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Evident and latent plasticity across the rice diterpene synthase family with potential implications for the evolution of diterpenoid metabolism in the cereals

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Abstract

The evolution of natural products biosynthetic pathways can be envisioned to occur via a number of mechanisms. Here we provide evidence that latent plasticity plays a role in such metabolic evolution. In particular, rice (Oryza sativa) produces both ent- and syn-copalyl diphosphate (CPP), which are substrates for downstream diterpene synthases. Here we report that several members of this enzymatic family exhibit dual reactivity with some pairing of ent-, syn-, or normal CPP stereochemistry. Evident plasticity was observed, as a previously reported entsandaracopimaradiene synthase also converts syn-CPP to syn-labda-8(17),12E,14-triene, which can be found in planta. Notably, normal CPP is not naturally found in rice. Thus, the presence of diterpene synthases that react with this non-native metabolite reveals latent enzymatic/metabolic plasticity, providing biochemical capacity for utilization of such a novel substrate (i.e., normal CPP) that may arise during evolution, the implications of which are discussed.

Keywords

Metabolic evolution, terpenoid natural products, terpene synthases, substrate specificity, phytoalexins

Comments

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Evident and latent plasticity across the rice diterpene synthase family with potential implications for the evolution of diterpenoid metabolism in the cereals

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SYNOPSIS

The evolution of natural products biosynthetic pathways can be envisioned to occur via a number of mechanisms. Here we provide evidence that latent plasticity plays a role in such metabolic evolution. In particular, rice (*Oryza sativa*) produces both *ent*- and *syn*-copalyl diphosphate (CPP), which are substrates for downstream diterpene synthases. Here we report that several members of this enzymatic family exhibit dual reactivity with some pairing of *ent*-, *syn*-, or normal CPP stereochemistry. Evident plasticity was observed, as a previously reported *ent*-sandaracopimaradiene synthase also converts *syn*-CPP to *syn*-labda-8(17),12*E*,14-triene, which can be found *in planta*. Notably, normal CPP is not naturally found in rice. Thus, the presence of diterpene synthases that react with this non-native metabolite reveals latent enzymatic/metabolic plasticity, providing biochemical capacity for utilization of such a novel substrate (i.e., normal CPP) that may arise during evolution, the implications of which are discussed.

Keywords

Metabolic evolution; terpenoid natural products; terpene synthases; substrate specificity; phytoalexins

INTRODUCTION

Terpenoids, with over 40,000 known individual compounds, form the largest class of natural products [1]. Prominent among these are the ~7,000 labdane-related diterpenoids, which are particularly prevalent in plants, where the affiliated gibberellin phytohormone production required for normal growth and development provides a genetic reservoir that has been repeatedly drawn upon for the evolution of novel such natural products [2, 3]. The unifying biogenetic feature of labdane-related diterpenoid biosynthesis is its initiation by a sequential pair of cyclization and/or rearrangement reactions, and the corresponding enzymes are often found as functionally diverse gene families in plants [4, 5]. The characteristic decalin ring structure is formed from the universal diterpenoid precursor (*E,E,E*)-geranylgeranyl diphosphate (GGPP) by initially acting class II diterpene cyclases, which most commonly produce a labdadienyl/copalyl diphosphate (CPP) of normal (9*S*,10*S*), *syn* (9*S*,10*R*), or *ent* (9*R*,10*R*) configuration (Scheme 1), with the corresponding enzymes termed CPP synthases (CPS). The resulting CPP is subsequently utilized by class I diterpene synthases, which lyse the allylic diphosphate ester linkage to initiate carbocationic cyclization and/or

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rearrangement reactions. Because the corresponding enzyme in gibberellin biosynthesis is *ent*-kaurene synthase (KS), the derived class I diterpene synthases are often termed KS-like (KSL). However, while these enzymes do not react with GGPP and are typically assumed to be specific for particular stereoisomers of CPP, comprehensive investigation of such substrate stereospecificity (e.g., with all three commonly found CPP stereoisomers) has not been reported.

Rice (*Oryza sativa*) has become a model system for investigation of labdane-related diterpenoid biosynthesis, as it has long been appreciated that it produces at least 20 such natural products as antimicrobial phytoalexins, and relatively early sequencing of the rice genome has led to functional characterization of the corresponding diterpene synthase gene families [4, 6]. However, since rice produces only *ent*- and *syn*-CPP, as first suggested by substrate studies with cell-free enzyme assays [7, 8], and then more conclusively demonstrated by functional genomics based investigations of the rice CPS family [9-11], the rice KSL (OsKSL) were only assayed with these two stereoisomers [12-18]. This OsKSL family, with eight functional members (Scheme 2), provided an opportunity to comprehensively explore diterpene synthase substrate stereospecificity not only for individual enzymes, but also across an entire organism/genome, providing insight into the encoded metabolic plasticity. Accordingly, we have utilized our modular metabolic engineering system [19], to investigate the CPP substrate stereospecificity of the OsKSL family. The results of this substrate specificity screen revealed not only evident, but also latent metabolic plasticity, as well as offering some insight into diterpene synthase substrate recognition, all of which are discussed below.

EXPERIMENTAL

General

Unless otherwise noted, chemicals were purchased from Fisher Scientific (Loughborough, Leicestershire, UK), and molecular biology reagents from Invitrogen (Carlsbad, CA, USA). All recombinant expression was carried out using the OverExpress C41 strain of *E. coli* (Lucigen, Middleton, WI), which is well suited to T7 promoter based expression of plant derived labdane-related diterpene synthases [20]. GC-MS was performed with a Varian (Palo Alto, CA) 3900 GC with Saturn 2100 ion trap mass spectrometer in electron ionization (70 eV) mode using a HP-5ms column. Samples (1 μL) were injected in splitless mode at 50 °C and, after holding for 3 min. at 50 °C, the oven temperature (unless otherwise noted) was raised at a rate of 14 °C/min. to 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.

Functional characterization via metabolic engineering

Functional characterization of the substrate stereospecificity of the OsKLS was accomplished by use of our previously described modular metabolic engineering system [19]. Briefly, using this system it possible to co-express class I labdane-related diterpene synthases such as the OsKSL from pET based pDEST expression vectors with GGPP and CPP synthases carried on a compatible pACYC-Duet (Novagen/EMD) derived plasmid, where pGG*n*C carries the D621A mutant of abietadiene synthase from *Abies grandis* that produces CPP of normal stereochemistry [21], while pGG*e*C carries the An2/ZmCPS2 *ent*-CPP synthase from *Zea mays* [22], and pGG*s*C carries the OsCPS4 *syn*-CPP synthase from rice [10]. Accordingly, C41 cells were transformed with the appropriate plasmid enabling functional recombinant expression of each OsKSL, as previously described [18], along with one of the three pGG*x*C vectors described above, in all possible pairings. These recombinant bacteria were then grown in 20-mL liquid media cultures with shaking at 37 °C to A_{600} ~ 1.0, then shifted to 16 °C for 1 hr prior to induction with 0.5 mM IPTG, with the cultures

then fermented for an additional 72 hrs at 16 °C with continued shaking. The cultures were then extracted with an equal volume of hexane, with 1:50 (v/v) ethanol added to disrupt emulsions. Diterpene production was quantified by GC-FID analysis, with GC-MS comparison to authentic standards enabling identification of all resulting compounds but that from co-expression of OsKSL10 with pGG*s*C (i.e., *syn*-CPP).

To directly determine the structure of the OsKSL10 product from *syn*-CPP, we utilized previously described methodology to improve production levels in our metabolic engineering system [23], which enabled facile generation of the milligram quantities needed for structural characterization by NMR spectroscopy. Specifically, we co-transformed pDEST15/OsKSL10 and pGG*s*C with the further co-compatible pIRS plasmid that increases flux into the endogenous methyl erythritol phosphate (MEP) pathway by overexpression of the *idi, dxs,* and *dxr* genes from *E. coli*. The resulting recombinant bacteria were grown and extracted as described above, but as a 1-L culture supplemented with the MEP pathway precursors pyruvate (50 mM) and 2% (v/v) glycerol. The resulting 1-L of hexane extract was reduced to ~10-mL by rotary evaporation, passed over a ~5-mL silica gel column to remove any polar contaminants, and then dried under nitrogen gas, yielding ~5 mg of the unknown diterpene, which was then re-dissolved in 0.4 mL $CDCl_3$ for NMR analysis.

Chemical structure identification

NMR spectra for the unknown *syn*-CPP fed OsKSL10 product were recorded at 25 °C on a Bruker Avance 500 spectrometer equipped with a cryogenic probe for ^{1}H and ^{13}C. Structural analysis was performed using 1D ^{1}H, 1D, DQF-COSY, HSQC, HMQC, HMBC and NOESY experiment spectra acquired at 500 MHz and ^{13}C and DEPT135 spectra (125.5 MHz) using standard experiments from the Bruker TopSpin v1.3 software. Chemical shifts were referenced to the known chloroform-d (^{13}C 77.23, ^{1}H 7.24 ppm) signals offset from TMS. Correlations from the HMBC spectra were used to propose a partial structure, while connections between protonated carbons were obtained from DQF-COSY to complete the partial structure and assign proton chemical shifts. The configuration of the A and B rings is predetermined by the configuration of the CPP enzyme substrate, since chemical bonds in that portion of the molecule are not altered. The configuration of the acyclic portion of the molecule (C11-C16) was determined by reference to spectra for normal labdatrienes, which has been previously characterized [24]. Specifically, the C12-C13 double bond configuration was determined to be *E* based on the C14 ^{13}C chemical shift for *syn*-labda-8(17),12*E*,14-triene (142.01ppm). The comparison of *E* and *Z* configuration of normal labda-8(17),12,14-trienes shows a dramatic ~10 ppm difference in the C14 chemical shift in *E* versus *Z* configurations (~131 ppm (*Z*) vs. ~141 (*E*) ppm). This reference data and our NMR analysis support the presented *syn*-labda-8(17),12*E*,14-triene double bond configuration for the OsKSL10 product. The assignments for the annotated ^{13}C and ^{1}H 1-D spectra are presented in Table 1.

Enzymatic assays

Assays were performed with purified GST-OsKSL10 fusion protein using enzymatic coupling, as previously described [25, 26]. Briefly, GGPP was completely converted to either *ent*- or *syn*- CPP by incubation with the appropriate CPS for 5 hrs at RT. Assays were performed in duplicate with varying concentrations of GGPP ranging from 0.1 μM to 100 μM, with time and GST-OsKSL10 concentration varied to ensure measurements were taken in the linear portion of the reaction progress curve. The assays were inactivated by heating the solution to 65 °C for 5 min, with the remaining substrate then dephosphorylated by treatment with phosphatase (CIP, New England Biolabs), enabling extraction of both the diterpene product and copalol (derived from dephosporylation of CPP). This was done to enable correction for handling errors by measurement of the fractional turnover (i.e.,

comparison of the relative amounts of diterpene product and copalol by GC-FID), which was used in combination with the known starting concentration of substrate to calculate the corresponding catalytic rate.

Extraction of Rice Leaves

Rice leaves that had been treated with methyl jasmonate for 36 hours, as previously described [10], were frozen at −80 °C. Approximately 100 g of these frozen leaves were cut up and then homogenized by grinding in a mortar and pestle under liquid nitrogen in the presence of glass beads. After grinding, the resulting powder was extracted with 75 mL of hexanes, then 75 mL of ethyl acetate, and finally 75 mL of tetrahydrofuran. The combined organic extracts were reduced by rotary evaporation, passed over silica gel, and dried under N_2 as above. The resulting material was then resuspended in 1 mL of ethyl acetate, with 20 μL aliquots being removed and then dried under N_2, and resuspended in 20 μl hexane for analysis by GC-MS, using an extended temperature ramp wherein, after the initial 3 min. hold at the 50 °C injection temperature, the oven temperature was raised at 18 °C/min. to 190 °C, then slowed to 2.5 °C/min until reaching 275 °C, following which it was raised at 20 °C/min. to 300 °C, where it was held for 2 min.

RESULTS

Screening OsKSL substrate specificity

To investigate the substrate stereospecificity of the entire OsKSL family, we turned to our previously developed modular metabolic engineering system [19]. In particular, using this system it was possible to co-express each of the OsKSL with a GGPP synthase and CPS producing one of the three commonly found stereoisomers of CPP (note that all the OsKSL have been demonstrated to not react with the acyclic GGPP precursor). Accordingly, the ability of each of the eight active OsKSL to react with CPP of *ent*-, *syn*-, or normal stereochemistry was determined by simply extracting the corresponding recombinant bacterial cultures and analyzing the resulting organic extract for the presence of diterpenes by GC-MS. The previously reported activity for each of the OsKSL, as summarized in Scheme 2, was evident. In addition, while the other OsKSL were specific for a single stereoisomer of CPP, OsKSL4 and OsKSL11 reacted with CPP of normal stereochemistry along with their previously reported activity with *syn*-CPP, and OsKSL10 was active with *syn*-CPP as well as its previously reported activity with *ent*-CPP. Surprised by this latter result, we purified OsKSL10 and verified its reactivity with *syn*-CPP, with the same product resulting from in vitro reactions as found in the context of the bacterial metabolic engineering system. In all three cases, diterpene yield with the “alternative” CPP stereoisomer approached that found with the “native” stereoisomer, being limited to less than three-fold decreases. As yield in our metabolic engineering system has been correlated with catalytic efficiency [25, 26], this suggests that these enzymes exhibit reasonable affinity for and activity with their “alternative” substrates.

Alternative substrate product identification

By comparison with available authentic standards, OsKSL4 activity with CPP of normal stereochemistry was found to produce a mixture of ~90% pimara-8(14),15-diene and ~10% isopimara-7,15-diene, while OsKSL11 reacted with the same substrate to yield essentially only isopimara-8(14),15-diene (also known as sandaracopimaradiene; Figure 1). However, it was not possible to identify the product of OsKSL10 activity with *syn*-CPP in this fashion, as it did not match any authentic standard on-hand. This diterpene was then identified by increasing flux towards terpenoid production, via up-regulation of the endogenous isoprenoid precursor supply pathway as previously described [23], to enable the corresponding recombinant bacteria to produce enough of the novel product for NMR

analysis (~5 mg, following purification, from 1 L of bacterial culture). The structure was assigned by analysis of collected HMBC, HSQC, and COSY spectra, and found to be *syn*-labda-8(17),12*E*,14-triene (Table 1), completing assignment of the observed alternative reactions (Scheme 3).

Physiological relevance of syn-labdatriene

Because rice produces both *ent*- and *syn*-CPP, it seemed possible that the dual substrate reactivity of OsKSL10 was physiologically relevant (i.e., both products can be found in planta). The labdane-related diterpenoid natural products of rice are largely thought to be anti-microbial phytoalexins [4, 6], whose biosynthesis, by definition [27], is induced as part of the plant microbial defense response. This includes transcriptional up-regulation of OsKSL10, as well as OsKSL4 [14]. Accordingly, the possibility that OsKSL10 produces *syn*-labdatriene in planta was examined by phytochemical analysis of methyl jasmonate induced and uninduced rice leaves. As previously reported [7], extracts from control (uninduced) rice leaves contained only small amounts of *ent*-kaurene, while numerous diterpenes were detected in extracts from induced rice leaves (Figure 2). These specifically included *syn*-labdatriene, as well as *syn*-stemodene, whose presence in planta had not been previously demonstrated, although the responsible OsKSL11 seems widely conserved [17]. Steady-state kinetic analysis also was consistent with physiological relevance of the ability of OsKSL10 to react with both *ent*- and *syn*-CPP, as it exhibited similar catalytic efficiency with each (Table 2). Notably, consistent with the lack of a normal CPP producing CPS in the rice genome, while the *syn*-CPP derived products of OsKSL11 (as noted above), as well as OsKSL4, were readily observed, the pimaradienes produced by these diterpene synthases from normal CPP were not evident.

DISCUSSION

Based on the available genome sequence [28], the entire family of rice diterpene synthases is known, and subsequent biochemical characterization has assigned unique functional roles to each of these [15, 18]. Hence, it is possible to define this early part of the labdane-related diterpene metabolic network for this important crop plant (Scheme 2), with the resulting/downstream natural products largely thought to be involved in the microbial defense response of the plant [4, 6]. One outcome of the studies reported here is the addition of the *syn*-labdatriene product of OsKSL10 to this metabolic network. Given the inducible nature of the transcription of *OsKSL10* [14], and the role of its already known *ent*-sandaracopimaradiene product as a precursor for the antifungal phytoalexins oryzalexins A – F, it seems likely that any *syn*-labdatriene derived natural products will similarly function as antifungal agents, although this remains to be demonstrated. This would be consistent with similar transcriptional induction of all the other diterpene synthases involved in biosynthesis of the >20 known rice diterpenoid phytoalexins [4, 6, 9-16]. Nevertheless, one intriguing alternative possibility is a role for *syn*-labdatriene in production of an inducible signaling molecule, as has been previously indicated for a similar labdatriene-derived diterpenoid in tobacco [29]. On the other hand, the ability of OsKSL4 and OsKSL11 to react with normal CPP is not directly relevant in rice, whose genome does not contain a CPS that produces this stereoisomer [9-11].

In any case, the ability of certain rice diterpene synthases to react with two different stereoisomers of CPP raises the question of how such dual substrate recognition is achieved. OsKSL4 and OsKSL11 react with both *syn*- and normal CPP, and the obvious structural similarity between these two stereoisomers is the occurrence of co-axial β-methyl substituents on C4 and C10, which provides steric bulk on this “upper face” of the *trans*-decalin nucleus (Figure 3). Presumably, it is this common feature that enables the dual reactivity exhibited by OsKSL4 and OsKSL11. In addition, the stereochemistry of the

observed products is consistent with similar conformations of both substrates in each active site (Figure 4). By contrast, the ability of OsKSL10 to react with both *ent*- and *syn*-CPP presumably arises from the common C9α connection of the isoprenyl diphosphate containing "tail" to the substrate nucleus (Figure 3), although the lack of cyclization with *syn*-CPP prevents any insights into relative conformation of the two substrates within the active site.

Notably, some plasticity in substrate specificity has been previously observed in other terpene synthases, leading to suggestions of evolutionary relevance. Perhaps most strikingly, work in strawberries has demonstrated a clear evolutionary derivation of a linalool/nerolidol synthase in cultivated strawberry from monoterpene synthases found in wild strawberry plants, wherein the loss of the plastid targeting pre-sequence from a presumably plastic monoterpene synthase ancestor gave rise to this novel sesqui-/mono-terpene synthase [30]. Similar mono-/sesqui-terpene synthase plasticity has been uncovered in other cases as well [31].

The ability of OsKSL4 and OsKSL11 to react with normal CPP demonstrates the presence of latent diterpene metabolic plasticity in rice. Given the production of normal CPP by a diverse array of plants, ranging from gymnosperms to angiosperms, with no obvious homology between the relevant known enzymes from these two broad groups [5, 32-34], it seems likely that neofunctionalization of the corresponding class II diterpene cyclases to production of this CPP stereoisomer occurs with some appreciable frequency. Indeed, recent results demonstrate that the closely related monocot cereal crop plant wheat (*Triticum aestivum*) produces normal rather than *syn*-CPP (Y. Wu, T. Toyomasu, & R.J.P.; manuscript in preparation). Accordingly, the latent metabolic plasticity evident here from the substrate promiscuity of these two rice diterpene synthases provides a means by which such emergence of stereochemically differentiated substrates could be immediately incorporated into 'secondary' metabolism, which seems pertinent to such biochemical evolution in the cereal plant family. In particular, coupled with the often-observed substrate plasticity of downstream tailoring enzymes, especially cytochrome P450 mono-oxygenases (CYP), this would then enable emergence of essentially complete biosynthetic pathways (e.g., the bioactive oryzalexins D – F are simply diterpenoid diols only two hydroxylations reactions removed from their diterpene precursor). For example, rice contains ~350 CYP [35], with the large CYP71 and CYP76 families (74 and 28 members, respectively) each containing members involved in labdane-related diterpenoid biosynthesis [36, 37]. Notably, at least one of these can hydroxylate pimaradienes of normal stereochemistry [38]. Thus, provided access to a novel metabolite substrate the latent plasticity of terpene synthases, such as that shown here, can immediately give rise to potentially bioactive natural products, providing a mechanism for rapid evolution of novel biosynthetic pathways. While not directly selectable in and of itself, such latent plasticity would be periodically uncovered upon the introduction of novel metabolites, presumably providing a corresponding selectable advantage, albeit in punctuate fashion.

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Abbreviations

CPP copalyl diphosphate

CYP	cytochromes P450
GC-MS	gas chromatography with mass spectral detection
GGPP	geranylgeranyl diphosphate
KSL	kaurene synthase-like

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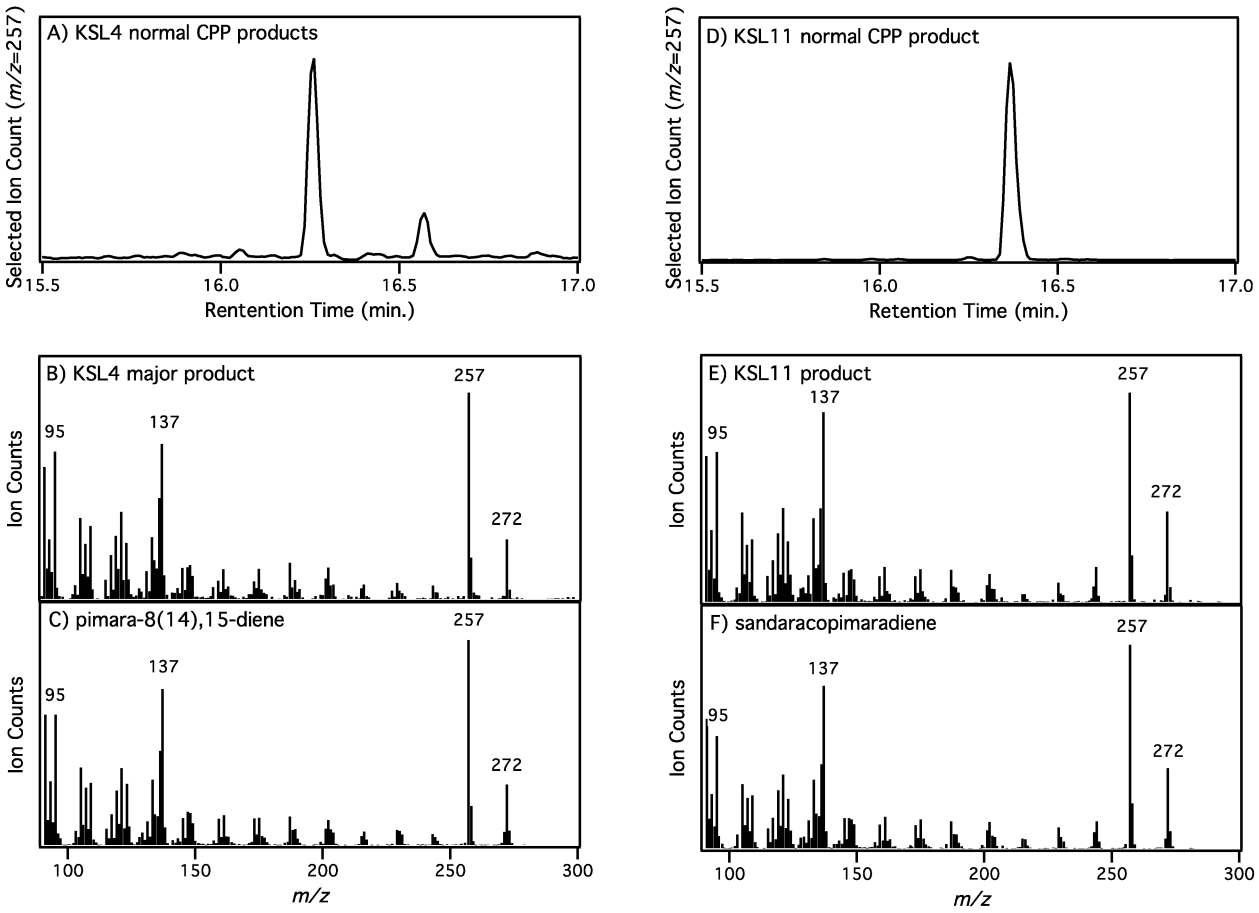


Figure 1.

Identification of alternative products by comparison to authentic standards. A) GC-MS selected ion chromatogram of OsKSL4 products from normal CPP. B) Mass spectra of OsKSL4 major product. C) Mass spectra of authentic pimara-8(14),15-diene. D) GC-MS selected ion chromatogram of OsKSL11 products from normal CPP. E) Mass spectra of OsKSL11 major product. F) Mass spectra of authentic sandaracopimaradiene.

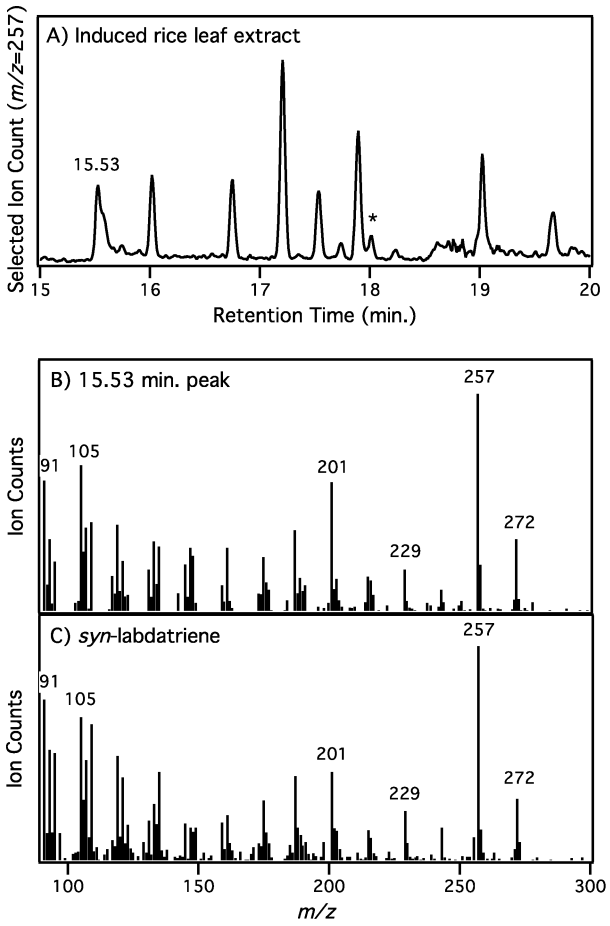


Figure 2.

Detection of *syn*-labda-8(17),12*E*,14-triene in induced rice plant tissue. (A) GC-MS selected ion chromatogram of induced rice leaf extract (* indicates peak corresponding to *syn*-stemodene product of OsKSL11). (B) Mass spectrum of 15.53 min. peak in induced rice leaf extract. (C) Mass spectrum of authenticated *syn*-labda-8(17),12,14-triene (RT = 15.51 min.).

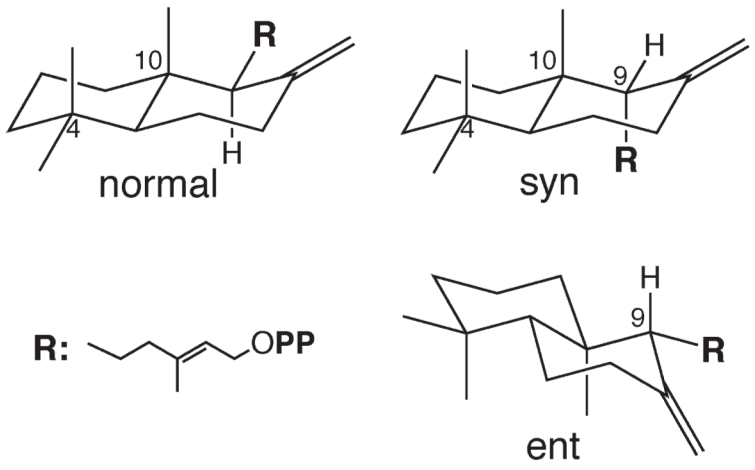


Figure 3.
Configurational comparison of CPP stereoisomers.

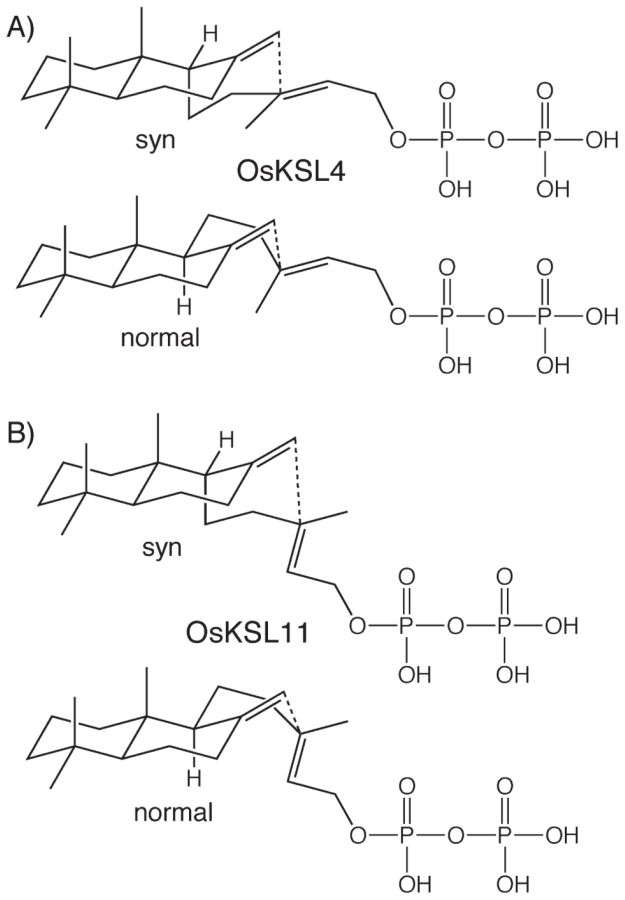
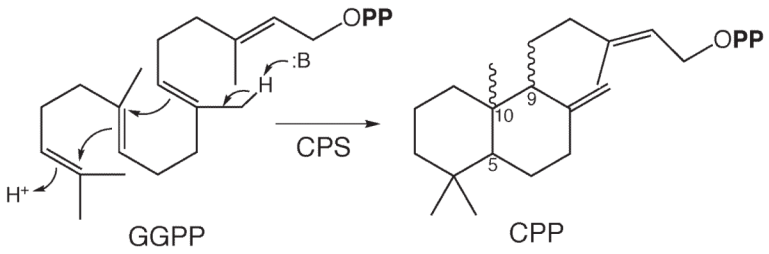
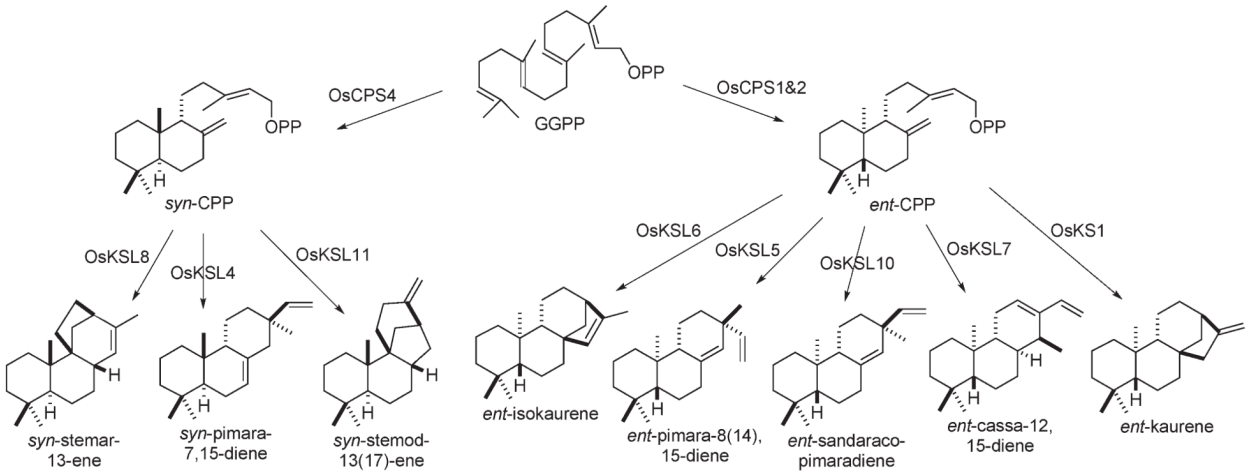


Figure 4.
Comparison of active site conformations of the alternative CPP stereoisomers indicated by catalyzed product outcome for A) OsKSL4 and B) OsKSL11.



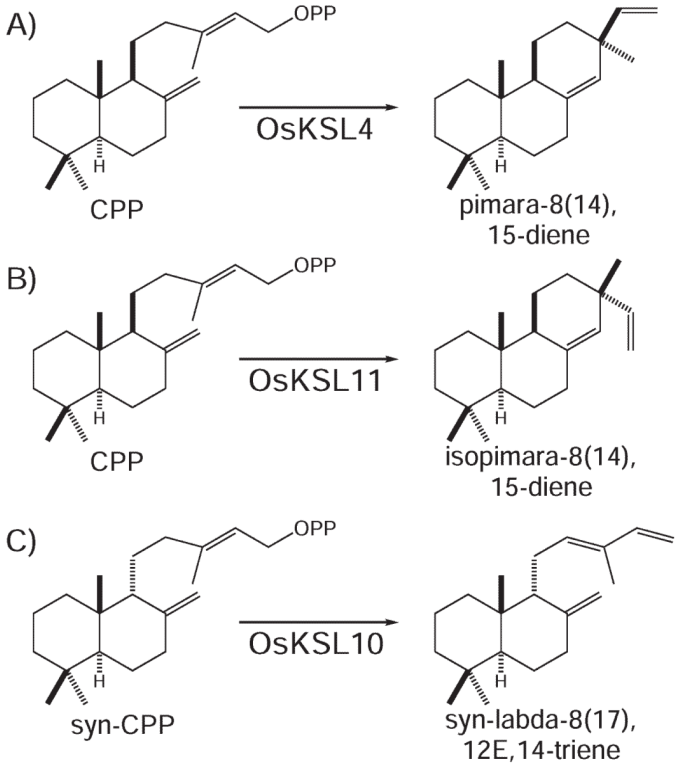
Scheme 1.

Cyclization of GGPP to stereoisomeric CPP by CPS. Note that the position of the C5 hydrogen is always anti to the C10 methyl due to the chair-chair conformation of the GGPP in this initial CPS catalyzed (i.e., class II) cyclization mechanism.



Scheme 2.

Rice diterpene metabolic map. Shown are the reactions catalyzed by the various rice diterpene cyclases [both class II, CPS, and class I, KS(L)]. Modified from [17, 18].



Scheme 3.
Alternative reactions catalyzed by A) OsKSL4; B) OsKSL11; C) OsKSL10.

Table 1

^{1}H and ^{13}C NMR assignments for *syn*-labda-8(17),12*E*,14-triene.

c	δ_C (ppm)	δ_H (ppm), multiplicity	*J*(Hz)
1	37.16	1.086(m), 1.550(m)	
2	19.39	1.434(m), 1.607(m)	
3	42.92	1.167(dt), 1.403(m)	3.6, 13.1
4	33.48		
5	46.04	1.298(m)	
6	23.85	1.296(m), 1.611(m)	
7	31.88	2.065(m),2.146(m)	
8	149.23		
9	58.42	1.635(m)	
10	38.25		
11	25.89	2.164(m), 2.409(dt)	15.0,5.6
12	133.36	5.365(t)	7.1
13	133.68		
14	142.01	6.330(dd)	10.7, 17.4
15	110.14	4.864(d), 5.020(d)	10.7; 17.4
16	12.12	1.707(s)	
17	109.72	4.474(t), 4.640(t)	1.6; 2.2
18	33.78	0.875(s)	
19	22.45	0.800(s)	
20	22.40	0.909(s)	

Table 2

OsKSL10 steady-state kinetic constants.

Substrate	K_M (μM)	k_{Cat} (min^{-1})	k_{Cat}/K_M ($M^{-1}s^{-1}$)
ent-CPP	0.6±0.1	0.02±0.01	5.6×10^3
syn-CPP	5±1	0.05±0.02	1.7×10^3