**ent-Kaurene Synthase from the Fungus Phaeosphaeria sp. L487**

cDNA ISOLATION, CHARACTERIZATION, AND BACTERIAL EXPRESSION OF A BIFUNCTIONAL DITERPENE CYCLASE IN FUNGAL GIBBERELLIN BIOSYNTHESIS*  

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**ent-Kaurene Synthase** is the first cyclic diterpene intermediate of gibberellin biosynthesis in both plants and fungi. In plants, ent-kaurene is synthesized from geranylgeranyl diphosphate via copalyl diphosphate in a two-step cyclization catalyzed by copalyl diphosphate synthase and ent-kaurene synthase. A cell-free system of the fungus Phaeosphaeria sp. L487 converted labeled geranylgeranyl diphosphate to ent-kaurene. A cDNA fragment, which possibly encodes copalyl diphosphate synthase, was isolated by reverse transcription-polymerase chain reaction using degenerate primers based on the consensus motifs of plant enzymes. Translation of a full-length cDNA sequence isolated from the fungal cDNA library revealed an open reading frame of 106-kDa polypeptide. The deduced amino acid sequence shared 24 and 21% identity with maize copalyl diphosphate synthase and pumpkin ent-kaurene synthase, respectively. A fusion protein produced by expression of the cDNA in Escherichia coli catalyzed the two-step cyclization of geranylgeranyl diphosphate to ent-kaurene. Amo-1618 completely inhibited the copalyl diphosphate synthase activity of the enzyme at $10^{-6}$ M, whereas it did not inhibit the ent-kaurene synthase activity even at $10^{-4}$ M. These results indicate that the fungus has a bifunctional diterpene cyclase that can convert geranylgeranyl diphosphate into ent-kaurene. They may be separate catalytic sites for the two cyclization reactions.

Gibberellins (GAs) are one of an important group of phytohormones regulating many aspects of plant growth and development. Some fungal species produce GAs as secondary metabolites (1). Gibberella fujikuroi is a rice pathogenic fungus producing high amount of GAs. The fermentation and biosynthesis of GA in G. fujikuroi were well characterized, since some GAs are produced industrially using the fungus (2, 3).

GA is unequivocally synthesized from ent-kaurene in both fungi and plants (4, 5). ent-Kaurene is a tetracyclic diterpene hydrocarbon formed from geranylgeranyl diphosphate (GGDP) via copalyl diphosphate (CDP). The pathway of ent-kaurene biosynthesis was first confirmed using cell-free systems from G. fujikuroi (6). The two-step cyclization was thought to involve two different enzymes: copalyl diphosphate synthase (CPS, formerly ent-kaurene synthase A) and ent-kaurene synthase (KS, formerly ent-kaurene synthase B). These enzymes were partially purified from Fusarium moniliforme, an anamorph of G. fujikuroi. (7). It was suggested that CPS and KS in the fungus might be tightly associated, because the two activities could not be separated. On the other hand, the two activities were successfully separated from a plant enzyme preparation from Marah macrocarpus by chromatographic methods (8). The two enzymes are possibly localized in plastids (9). Quite recently, genes encoding both enzymes have been cloned from plants: CPS from Arabidopsis (10), maize (11) and pea (12), and KS from pumpkin (13). Several other GA biosynthetic enzymes have been cloned from plants as well (14). In contrast, no GA biosynthetic enzymes have been cloned from fungi.

GA biosynthesis in another GA-producing fungus, Phaeosphaeria sp. L487 has been characterized recently (15). This fungus produces significant amounts of GA$_1$ through a pathway similar to that in higher plants: namely, 3β-hydroxylation of GA$_9$ and GA$_{19}$ occurs to form GA$_{1}$ and GA$_{3}$, respectively (15, 16). This contrasts to G. fujikuroi, in which 3β-hydroxylation occurs early in the pathway of GA$_{12}$-aldehyde and GA$_{9}$ is not converted to GA$_{1}$. Although ent-kaurene is an intermediate of GA biosynthesis in both fungi and plants (17), we were unable to compare the characteristics of the plant CPS and KS with fungal CPS and KS at molecular levels. Little is known about enzymes and genes involved in ent-kaurene biosynthesis of Phaeosphaeria sp. L487. In addition, none of the diterpene cyclases from fungi have been isolated yet. Therefore, we focused on fungal-type CPS (FCPS) of Phaeosphaeria sp. L487. In this paper we report cDNA cloning and characterization of an enzyme involved in ent-kaurene biosynthesis in the fungus Phaeosphaeria sp. L487.

**EXPERIMENTAL PROCEDURES**

Chemicals—[1-3H]GGDP was purchased from Amersham. [3H]CDP was a gift from Dr. Saito of the Institute of Physical and Chemical Research (18). Amo-1618 was obtained from Serva (Heidelberg).

Preparation of Cell-free Extracts—Eleven-day-old mycelium of Phaeosphaeria sp. L487 grown as described (15) was harvested by filtration and homogenized in potassium phosphate buffer (50 mM, pH 8.0) containing dithiothreitol (2 mM), pepstatin A (0.1 mM), and (p-amidophenyl) methanesulfonyl fluoride hydrochloride (10 mM) using Dyno-Mill (Willy A. Bachofen, Basel, Switzerland). The supernatant after centrifuga-
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**Table I**

| Substrates          | S-200 + Mg<sup>2+</sup> | S-200 + Mg<sup>2+</sup> + EDTA | Denatured enzyme + Mg<sup>2+</sup> |
|---------------------|------------------------|-------------------------------|-----------------------------------|
| [3H]GGDP            | 305 ± 27               | 4 ± 1                         | 4 ± 1                             |
| [3H]CDCP            | 198 ± 20               | 4 ± 1                         | 4 ± 2                             |

**Feasibility of the S-200 fraction**

- **S-200** fraction can be used to prepare cell-free systems for diterpene cyclase activity.
- Cells were collected after 24-h induction at 20°C and washed with 50 mM phosphate buffer.
- The S-200 fraction from the n-hexane extract was separated by gel filtration chromography and analyzed by liquid scintillation counting.

**Conversion of [3H]GGDP, [3H]CDCP, and Unlabeled GGDP—CPS and KS activities**

Conversion of [3H]GGDP and [3H]CDCP was measured as described previously (15, 18). In the case of the cell-free system, each substrate was incubated in 100 μl of the S-200 enzyme solution containing Mg<sup>2+</sup> (5 mM) and uniconazole (1 mM), as ent-kaurene oxidase inhibitor (22), at 30°C for 30 min. A ent-kaurene fraction after incubation was extracted with n-hexane and separated on silica gel TLC with n-hexane. The radioactivity of the ent-kaurene region was measured as described above. The soluble protein was analyzed by SDS-polyacrylamide gel electrophoresis with Coomassie Brilliant Blue staining (21). The GST-fusion protein was detected by Western blot analysis using GST detection module (Pharmacia).

**RESULTS**

**CPS and KS activities in a cell-free system of Phaeosphaeria sp. L487**

- [3H]GGDP (1 KBq, 75 GBq mol<sup>−1</sup>) or [3H]CDCP (1.5 KBq, 74 GBq mol<sup>−1</sup>) was added to the soluble enzyme preparation (S-200, 1.0 μg ml<sup>−1</sup>, 100 μl total), which was then incubated at 30°C for 30 min. The ent-kaurene fraction from a n-hexane extract was separated by gel filtration chromography and analyzed by liquid scintillation counting.

**Isolation and Characterization of cDNA Clone**

To design...
degenerate primers for reverse transcription-PCR, plant CPS sequences were compared. Several combinations of primers were designed based on the consensus motifs. Double-stranded cDNA was synthesized from poly(A)$^+$ RNA prepared from 6-day-old mycelium, and PCR was carried out. Only one combination of primers encoding AYDTAW and DDTAMAF motifs yielded a specific DNA fragment of the anticipated size. Sequence analysis of the DNA fragment revealed 873 bp encoding

![FIG. 1. The nucleotide sequence of FCPS cDNA and the predicted sequence of the encoded polypeptide. The possible translation start codon is shown in boldface. The element amplified by reverse transcription-PCR is shown between arrowheads. Aspartate- and aspartate/glutamate-rich motifs are double underlined. QXQGSW motifs are underlined with dots. Gene-specific primers used for reverse transcription of 5'-rapid amplification of cDNA ends are underlined.](image-url)
291 amino acid residues that showed significant similarity to sequences of CPS (10–12), KS (13), and abietadiene synthase (AS) (25). RNA blot analysis was performed using poly(A) RNA prepared from 6-day-old mycelium of Phaeosphaeria to detect a 3-kilobase transcript that hybridized with the PCR product (data not shown).

Using this PCR product as a hybridization probe, $2.5 \times 10^5$ plaques from a cDNA library constructed in λZAP II were screened. Four positive clones were isolated and further purified. One of the clones, pFCPS, was further characterized.

![Comparison of the amino acid sequence of FCPS from Phaeosphaeria sp. LA87 with those of maize copalyl diphosphate synthase (ZmCPS), pumpkin ent-kaurene synthase (CmKS), and abietadiene synthase (AS). Identities are shown by boldface. Gaps are indicated by dots. The aspartate- and aspartate/glutamate-rich motifs are indicated with a box.](image)
Sequence analysis revealed that the 3,034 bp pFCPS cDNA insert contained an open reading frame of 2,838 bp encoding 946 amino acid residues. To determine the 5’-end of the FCPS cDNA, 5’-rapid amplification of cDNA ends experiments were performed. The 5’-end of the FCPS transcript was mapped at 24 bp upstream from the 5’-end of pFCPS. Since there was no ATG codon in the extended sequence, we concluded that the 2,838-bp open reading frame encodes FCPS. The complete sequence of FCPS cDNA is shown in Fig. 1.

The predicted amino acid sequence of FCPS has significant homology with those of diterpene cyclases from plants: 20% identity with AS (grand fir, Ref. 25), 20% with casbene synthase (castor bean, Ref. 26), 24% with maize CPS (11), 22% with pumpkin KS (13), and 21% with taxadiene synthase (Pacific yew, Ref. 27). The FCPS sequence is not closely related (19% identity) to any of the microbial sesquiterpene cyclases (28–30). Sequence alignment with plant diterpene cyclases revealed that the motifs, YDTAW, QXXDGSW, and DVDDTA, were conserved in the FCPS sequence (Fig. 2). The electron-rich aromatic residue of the QXXDGSW motif is proposed to stabilize intermediate cation during the cyclization process (31). FCPS contains aspartate- and aspartate/glutamate-rich motifs (DDVLD: amino acids 132–136 and DEVIDEVVD: amino acids 683–691, Fig. 2). These functions have been strongly suggested to mediate substrate binding by chelation of the divalent metal ion (32, 33). The transit peptide in plant diterpene cyclases is rich in serine and threonine residues, with a net positive charge (34), is found in the amino-terminal region. However, the amino-terminal region of FCPS was not rich in serine and threonine. The calculated pI of the first 30 amino acids of the FCPS was 4.8 (complete amino acid sequence: calculated pI = 5.2). Therefore, the transit peptide region is not present in FCPS (Fig. 1).

Expression and Functional Analysis of the Recombinant Protein in E. coli—The pFCPS was introduced into pGEX 4T-3 and the GST-FCPS fusion protein was expressed in E. coli after induction with 1 mM IPTG. The IPTG-induced protein was detected in the soluble fraction as a 132-kDa band on SDS-polyacrylamide gel electrophoresis. It was confirmed to be GST-FCPS by Western blot analysis using GST detection module (data not shown).

The soluble enzyme fraction containing GST-FCPS was used for enzyme assays. Interestingly, when [3H]GGDP was incubated with the soluble enzyme, radioactivity showing formation of a diterpene hydrocarbon was detected by TLC analysis. Furthermore, when [3H]CDP was incubated with the enzyme, radioactivity associated with the ent-kaurene-like compound was also detected. To confirm production of ent-kaurene from GGDP by the FCPS, full-scan GC-MS analysis was carried out. To identify residual GGDP and CDP as geranylgeraniol and copalol by GC-MS, respectively, the reaction mixture after incubation of unlabeled GGDP with the enzyme was treated with alkaline phosphatase. After the hydrolysis, nonpolar compounds were extracted with n-hexane. After full-scan GC-MS analysis, ent-kaurene was identified. At the same time, copalol was also detected in the reaction mixture (Fig. 3). When pea CPS (PsCPS) fusion protein was incubated under the same condition, conversion of GGDP to CDP, but not to ent-kaurene, occurred (Fig. 3). Furthermore, when pumpkin KS (CmKS) fusion protein was added to PsCPS fusion protein, ent-kaurene was produced (Fig. 3). The KS activity of the FCPS was confirmed using [3H]CDP (Table II). Thus, it is clear that the FCPS fusion protein catalyzes both the cyclization of GGDP to CDP and that of CDP to ent-kaurene (Fig. 4). Hence, here we changed the name of FCPS to fungal-type ent-kaurene synthase or FCPS/KS as a bifunctional enzyme.

Amo-1618 is a quaternary ammonium compound that inhibits CPS activity (7, 35). When Amo-1618 was added to the

![Mass chromatograms of products after incubation of GGDP with recombinant proteins.](image-url)
the two-step cyclization of GGDP to ent-kaurene (3), which was then incubated at 30 °C for 30 min. The reaction mixture at a concentration of 10^{-6} M, conversion of [^{3}H]GGDP to ent-kaurene by FCPS/KS was completely inhibited. However, conversion of [^{3}H]CDP to ent-kaurene was not inhibited, even at 10^{-4} M (Table II). This suggested that sites for CPS and KS activities are distinct in FCPS/KS.

**DISCUSSION**

We report the isolation and characterization of a cDNA encoding the fungal ent-kaurene synthase, FCPS/KS, from *Phaeosphaeria* sp. L487. The genes for the fungal sesquiterpene cyclases, trichodiene synthase from *Fusarium sporotrichoides* (28) and aristolochene synthase from *Penicillium roqueforti* sp. L487, have been characterized (29). However, although many fungal species produce interesting bioactive diterpene compounds, such as the GAs and fusicoccins (36), none of the genes encoding diterpene cyclases have been isolated from fungi. Thus, this is the first report of the cloning of a diterpene cyclase of fungal origin. We initially focused on the cDNA cloning of fungal CPS, since the information about the sequence of CPS was obtained from various plants. The predicted amino acid sequence of FCPS/KS isolated from *Phaeosphaeria* sp. L487 has homology with the plant CPS and KS. The phylogenetic tree (Fig. 5) indicates that FCPS/KS has a significant evolutionary relationship with plant CPS. However, whereas both CPS and KS have a transit peptide in their amino-terminal regions, this is absent in FCPS/KS. FCPS/KS contains neither membrane translocation sequences nor membrane-spanning regions. Therefore, FCPS/KS is likely to be localized in the cytoplasm, as are the fungal sesquiterpene cyclases (29). This is also consistent with the presence of enzyme activity in the soluble fraction of the cell-free preparation from *Phaeosphaeria* sp. L487. In plants, all GGDP-derived carbon skeletons are synthesized within plastids and sesquiterpene hydrocarbons within the cytoplasm or endoplasmic reticulum (37).

We revealed that FCPS/KS is a bifunctional cyclase having both activities of CPS and KS in plants. FCPS/KS catalyzes the two-step cyclization of GGDP to ent-kaurene via CDP with a single polypeptide. Amo-1618 inhibits CPS activity in FCPS/KS at low concentrations. In contrast, it does not inhibit the KS activity, even at high concentrations. Similar inhibition characteristics are observed in enzyme preparations of *G. fujikuroi* and *Marah macrocarpus* (35). It has been suggested that, in plants, CPS and KS may interact with each other to synthesize ent-kaurene from GGDP (6). This suggests that the catalytic sites for the two cyclizations are separated. Furthermore, the similar modes of action of Amo-1618 on ent-kaurene biosynthesis in fungi and plants indicate that FCPS/KS of *Phaeosphaeria* sp. L487 and CPS-KS complex of plants have similar structure. At present, the two active sites responsible for CPS and KS activities within the FCPS/KS cannot be determined by sequence comparison. In contrast, AS is also a bifunctional enzyme which catalyzes the cyclization of GGDP to abietadiene via labdadienyl diphosphate (25). The amino-terminal region of AS, containing the DDDTA motif, resembles CPS, whereas the carboxyl-terminal region, containing the DBXXD motif, resembles KS (Fig. 2). The two active sites within the AS suggested a fusion of elements of CPS-type enzymes and KS-type enzymes (25). Thus, there are some examples where biosynthetic genes are separate in one organism but linked in another (38–40). FCPS/KS is an attractive model for understanding of structure-activity relationship of the enzymes involved in ent-kaurene biosynthesis between fungi and plants. To understand the catalytic sites of FCPS/KS, more detailed studies will be required. Comparison of FCPS/KS with diterpene cyclases from *G. fujikuroi* and other fungi will be necessary for understanding the evolution of GA biosynthesis in fungi.

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