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Gibberellin biosynthesis in bacteria: Separate *ent*-copalyl diphosphate and *ent*-kaurene synthases in *Bradyrhizobium japonicum*

Dana Morrone [a], Jacob Chambers [b], Luke Lowry [a], Gunjune Kim [c], Aldwin Anterola [c], Kelly Bender [b], Reuben J. Peters [a,*]

[a] Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA 50011, USA
[b] Department of Microbiology, Southern Illinois University, Carbondale, IL 62901, USA
[c] Department of Plant Biology, Southern Illinois University, Carbondale, IL 62901, USA

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ABSTRACT

Gibberellins are *ent*-kaurene-derived diterpenoid phytohormones produced by plants, fungi, and bacteria. The distinct gibberellin biosynthetic pathways in plants and fungi are known, but not that in bacteria. Plants typically use two diterpene synthases to form *ent*-kaurene, while fungi use only a single bifunctional diterpene synthase. We demonstrate here that *Bradyrhizobium japonicum* encodes separate *ent*-copalyl diphosphate and *ent*-kaurene synthases. These are found in an operon whose enzymatic composition indicates that gibberellin biosynthesis in bacteria represents a third independently assembled pathway relative to plants and fungi. Nevertheless, sequence comparisons also suggest potential homology between diterpene synthases from bacteria, plants, and fungi.

1. Introduction

Gibberellins (GAs) are complex diterpene-derived compounds sharing a common tetracyclic 6-5-6-5 fused hydrocarbon ring (gibberellane) skeletal structure. The first GA to be characterized was gibberellin A (GA_3), which was originally isolated as the bioactive component of *Gibberella fujikuroi* that causes foolish rice seedling disease [1]. Since then, more than 130 additional GAs have been identified from other fungi [2] and numerous vascular plants [3], as well as bacteria [4], and are now simply designated by number, based on the order in which they were discovered [3]. Some of these gibberellins (including GA_3) serve as plant (phyto)hormones, and mediate a variety of developmental processes, such as seed germination, stem elongation and flower induction [5]. The function of gibberellins in fungi and bacteria is less understood, although it is thought that they have roles in influencing the growth and development of their host plants [4,6].

Plant growth promoting rhizobacteria (PGPR) are root-colonizing bacteria that exert beneficial effects on the host plant. Perhaps the best known PGPR is the legume-associated *Bradyrhizobium japonicum*, which produces the same gibberellin (GA_3) as *G. fujikuroi* [7]. Besides GA_3, other gibberellins with phytohormone activity also have been found in PGPR, such as GA_1 in *Azospirillum* spp. [8–10], and GA_1 and GA_4 in *Rhizobium phaseoli* [11] and *Bacillus* spp. [12]. However, while the biosynthetic pathways leading to gibberellin production in both plants [13] and fungi [2] are already known, that in bacteria remains obscure [14].

Gibberellins are formed from geranylgeranyl diphosphate (GGPP) via a set of reactions catalyzed by different enzymes, including consecutively acting diterpene cyclases, cytochromes P450 and, in plants, 2-oxoglutarate dependent dioxygenases (2ODDs) [15]. In higher plants, cyclization of GGPP into *ent*-copalyl diphosphate (*ent*-CPP) and then to *ent*-kaur-16-ene is catalyzed by two distinct enzymes, *ent*-copalyl diphosphate synthase (E.C. 5.5.1.13) [16] and *ent*-kaurene synthase (E.C. 4.2.3.19) [17], respectively. In fungi [18,19], and in the moss *Physcomitrella patens* [20], the two cylization steps that form *ent*-kaurene from GGPP are

Abbreviations: aa, amino acid; CPP, copalyl diphosphate; CPS, copalyl diphosphate synthase; GA, gibberellin; GC, gas chromatography; GGPP, geranylgeranyl diphosphate; KS, *ent*-kaurene synthase; MS, mass spectrometer; PGPR, plant growth promoting rhizobacteria; 2ODD, 2-oxoglutarate dependent dioxygenases

* Corresponding author. Fax: +1 515 294 8580.
E-mail address: rjpeters@iastate.edu (R.J. Peters).

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catalyzed by bifunctional diterpene synthases (although the corresponding amino acid sequences in fungi and moss are not closely related). P450s then catalyze subsequent oxidation of *ent*-kaurene, first to *ent*-kaurenoic acid in both types of organisms, then in plants to GA_{12} and subsequently GA_{53} [21], while *G. fujikuroi* produces GA_{14} [22]. To form bioactive nor-diterpenoid (C_{19}) gibberellins, plants then utilize 2ODDs [23], while fungi rely on additional P450s [2,24,25], although formation of the 1,2-double bond of GA_3 in *G. fujikuroi* is mediated by a desaturase (des)[26]. These clear differences between plant and fungal GA biosynthesis, particularly in enzymatic type (e.g., Fig. 1), demonstrate that the corresponding pathways in these two broad groups of organisms were independently assembled [15].

In *B. japonicum*, an operon consisting of genes putatively encoding a ferredoxin, a short chain alcohol dehydrogenase, three P450s, a GGPP synthase, and two (di)terpene synthases, has been proposed to be involved in gibberellin biosynthesis [27]. Analysis of the subsequently reported genomic sequence [28], indicates that this is the only recognizable diterpenoid operon encoded by *B. japonicum*, consistent with the hypothesis that these enzymatic genes are involved in GA biosynthesis. To more directly investigate this hypothesis, we have biochemically characterized the two putative diterpene synthases and determined that they are separate *ent*-copalyl diphosphate and *ent*-kaurene synthases. Hence, we provide the first direct evidence that this operon may indeed be involved in GA production, and discuss the implications of these findings for the evolution of diterpene synthases and gibberellin biosynthesis in bacteria, plants, and fungi.

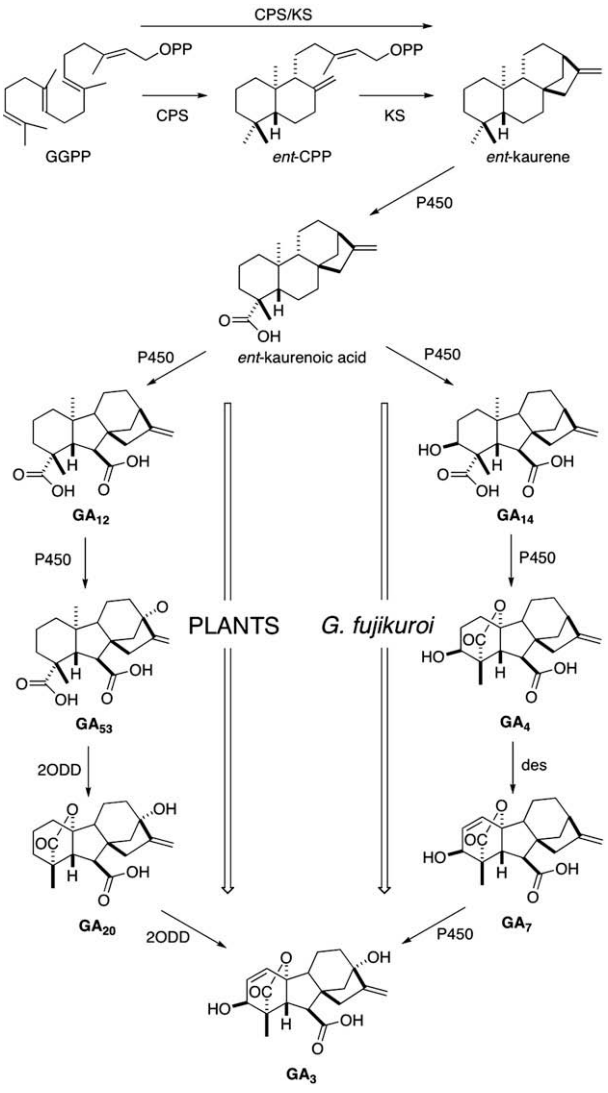

Fig. 1. GA biosynthesis in plants versus the fungus *G. fujikuroi*. Each arrow represents a single enzymatic step, with the corresponding enzymatic type indicated (as defined in text).

2. Materials and methods

2.1. General

Bradyrhizobium japonicum strain USDA110 was obtained from Michael Sadowsky (University of Minnesota). Molecular biology reagents were from Invitrogen, and all other chemicals were from Fisher Scientific, unless otherwise stated. Bioinformatic analyses were carried out with the Vector NTI software package (Invitrogen) using standard parameters.

2.2. Cloning of putative B. japonicum diterpene synthases

Genomic DNA from *B. japonicum* strain USDA110 was prepared using the Wizard Genomic DNA Purification Kit (Promega), and used as template to amplify the two putative terpene synthase genes (labeled blr2149 and blr2150 in Rhizobase) by PCR with *Pfu* DNA polymerase (Stratagene) with 4% DMSO. The oligonucleotides 5′-CACCATGAACGCGCTGTCCGAACATATCC-3′ (primer changes native GTG Val start codon to more common ATG Met start codon) and 5′-TCATGGCGCCGCTCCTGCCCCCT-3′ were used as primers for blr2149 amplification, and 5′-CACCATGATCCAGACTGAACGCGCGGTGC-3′ and 5′-TCCGGAATGGCGCCCAGGTATTCC-3′ (primer corresponds to 191 bp downstream of the native stop codon) for blr2150. Due to the high G + C nature of the *B. japonicum* genome, PCR cycling conditions consisted of a hotstart at 94 °C for 2 min, followed by 30 cycles of 94 °C for 45 s, 70 °C for 45 s, and 72 °C for 2 min, ending with a 72 °C incubation for 10 min. The blunt-ended PCR products were each purified by agarose gel electrophoresis (Qiaquick Gel Extraction Kit, Qiagen) and cloned into the Gateway system vector pENTR/SD/D-TOPO using a topoisomerase-mediated procedure. The resulting pENTR:blr2149 and pENTR:blr2150 constructs were each transformed into chemically competent TOP10 *Escherichia coli*, from which the corresponding plasmids were subsequently purified using a QIAprep Miniprep kit (Qiagen).

2.3. Metabolic engineering expression constructs

For analysis of the putative copalyl diphosphate synthase (CPS) from *B. japonicum*, a previously described pGG vector [29], which carries a GGPP synthase, was modified to carry an in-frame DEST cassette into the first multiple cloning site of the pACYCDuet (Novagen) parent vector. This was accomplished via introduction of a 5′ NcoI and 3′ NotI site onto the DEST cassette, followed by digestion of the PCR product and pGG with NcoI, followed by NotI, with the resulting large fragments gel purified and ligated together to create pGG-DEST. Clones can then be transferred from pENTR to pGG-DEST, enabling their co-expression with a GGPP synthase, which was done for blr2149. The putative kaurene synthase (KS) from *B. japonicum* (blr2150) was transferred by directional recombination to the Gateway system N-terminal GST fusion expression vector pDEST15.

2.4. Functional analysis of diterpene synthase activity

The putative *B. japonicum* diterpene synthases were functionally characterized using a previously described modular metabolic engineering system [29]. This was carried out using the BL21-de-

rived *E. coli* strain C41 (Lucigen), with the resulting recombinant strains grown in 50 mL cultures to mid-log phase (OD_{600} = 0.6) at 37 °C. After further incubation at 16 °C for 1 h, IPTG was added to the medium to a final concentration of 0.5 mM. These were fermented for an additional ~72 h and the cultures extracted with an equal volume of hexanes, which was then separated and dried under a gentle stream of nitrogen and re-dissolved in 100 μL hexanes for analysis by gas chromatography (GC), performed with a Varian (Palo Alto, CA) 3900 GC with Saturn 2100 ion trap mass spectrometer (MS) in electron ionization (70 eV) mode. Samples (1 μL) were injected in splitless mode at 50 °C and, after holding for 3 min. at 50 °C, the oven temperature was raised at a rate of 14 °C/min–300 °C, where it was held for an additional 3 min. MS data from 90 to 600 *m/z* were collected starting 12 min. after injection until the end of the run. Diterpene products were identified by comparison of retention time and mass spectra with that of authentic samples.

3. Results

In order to begin investigating GA biosynthesis in *B. japonicum*, characterization of the putative diterpene synthases encoded by blr2149 and blr2150 was undertaken. While annotation of the *B. japonicum* genome suggested that blr2149 encoded a 587 amino acid (aa) residue protein, this is significantly longer than other bacterial enzymes with analogous (i.e., class II diterpene cyclase) activity. In particular, the *ent*-CPP synthase from *Streptomyces* sp. strain KO-3988 (SkoCPS) [30], the terpentediene diphosphate synthase from *Streptomyces griseolosporeus* (SgTPS) [31], and the halimadienyl diphosphate synthase from *Mycobacterium tuberculosis* (MtHPS) [32], which are 499–511 aa in length. Further, in the initial characterization of the associated operon it was suggested that the corresponding gene encoded a 516 aa length protein [27], and this was the construct analyzed here.

Sequence comparisons with this corrected/original blr2149 encoded protein demonstrated that it exhibits ~27% aa sequence identity to the known bacterial class II diterpene cyclases, as well as more limited homology with both fungal and plant enzymes of this type (Fig. 2). Critically, this includes conservation of a DXDD motif required for class II diterpene synthase activity (i.e., protonation-initiated cyclization) [33], consistent with the hypothesis that blr2149 encodes such an enzyme.

The 300 aa protein encoded by blr2150 exhibits only ~16% aa sequence identity with the two known class I diterpene synthases from bacteria, and even more limited homology with plant enzymes of this type (Fig. 3). However, the two previously identified bacterial class I diterpene synthases, terpentetriene synthase from *Streptomyces griseolosporeus* (SgTS) [31] and pimaradiene synthase from *Streptomyces* sp. strain KO-3988 (SkoPS) [34], similarly exhibit only 16% aa sequence identity to each other. More importantly, the canonical DDXXD and $NDX_2(S/T)X_3E$ divalent metal cation binding motifs required for class I terpene synthase activity (i.e., allylic diphosphate ester ionization) are conserved in the blr2150 gene product [35]. Note that, while the blr2150 encoded aa sequence contains a Gly in place of the S/T position in the second motif, such substitution has been observed in plant KS and recently been shown to be functional [36]. Thus, we hypothesized that blr2150 encodes a class I diterpene synthase.

PCR amplification of blr2149 and blr2150 gave 1767 and 1097 (includes 191 bases downstream of stop codon) bp bands,

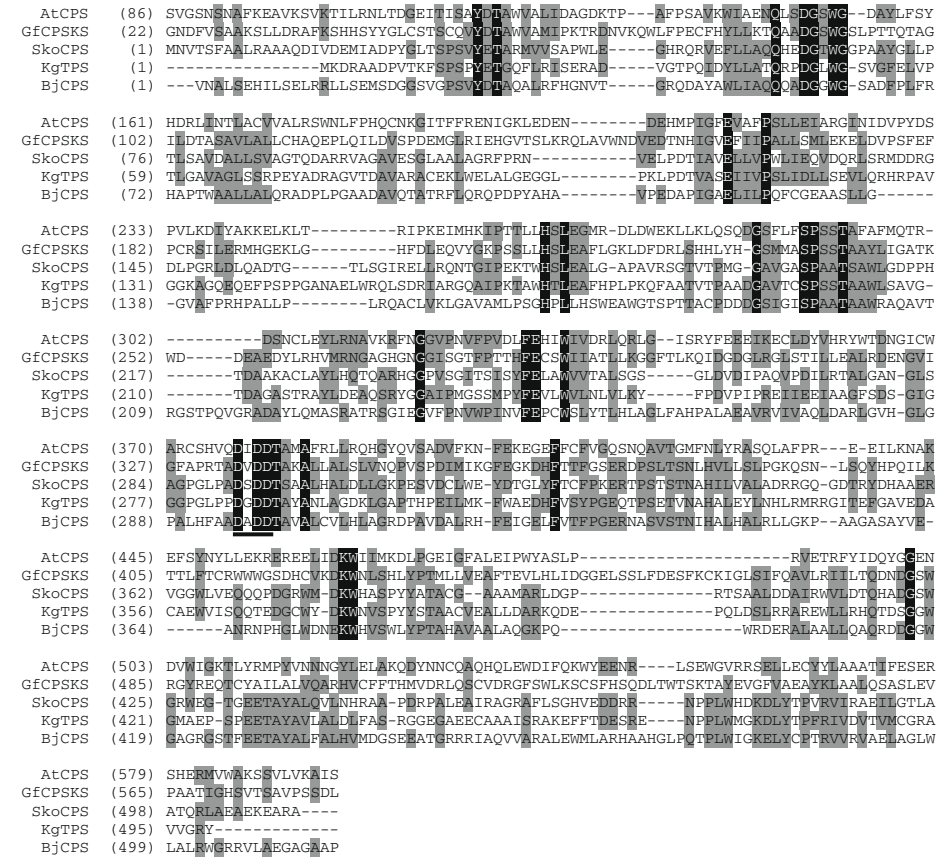

Fig. 2. Alignment of BjCPS with other bacterial class II diterpene cyclases and a representative plant CPS (AtCPS) and the fungal bifunctional CPS/KS from *G. fujikuroi* (GfCPSKS). While only the homologous regions of the fungal and plant enzymes are shown, the alignment was generated with their full-length sequences. The DXDD motif required for class II terpene synthase activity is underlined.

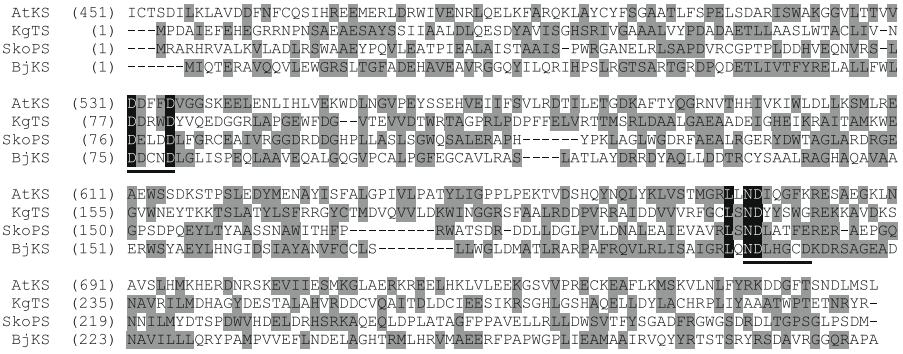

Fig. 3. Alignment of BjKS with other bacterial class I terpene synthases and a representative plant KS (AtKS). Although only the homologous region is shown, the alignment was generated with the full-length AtKS sequence. The divalent metal cation binding D(D/E)XXD and $NDX_2(S/T/G)X_3(E/D)$ motifs required for class I terpene synthase activity are underlined. While fungal enzymes do contain the same motifs, albeit with somewhat different spacing (~180 versus ~130–140 aa apart), alignments with their full lengths sequences do not readily detect homology in the C-terminal region to plant or bacterial diterpene synthases.

respectively, among other non-specific products, which necessitated the use of gel purification to clone these genes into an entry vector pENTR/SD/D-TOPO. After confirmation by DNA sequencing, the genes were transferred to expression vectors (pGG-DEST and pDEST15, respectively).

The putative *B. japonicum* diterpene synthases were characterized using a previously described modular metabolic engineering system to co-express these with a GGPP synthase (GGPS) and/or other diterpene synthases [29]. Co-expression of either blr2149 or blr2150 with GGPS alone did not lead to any detectable diterpene production. However, when blr2149 was co-expressed with GGPS and the *ent*-CPP specific KS from *Arabidopsis thaliana* (AtKS), or blr2150 was co-expressed with GGPS and the *ent*-CPP specific CPS from *A. thaliana* (AtCPS), the resulting recombinant *E. coli* strains were both found to specifically produce *ent*-kaur-16-ene. Thus, blr2149 and blr2150 can functionally substitute for *ent*-CPP specific CPS and KS involved in plant GA biosynthesis, respectively. Further, co-expression of blr2149 and blr2150 together with GGPS also led to the production of *ent*-kaur-16-ene (Fig. 4). In contrast, co-expression of blr2149 with GGPS and diterpene synthases that only react with CPP of *syn*-or normal stereochemistry did not result in the production of the corresponding diterpenes. Similarly, co-expression of blr2150 with GGPS and a CPS producing CPP of normal stereochemistry did not lead to any diterpene production, while co-expression with GGPS and a CPS producing *syn*-CPP only resulted in the production of trace amounts of *syn*-pimara-7,15-diene (data not shown).

4. Discussion

The plant growth promoting bacterium *B. japonicum* produces GA_3 [7], and contains an operon that has been hypothesized to encode GA biosynthetic enzymes [27], wherein the last two genes (blr2149 and blr2150) show some homology to known bacterial diterpene synthases. The functional analysis presented here demonstrates that blr2149 encodes an *ent*-CPP specific CPS (BjCPS) and blr2150 a KS (BjKS). These represent the first *ent*-copalyl diphosphate synthase identified in proteobacteria and the only *ent*-kaurene synthase to have been identified from bacteria. More critically, their combined activity is sufficient to produce the *ent*-kaur-16-ene intermediate expected for GA biosynthesis.

While the fact that *B. japonicum* contains separate CPS and KS superficially resembles plant rather than fungal GA biosynthesis, there are some obvious differences. In particular, both BjCPS and BjKS are significantly smaller than either the plant CPS or KS, as well as the fungal bifunctional CPS/KS. Nevertheless, more careful analysis suggests potential homology between all of these. The

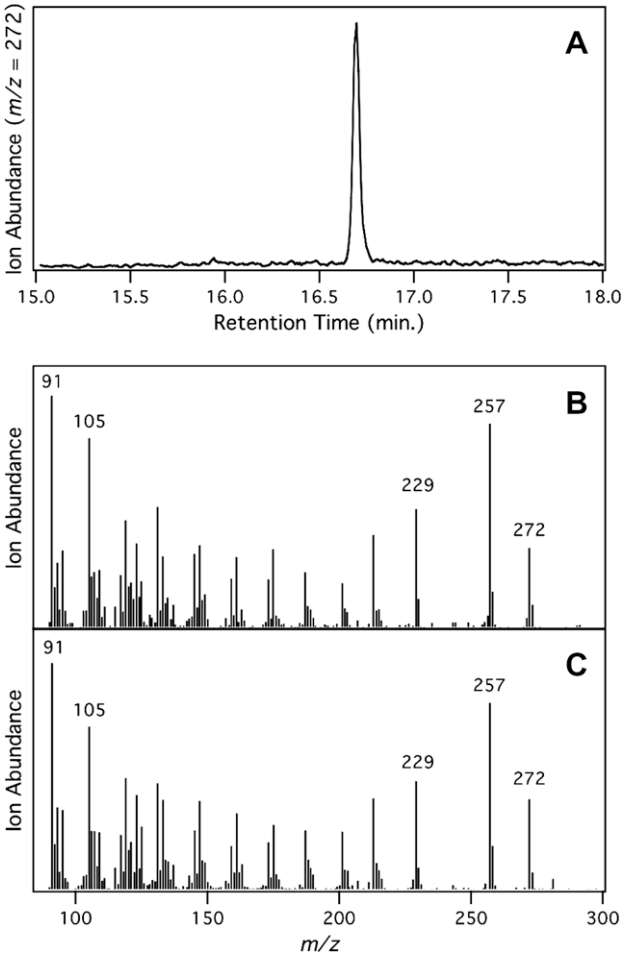

Fig. 4. GC–MS data demonstrating production of *ent*-kaurene from GGPP by blr2149 (BjCPS) and blr2150 (BjKS). (A) Chromatogram from extract of BjCPS + BjKS co-expressing strain. (B) Mass spectra of BjCPS + BjKS product (RT = 16.70 min). (C) Mass spectra of authentic *ent*-kaur-16-ene (RT = 16.70 min).

plant CPS and KS are of equivalent length, and seem to represent loss of function from an ancestral bifunctional enzyme, with the fungal bifunctional CPS/KS enzyme being of roughly similar length and exhibiting distant homology at least to plant CPS [37]. Truncation analysis of one such bifunctional fungal enzyme has associated the CPS and the KS activity with its N-terminal and C-terminal regions, respectively [38]. In addition, mutational

analysis suggests that the catalytic activity of plant class II diterpene cyclases (e.g., CPS) is associated with their N-terminal region, while the catalytic activity of plant class I diterpene synthases (e.g., KS) is associated with their C-terminal domain [39–41]. Intriguingly, BjCPS and other bacterial class II diterpene cyclases are roughly equivalent in length and exhibit some similarity to the CPS activity associated N-terminal region of the plant and fungal enzymes (Fig. 2). On the other hand, BjKS and other bacterial class I diterpene cyclases also are roughly equivalent in length and exhibit some similarity to the KS activity associated C-terminal domain, at least of the plant enzymes (Fig. 3). Further, there is previously noted structural homology between class I terpene synthases from microbes and the C-terminal domain of plant class I terpene synthases [35]. Thus, we hypothesize that the diterpene synthases associated with GA biosynthesis, as well as that of related diterpenoid natural products, in plants, fungi, and bacteria may share a common origin/ancestry (Fig. 5).

Regardless of the relationship between the diterpene synthases involved in production of the common *ent*-kaur-16-ene intermediate, the profound differences in downstream oxidation steps provide a clear distinction between at least plant and fungal GA biosynthesis. While plants use P450s and 20DDs to produce bioactive GAs, fungi use only P450s, and in some species a desaturase, demonstrating that the plant and fungal pathways were independently assembled via a convergent evolutionary process [15,24,25]. Intriguingly, the BjCPS and BjKS containing operon contains only three full-length P450s, as well as a short chain alcohol dehydrogenase, suggesting some similarities with fungal GA biosynthesis (i.e., no use of 20DDs). However, there is no significant homology between the fungal and *B. japonicum* P450s ($\leqslant$15% identity at the amino acid sequence level). Furthermore, while GA_3 biosynthesis in *G. fujikuroi* utilizes four P450s [2], *B. japonicum* presumably only requires the three found in the BjCPS and BjKS containing operon to produce the same GA_3. Consistent with this hypothesis, several other GA producing rhizobacteria contain a homologous operon that, in each case, also contains only three P450s. Also conserved in these operons is the short chain alcohol dehydrogenase, whose expected activity differs from the hydrocarbon targeting desaturase operating in *G. fujikuroi* GA_3 biosynthesis. Thus, there appear to be clear differences in the oxidation of *ent*-kaurene to the observed bioactive GA_3 in bacteria relative to either plants or fungi, which would then represent yet another convergent evolutionary solution to GA biosynthesis.

In conclusion, the results reported here provide the first biochemical characterization of enzymes potentially involved in bacterial GA production. Intriguingly, sequence comparisons with the identified diterpene synthases suggest a potential common origin for such enzymes in plants, fungi, and bacteria. By contrast, the composition of the associated operon indicates that bacterial GA biosynthesis represents a third independently assembled pathway for production of this highly bioactive and phylogenetically widespread metabolite.

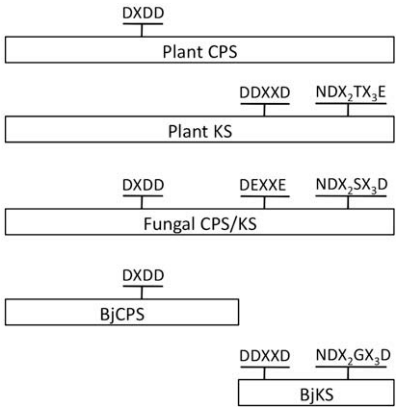

Fig. 5. Schematic comparison of plant, fungal and bacterial diterpene synthases, with conservation of catalytic motifs as indicated.

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