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 diterpenoid A₄-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 also produce GAs, G. fujikuroi was historically paramount in the discovery of GAs; GAs were first isolated from its culture filtrate by Kurosawa and crystallized by Yabuta and Sumiki. 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, and is formed by the two-step cyclization of geranylgeranyl diphosphate (GGDP) via copalyl diphosphate (CDP). Stepwise, this cyclization is catalyzed by two enzymes, CDP synthase and ent-kaurene synthase. 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 Gcps/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 designed from the deduced amino acid sequence as the template.

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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. 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, especially to Pcps/ks (45% identity). Figure 2 shows the alignment of Gcps/ks with Pcps/ks. Indepen-
dently, Tuzynski et al. 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 Met1-Pro2 and Leu359 in our sequence are replaced by Met1-Ser4 and Pro189-Gly490 in their sequence. A comparison of the sequence of our cDNA with that of their genomic DNA (Fig. 3) suggests that G190-G148 in their genomic sequence is the first intron and that G169-G178, which they deduced as the first intron, is in fact the second intron. We further suggest that the 5' end of our cDNA is C721 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.
A

(1) 761 TGGCAATAGGATCTGGACTTCTCTCTCTGATACAAAAAACATTTCTGAAAAGTT 760
(2) 38
(3) 7  
(4) 4

(1) 1  M H I L T Y P S G K I E N G T P K D 18
(2) 821 AGCTGATGACACATTCTAACCTATCAAATCAGAAAGATGCCGTACGTATGAAILCACCTCTTT 879
(3) 78  
(4) 3

B

(1) 386 L S L P G K 391
(2) 2082 CTGAGCTCTCTAAGAC 2099
(3) 1210 CTGAGTCT--CTG---AAG 1224
(4) 380 L S L L K 384

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; (2) genomic DNA sequence (No. Y15013); (3) cDNA sequence (No. AB012203); (4) amino acid sequence deduced from the cDNA sequence.

cated at position A705-G707 (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 Tuzynski et al. missed the first intron when they deduced the mRNA sequence. The second difference is Leu383 in our sequence (Fig. 2), this being well conserved in Pcps/ks as Leu385 (Fig. 2). It is also suggested that T2096, G2095 and A2096 in their genomic sequence should be omitted (Fig. 3B). We

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.
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 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, 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.3

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) NH₄NO₃, 0.5% (w/v) KH₂PO₄, 0.1% (w/v) MgSO₄·7H₂O, 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) NH₄NO₃, 0.5% (w/v) Pharmamedia, 0.5% (w/v) KH₂PO₄, and 0.1% (w/v) MgSO₄·7H₂O). The mycelia were filtered, and 2 g was stored in liquid nitrogen. The culture filtrate was used for a GA analysis, contents of GA, being estimated from the peak area profiled by gas chromatography.

RT-PCR. The same degenerate primers which were described in Kawaide et al.10 were used for cloning ent-kaurene synthase: forward primer (sense), 5'-GCITA(CT)GAI(CT)ACIGGTTG-3'; reverse primer (antisense), 5'-AGAAIGCATGIGCIGT(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.10 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 MgCl₂, 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 previously12 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'-CTCGAGTCATCCGTCGGTTA-3' (antisense). Both primers consisted of a gene-specific sequence and incorporated a restriction enzyme site at the 5' end: BamHI for the 5'-end primer and XhoI 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'-GGATCCATGCACATCTCAACTTATCC-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). Homologous 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, BamHI and Xhol, 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 OD₆₀₀=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 protease 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 MgCl₂ in 500 μl of a reaction buffer of 50 mM Tris-HCl (pH 8), 5 mM DTT, 0.5 mM EDTA, 10% glycerol and protease 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 N₂ gas and finally subjected to GC-MS (GCQ, Thermoquest, California, U.S.A.).

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