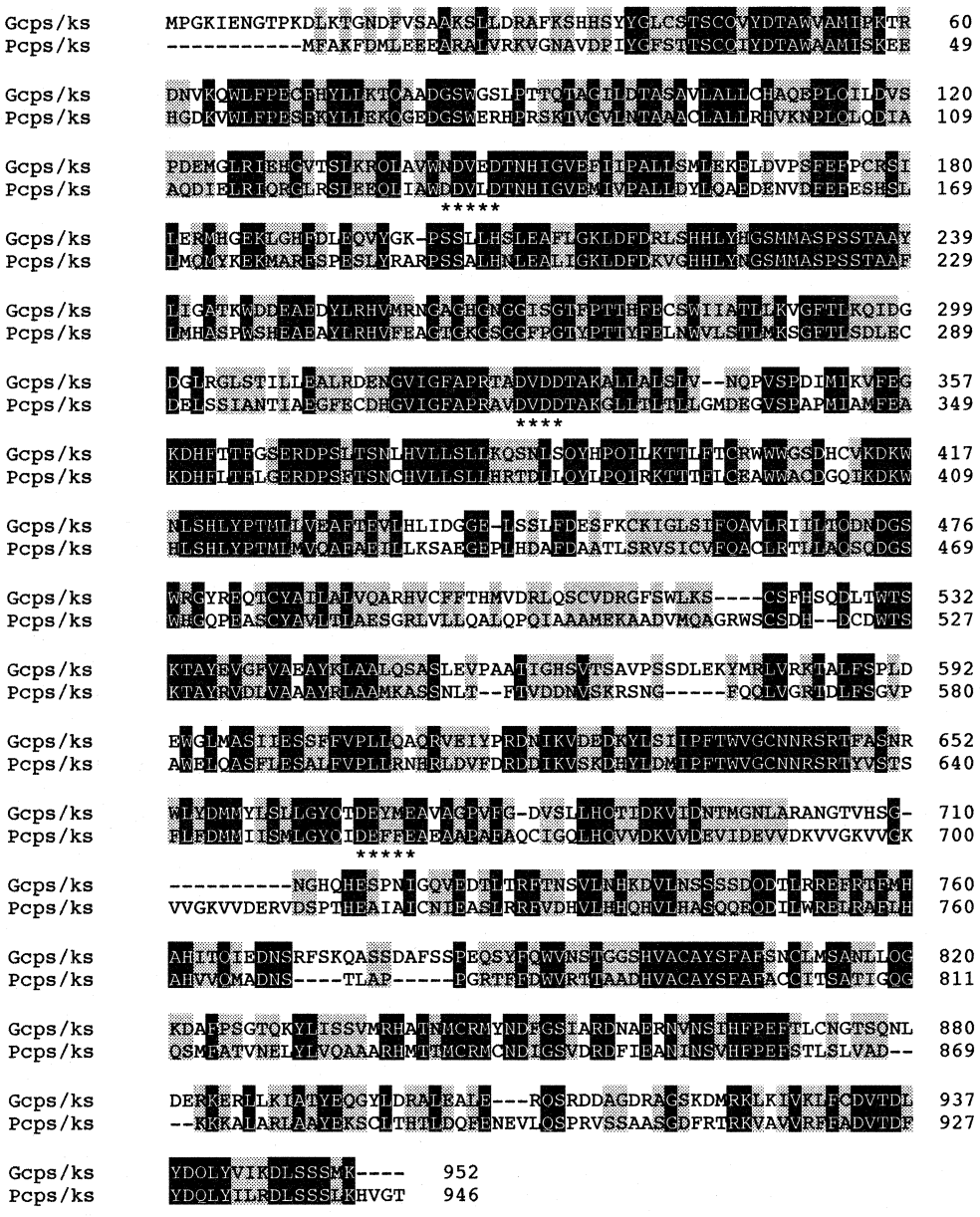

Fig. 2. Alignment of the Deduced Amino Acid Sequence of Gcps/ks with That of Pcps/ks.

The sequence data for Gcps/ks appears in the EMBL, DDBJ and GenBank nucleotide databases with the accession number, AB012203. The accession number of Pcps/ks is AB003395. Black and shaded boxes indicate identical and similar residues, respectively. Asterisks indicate the aspartate- and aspartate/glutamate-rich motifs: D(N)DxxD, DxDD and DExxE.

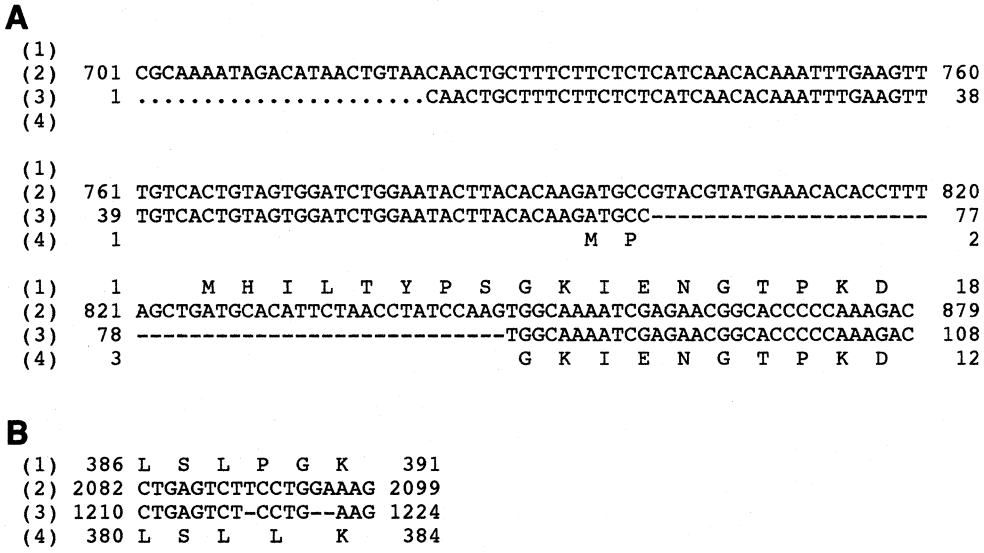

Fig. 3. Comparison between the Sequences of Gcps/ks cDNA and Genomic DNA.
A and B indicate the 5′ end and middle part, respectively. (1) amino acid sequence deduced by Tuzynski;[10] (2) genomic DNA sequence (No. Y15013); (3) cDNA sequence (No. AB012203); (4) amino acid sequence deduced from the cDNA sequence.

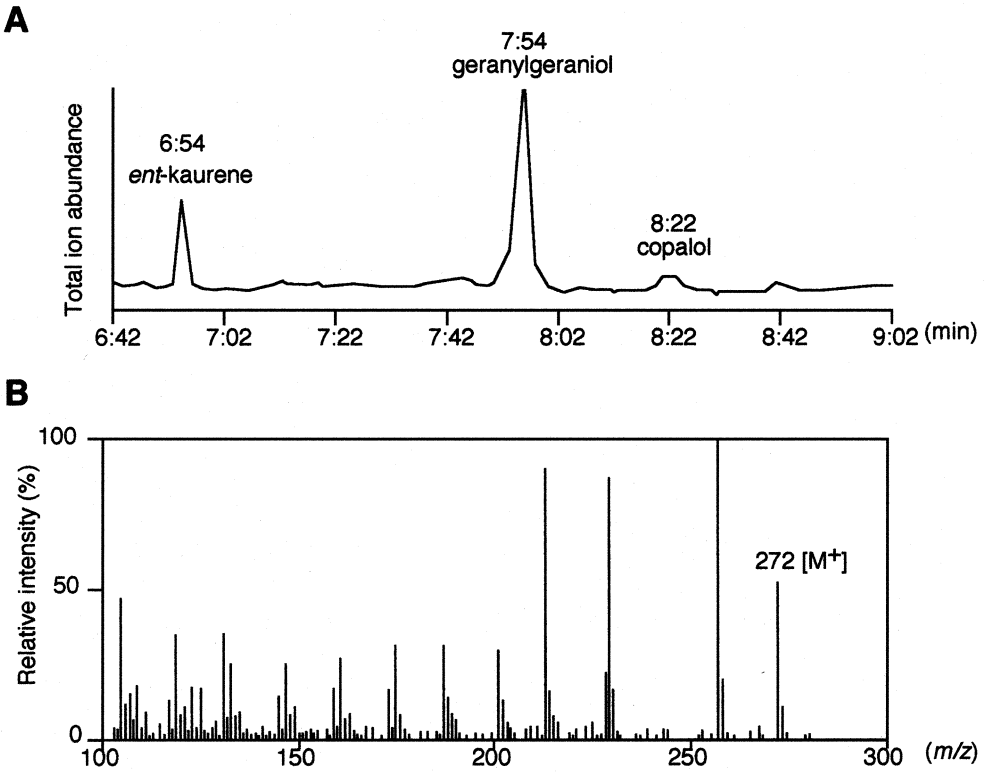

Fig. 4. Two-step Cyclization Reaction from GGDP to *ent*-Kaurene Catalyzed by the Recombinant Gcps/ks Fusion Protein.
A; Total ion chromatogram of the *n*-hexane extract after incubating GGDP with the protein. B; Full-scan mass spectrum of the peak at 6:54. This shows the same features as that of authentic *ent*-(−)-kaurene.

cated at position A^{795}-G^{797} (Fig. 3A). According to their nucleic acid sequence, another 5′-end gene-specific primer, whose partial sequence might be located in the first intron, was designed. However, no band could be amplified by RT-PCR when using this as the sense primer (data not shown). It is possible that Tudzynski *et al.*[10] missed the first intron when they deduced the mRNA sequence. The second difference is Leu^{383} in our sequence (Fig. 3B), this being well conserved in Pcps/ks as Leu^{375} (Fig. 2). It is also suggested that T^{2090}, G^{2095} and A^{2096} in their genomic sequence should be omitted (Fig. 3B). We

report here the correct nucleotide and deduced amino acid sequences of Gcps/ks cDNA.

This polypeptide has an estimated relative molecular mass of 107 kDa, which implies that the 430–490 kDa protein partially purified by Fall and West[7] might be a tetramer of the 107-kDa polypeptide. Aspartate- and aspartate/glutamate-rich motifs are well conserved in the predicted amino acid sequences of both Gcps/ks and Pcps/ks, these being marked in Fig. 2. These three motifs show that Gcps/ks is probably a bifunctional enzyme like Pcps/ks, as described by Kawaide *et al.*[11] We carried out a functional analysis by using the recombinant protein expressed in *Escherichia coli*. RT-PCR was performed by using the 5′-end and 3′-end gene-specific primers to amplify the open reading frame, including the stop codon. A full-length cDNA was successfully amplified and ligated into a pGEX 4T-3 vector for a protein-expression analysis. Figure 4 shows the results for the GC-MS analysis of GGDP conversion with the recombinant Gcps/ks fusion protein. Figure 4A shows two major peaks and one minor peak on the total ion chromatogram. A comparison of their full-scan mass spectra and retention times with those of authentic compounds (data not shown) suggests that the peaks at 6:54, 7:54 and 8:22 after injection are *ent*-kaurene, geranylgeraniol derived from a residual substrate, and copalol, respectively. The full-scan mass spectrum of the peak at 6:54 (*ent*-kaurene) is shown in Fig. 4B, while the peak at 8:22 was also identified as a copalol; *m/z* (relative intensity): 290 ($[M]^+$, 8), 275 (73), 257 (100). Geranylgeraniol and copalol were derived from GGDP and CDP by treating with alkaline phosphatase. Although strong peaks for the substrate and *ent*-kaurene were detected, the peak derived from CDP was only at a trace level. This confirms that the Gcps/ks protein catalyzed a two-step cyclization reaction of GGDP to *ent*-kaurene via CDP, similar to Pcps/ks.[8]

Experimental

Culture of the mycelia. Wild-type strain IFO30336 of *Gibberella fujikuroi* from the Institute for Fermentation (Osaka, Japan) was used. A mycelial agar plug of *ca.* 4×4 mm from a potato-glucose-agar slant of the fungus was inoculated into 500-ml Sakaguchi flasks containing 100 ml of a yeast extract medium (8% (w/v) glucose, 0.12% (w/v) NH_4NO_3, 0.5% (w/v) KH_2PO_4, 0.1% (w/v) $MgSO_4 \cdot 7H_2O$, and 0.5% (w/v) yeast extract). After incubating it by shaking for 4 days at 25°C, 3 ml of this culture broth was transferred to a 500-ml Sakaguchi flask containing 100 ml of a Pharmamedia medium (8% (w/v) glucose, 0.12% (w/v) NH_4NO_3, 0.5% (w/v) Pharmamedia, 0.5% (w/v) KH_2PO_4, and 0.1% (w/v) $MgSO_4 \cdot 7H_2O$). The mycelia were filtered, and 2 g was stored in liquid nitrogen. The culture filtrate was used for a GA analysis, contents of GA_3 being estimated from the peak area profiled by gas chromatography.

RT-PCR. The same degenerate primers which were described in Kawaide *et al.*[8] were used for cloning *ent*-kaurene synthase: forward primer (sense), 5′-GCITA(CT)GA(CT)ACIGCITGG-3′; reverse primer (antisense), 5′-(AG)AAIGCATGIGCIGT(AG)TC(AG)TC-3′. Total RNA was extracted from the frozen mycelia, cultured for 3 days by the SDS-phenol method, and double-stranded cDNA was synthesized according to the method described previously.[8] Twenty nanogram of each double-stranded cDNA was used as a template for PCR. The reaction mixture (50 μl) contained 200 μM dNTPs, 1.5 mM $MgCl_2$, 1 μM of each primer, and 2.5 U of Expand HF (Boehringer, Indianapolis, IN, U.S.A). Samples were heated to 95°C for 2 min, and then subjected to 40 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min, with a final extension for 7 min. 5′- and 3′-RACE were carried out according to the method described previously[12] to determine the nucleotide sequence of the full-length cDNA. The design of the gene-specific primers was based on the nucleotide sequence of the PCR fragment. To determine the 5′ end of cDNA in 5′-RACE, two clones with the longest fragments were selected from among six clones.

Amplification of a full-length cDNA. The double-stranded cDNAs just described were used as templates. PCR was carried out with the 5′- and 3′-end primers to amplify the coding region: forward primer, 5′-GGATCCATGCCTGGCAAAATCGAG-3′ (sense) and reverse primer, 5′-CTCGAGTCACTTCATGCTGCTTGA-3′ (antisense). Both primers consisted of a gene-specific sequence and incorporated a restriction enzyme site at the 5′ end: *Bam*HI for the 5′-end primer and *Xho*I for the 3′-end primer. According to the putative sequence of cDNA encoding *ent*-kaurene synthase that has been registered by Tudzynski *et al.*,[10] another forward primer, 5′-GGATCCATGCACATTCTAACCTATCC-3′, was synthesized. The PCR conditions were as already described, except that the annealing temperature was 50°C and the extension time was 2 min. The fidelity of the full-length nucleotide sequence was confirmed by comparing the sequences of three independently amplified fragments.

Cloning and sequence analysis of the PCR products. The PCR products were purified by agarose gel electrophoresis and ligated into a pCRII vector by using the TA cloning kit (Invitrogen, San Diego, CA, U.S.A.). The ligation products were introduced into *E. coli* JM109 and recombinant clones

were selected. The nucleotide sequence of each clone was determined with a Taq DyePrimer cycle sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.) and an ABI 377 DNA sequencer (Applied Biosystems). Homology searches of the EMBL, GenBank, PIR and SwissProt data bases were performed by using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and the amino acid sequences were aligned with Clustal W (http://www.clustalw.genome.ad.jp/).

Expression of the Gcps/ks gene in E. coli and purification of the recombinant protein. A full-length cDNA insert was excised from a plasmid by using the restriction enzymes, *Bam*HI and *Xho*I, and inserted into the pGEX 4T-3 vector (Pharmacia). *E. coli* strain JM109 harboring the pGEX-Gcps/ks plasmid was incubated overnight in 5 ml of 2×YT broth containing ampicillin (100 μg ml^{-1}) and glucose (0.1% w/v) at 37°C. The culture broth was transferred into 1 liter of the fresh medium and incubated at 30°C for 4 h. When the growth of *E. coli* had reached $OD_{600}=0.7$, the flask was kept on ice for 30 min. After adding isopropyl-thio-β-D-galactopyranoside (IPTG, 1 mM final conc.), the culture was started again at 17°C for 24 h. Cells were collected by centrifuging at 2000×*g* and 4°C for 15 min and washed with 40 ml of a 50 mM Tris-HCl buffer (pH 8.0). The washed cells (3.5 g wet weight) were resuspended in 17.5 ml of a lysis buffer of 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 0.5 mM EDTA, 10% glycerol and proteinase inhibitor cocktail tablets (Complete, Boehringer Manheim), before being treated with 10 mg of lysozyme (egg white, Seikagaku Corp.). The digested cells were disrupted by sonication (2×30 s treatment) and then centrifuged at 10000×*g* for 40 min at 4°C to obtain a soluble protein fraction. The recombinant GST-Gcps/ks fusion protein was purified by using Sepharose 4B glutathione affinity resin (Amersham Pharmacia Biotech) in accordance with the supplier's instructions.

Enzyme assay. GGDP (1 μg) was incubated with 5 μg of purified GST-Gcps/ks and 5 mM $MgCl_2$ in 500 μl of a reaction buffer of 50 mM Tris-HCl (pH 8), 5 mM DTT, 0.5 mM EDTA, 10% glycerol and proteinase inhibitor cocktail tablets at 30°C for 1 h. The enzyme reaction was terminated by heating to 60°C for 20 min. CDP as the product and residual GGDP were dephosphorylated with 6 units of bacterial alkaline phosphatase (Nippon Gene, Japan) at 37°C for 2 h. The reaction was stopped by adding 500 μl of acetone, and the product was extracted twice with 1 ml of *n*-hexane. The *n*-hexane fraction was evaporated with a gentle stream of dry N_2 gas and finally subjected to GC-MS (GCQ, Thermoquest, California, U.S.A.).[8]

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