An unexpected diterpene cyclase from rice: Functional identification of a stemodene synthase

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This manuscript is dedicated to Professor Rodney Croteau (Washington State University) on the happy occasion of his 60th birthday.

Abstract

We have cloned a novel diterpene synthase (OsKSL11) from rice that produces stemod-13(17)-ene from *syn*-copalyl diphosphate. Notably, this gene sequence was not predicted from the extensive sequence information available for rice, nor, despite extensive phytochemical investigations, has this diterpene or any derived natural product previously been reported in rice plants. OsKSL11 represents the first identified stemodene synthase, which catalyzes the committed step in biosynthesis of the stemodane family of diterpenoid natural products, some of which possess antiviral activity. In addition, OsKSL11 is highly homologous to the mechanistically similar stemarene synthase recently identified from rice, making this pair of diterpene cyclases an excellent model system for investigating the enzymatic determinants for differential product outcome. The unexpected nature of this cyclase and its product parallels recent observations of previously unrecognized natural products metabolism in *Arabidopsis thaliana*, suggesting that many, if not all, plant species will prove to have extensive biosynthetic capacity.

Keywords: Biosynthesis; Natural products; Functional genomics; Terpene synthases; Labdane-related diterpenoids

The rice (*Oryza sativa*) draft genome sequences have provided researchers with a valuable tool for investigating the biochemical machinery of this vital cereal crop plant [1,2]. One area of particular interest is the production of natural products, which govern essentially all aspects of plant biology, ranging from growth and development to defense [3]. Many of these low-molecular weight organic compounds are terpenoids, which comprise the largest class of natural products with nearly 50,000 known members [4]. The labdane-related diterpenoids, characterized by minimally containing the labdane bicyclic core structure, constitute over 10% of all terpenoids. Notably, rice has previously been shown to produce many such natural products and, thus, has become a model system for investigating their metabolism (Fig. 1).

In addition to the ubiquitous gibberellic acid phytohormones, rice produces more than 10 other labdane-related diterpenoids. These serve as phytoalexins [5–12], which are antibiotic compounds produced in response to microbial infection [13], and allelochemicals [14–16], which are secreted from the roots to suppress germination of neighboring seeds [17]. The known rice diterpenoids fall into five structurally related families; the gibberellins being derived from *ent*-kaurene, phytocassanes A–E from *ent*-cassa-12,15-diene, oryzalexins A–F from *ent*-sandaracopimaradiene, momilactones A and B from *syn*-pimara-7,15-diene, and oryzalexin S from *syn*-stemar-13-ene [18,19].

Biosynthesis of labdane-related diterpenoids is initiated by class II diterpene cyclases that produce specific

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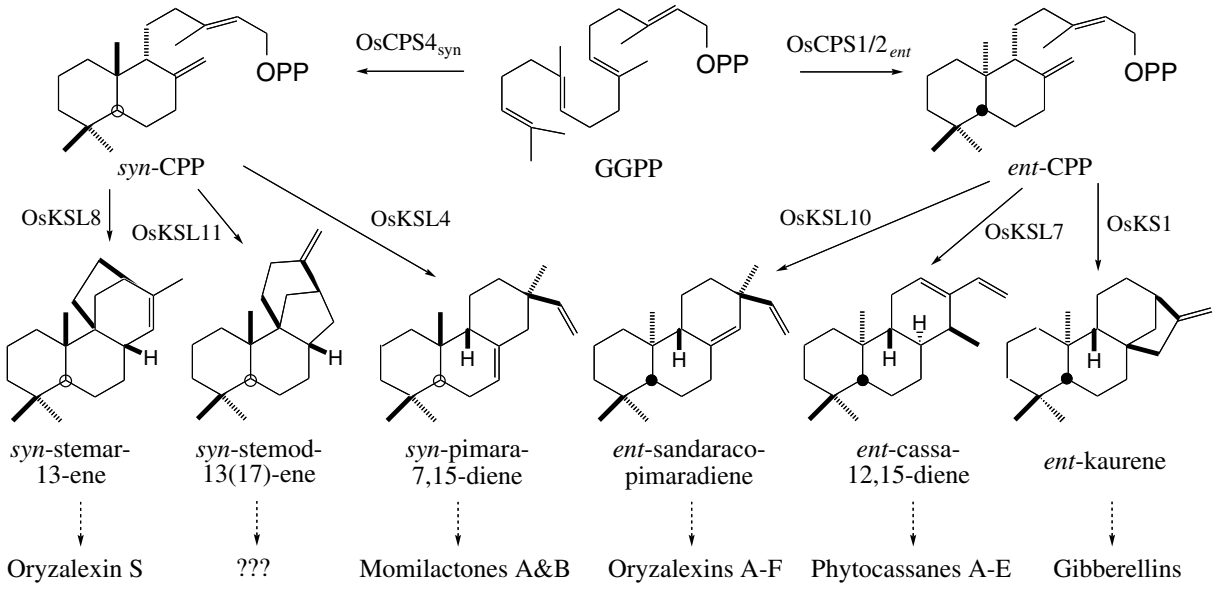


Fig. 1. Known cyclization steps in rice. The corresponding enzymes are indicated, along with their products and, where known, the derived natural products (dashed arrows indicate multiple enzymatic steps).

stereoisomers of labdadienyl/copalyl diphosphate (CPP)[1] from the universal diterpenoid precursor (*E*,*E*,*E*)-geranylgeranyl diphosphate (GGPP). This bicyclic core structure can then be further cyclized and/or rearranged by more typical (i.e., class I) CPP stereospecific terpene synthases to form various skeletal structures [20]. While prototypical plant class I terpene synthases contain two structurally defined domains [21,22], those involved in labdane-related diterpenoid metabolism invariably contain additional N-terminal sequence (~240 amino acids) termed the 'insertional' element [23]. However, whereas the class II cyclases contain a DXDD motif required for their protonation-initiated cyclization reactions [24], the class I enzymes contain a separately placed DDXXD motif involved in ligation of the divalent metal ion binding required for the corresponding diphosphate ionization initiated reaction [25].

From the known sequence information available for rice, four class II and 10 class I labdane-related diterpene synthases have been predicted. The class II genes were termed OsCPS1–4 [26], and all the active class II enzymes produce CPP. Specifically, OsCPS1 and 2 produce *ent*-CPP for gibberellin and phytoalexin biosynthesis, respectively, while OsCPS4 produces *syn*-CPP and OsCPS3 is a pseudo-gene [27–29]. The class I genes were assigned as OsKS1-10 [26,30], although only OsKS1 actually operates in gibberellin biosynthesis and, presumably, produces *ent*-kaurene. The synthases responsible for production of *ent*-cassa-12,15-diene, *syn*-pimara-7,15-diene, and *syn*-stemar-13-ene have also been termed OsDTC1, OsDTS2, and OsDTC2, respectively [23,31,32]. To avoid confusion, we suggest the use of OsKSL (rice kaurene synthase-like), with the corresponding number from Sakamoto et al. (2004) [26] where appropriate, for these non-kaurene producing class I genes. Thus, OsKS1 presumably produces *ent*-kaurene [26,33], while OsKSL4 (OsDTS2) produces *syn*-pimaradiene [23,30], OsKSL7 (OsDTC1) *ent*-cassadiene [31], OsKSL8 (OsDTC2) *syn*-stemarene [32], and OsKSL10 *ent*-sandaracopimaradiene [30] (Fig. 1). Accordingly, the novel stemodene synthase member of this family reported here has been designated OsKSL11.

Materials and methods

Chemicals

The preparations of GGPP, *ent*-and *syn*-CPP, and *syn*-stemarene have been previously described [18]. Stemodene standards were derived from a 1.5:1 mixture (ca. 5 mg) of synthetic racemic *exo*- and *endo*-stemodenes, kindly provided by Jim White [34]. This was fractionated by flash chromatography on a silica gel column (1 cm × 10 cm) using pentane and the later eluting fractions (enriched for the *exo* isomer) were pooled and concentrated under nitrogen to yield ~2 mg *exo*-stemodene (~85% purity by GC analysis). Unless otherwise noted, chemicals were purchased from Fisher Scientific (Loughborough, Leicestershire, UK) and molecular biology reagents from Invitrogen (Carlsbad, CA, USA).

Plant material

Rice plants (*O. sativa* L. ssp. *indica* cv IR24) and seedlings (ssp. *japonica* cv Nipponbare) were those previously described

[1] *Abbreviations used:* CPP, copalyl diphosphate; GC, gas chromatography; GGPP, (*E*,*E*,*E*)-geranylgeranyl diphosphate; KS, kaurene synthase; MS, mass spectrometry; NMR, nuclear magnetic resonance; OsCPS, rice CPP synthase; OsKSL, rice kaurene synthase-like; RT-PCR, reverse-transcription polymerase chain reaction; RACE, rapid amplification of cDNA ends.

[27]. Briefly, detached leaves from 4-week-old greenhouse grown plants were UV-irradiated (254 nm from 15-cm distance for 25 min.) and then incubated for 24 h under dark, humid conditions at 30 °C. Seedlings were germinated from surface sterilized seeds on filter paper in sterile 1.2% agar plates at 30 °C in the dark for a week, then sprayed with approximately 2 mL 0.1% Tween 20 with 0.5 mM methyl jasmonate per gram of plant weight, and incubated 24 h under the same conditions. The resulting tissues were frozen in liquid nitrogen and total RNA extracted using Concert Plant RNA Reagent, following the manufacturer's instructions.

Cloning

Using the predicted sequence from GenBank Accession No. AK108710 a 5′ primer (CACCATGATGCTGCTGA GTTCCTC) was designed and employed in a 3′ RACE reaction (GeneRacer), using RNA from UV-induced *indica* leaves, to amplify a cDNA product of 2703 nucleotides. This was cloned into pCR4-ZeroBlunt, and completely sequenced. The derived sequence was further confirmed by cloning an identical open reading frame (2451 nucleotides) from methyl jasmonate-induced *japonica* seedlings by RT-PCR using the 5′ primer described above and a 3′ primer (TTACTCTTGCAGGTGCAGTGGCTC). We have deposited this sequence as OsKSL11 in the various DNA databases as Accession DQ100373. Full-length and N-terminally truncated (by 60 residues) versions of OsKSL11 were constructed in pENTR/SD/D-TOPO (Gateway system) and verified by complete sequencing. These were then transferred by directional recombination to the T7-based, glutathione-*S*-transferase (GST) fusion expression vector pDEST15 (Gateway system).

Recombinant expression

Recombinant expression was carried out in the BL21-derived C41 strain (Avidis, France), as described earlier [27]. Briefly, NZY media cultures were grown to midlog phase at 37 °C ($OD_{600} \sim 0.6$) then shifted to 16 °C for 1 h prior to induction (with 1 mM IPTG) and overnight expression. Cells were harvested by centrifugation, resuspended in cold lysis buffer (50 mM Bis–Tris, pH 6.8, 1 mM DTT), lysed by mild sonication on ice (15 s, continuous output, setting 5), and clarified by centrifugation (40,000*g*, 20 min). The recombinant GST-tagged protein was purified using GST-agarose beads (Sigma–Aldrich). SDS–PAGE analysis demonstrated that only a single band of the appropriate molecular weight was obtained.

Functional characterization

Assays were generally carried out with 5 μg of substrate (GGPP, *syn*-CPP or *ent*-CPP) and 25 μL of recombinant protein in 0.5 mL of assay buffer (50 mM Hepes, pH 7.2, 10 mM $MgCl_2$, 10% glycerol, and 5 mM DTT) for 3–16 h at room temperature. The assay solutions were extracted three times with 0.5 mL portions of hexanes, which were pooled, dried under a gentle stream of nitrogen, and re-dissolved in 100 μL of hexanes. Gas chromatography–mass spectrometry (GC–MS) analysis was carried out using an HP-5MS column on an Agilent (Palo Alto, CA) 6890N GC instrument with a 5973N mass selective detector. Samples (5 μL) were injected at 40 °C in the splitless mode and, after holding 3 min at 40 °C, the temperature was increased at 20 °C/min to 300 °C, where it was also held for 3 min. MS data were collected from 50 to 500 *m*/*z* during the temperature ramp. Larger amounts of product were obtained from GGPP using coupled assays with purified GST–OsKSL11 and the truncated and GST-tagged version of OsCPS4 we have previously described [27], which was expressed and purified as described above for GST–OsKSL11. The resulting hexane–benzene extract (~1 mL) containing the diterpene products was shipped to the University of Illinois on dry ice for NMR spectroscopy. The solution was filtered through a short silica gel column with pentane and then concentrated under a N_2 stream to ca. 0.1 mL and re-dissolved in 1 mL of C_6D_6. The evaporation–dissolution was repeated four times to completely remove the hexane and protonated benzene solvents. Proton NMR spectra were recorded in C_6D_6 (to reduce the risk of air oxidation) using a Varian 500 MHz spectrometer in the School of Chemical Sciences NMR spectroscopy facility at the University of Illinois.

Sequence analysis

BLAST searches were carried out on-line at either GenBank (www.ncbi.nih.gov), TIGR (www.tigr.org), or Gramene (www.gramene.org). All other sequence analysis was performed with the AlignX program in the Vector NTI software package (Invitrogen) using standard parameters. OsKS1 was designated as the reference sequence in all alignments.

Results

Cloning an unexpected class I terpene synthase

A putative class I labdane-related diterpene synthase gene, predicted from the rice genome sequence, was found in GenBank Accession No. AK108710. Surprisingly, when we attempted to clone the corresponding cDNA, the isolated sequence did not correspond to that found in AK108710, although the two were ~92% identical at the nucleotide level. In fact, corresponding sequence could not be found in the currently available rice genome or expressed sequence tag data [35,36], although we were able to clone this cDNA from both spp. *indica* and *japonica* rice. The isolated cDNA does contain an open reading frame that encodes a class I diterpene synthase (i.e., had the large 'insertional' element and DDXXD motif) that was highly homologous (42–89% amino acid identity) to the known OsKSL enzymes (Fig. 2). Thus, this seems to be a novel

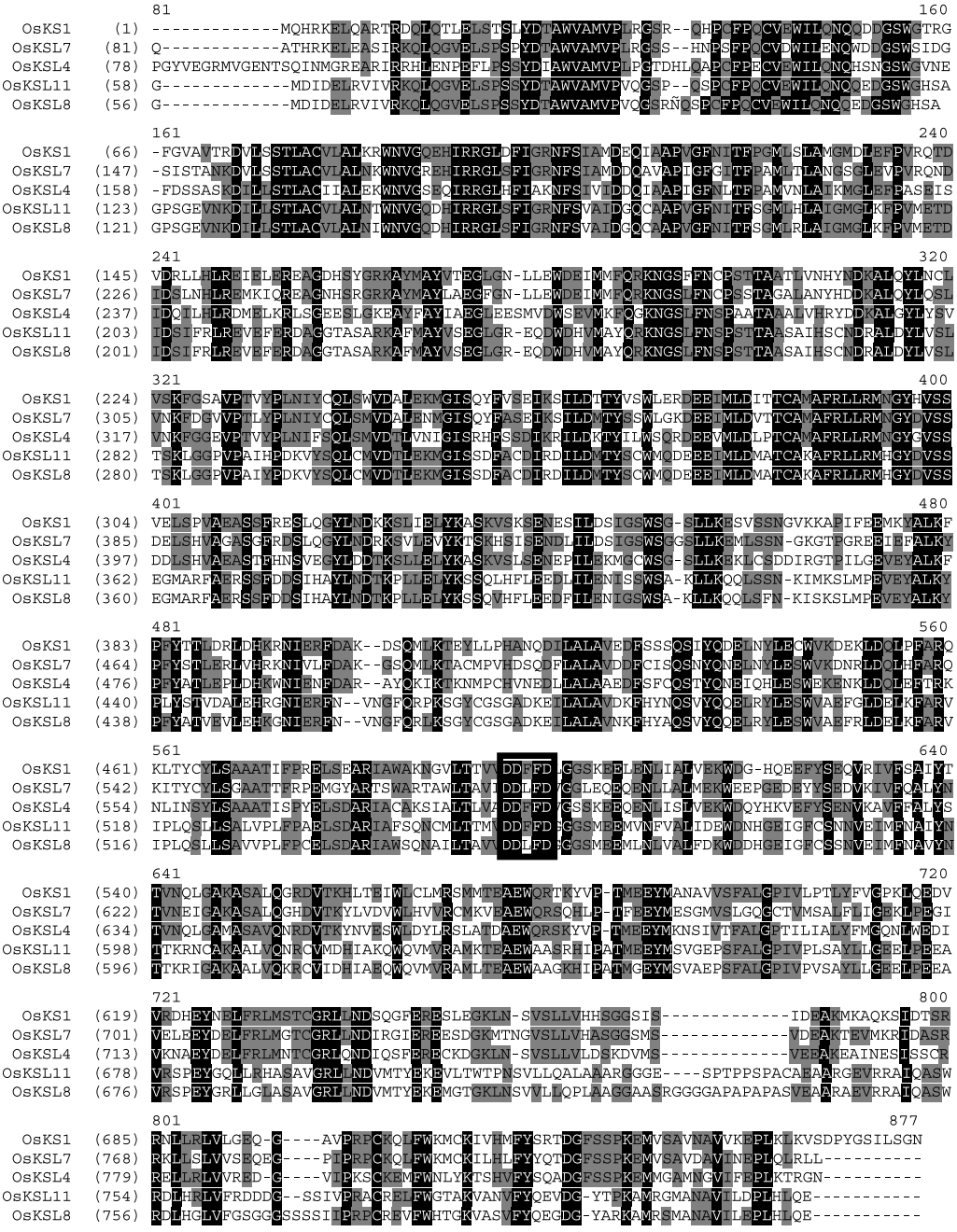


Fig. 2. Sequence comparison of OsKSL11 with other functionally identified rice kaurene synthase-like enzymes. Not shown are the extended transit peptide sequences typical of the plastid-targeted diterpene synthases. The DDXXD motif is boxed.

kaurene synthase-like gene, which we have designated OsKSL11. The originally targeted sequence contained in AK108710 corresponds to OsKSL8, which has recently been reported to encode a transcriptionally inducible *syn*-CPP specific stemarene synthase [32]. Unfortunately, we could not distinguish between OsKSL8 and OsKSL11 by RT-PCR analysis due to the extensive sequence similarity, despite repeated attempts with several primer combinations designed to discriminate between the two. Therefore, it was not possible to conclusively determine if transcription of OsKSL11 is likewise induced by UV-irradiation or application of the defensive signaling molecule methyl jasmonate.

Functional characterization of OsKSL11 as a syn-CPP specific stemodene synthase

Full-length and truncated OsKSL11 proteins were expressed and purified as fusions to GST. The recombinant protein was then assayed with GGPP, *ent*-CPP, or *syn*-CPP as substrate, and enzymatic product formation assessed by GC–MS analysis of organic extracts. While the truncated construct exhibited better activity, both constructs were catalytically active only with *syn*-CPP, and enzymatic products were not observed with GGPP or *ent*-CPP. Intriguingly, GC–MS analysis demonstrated

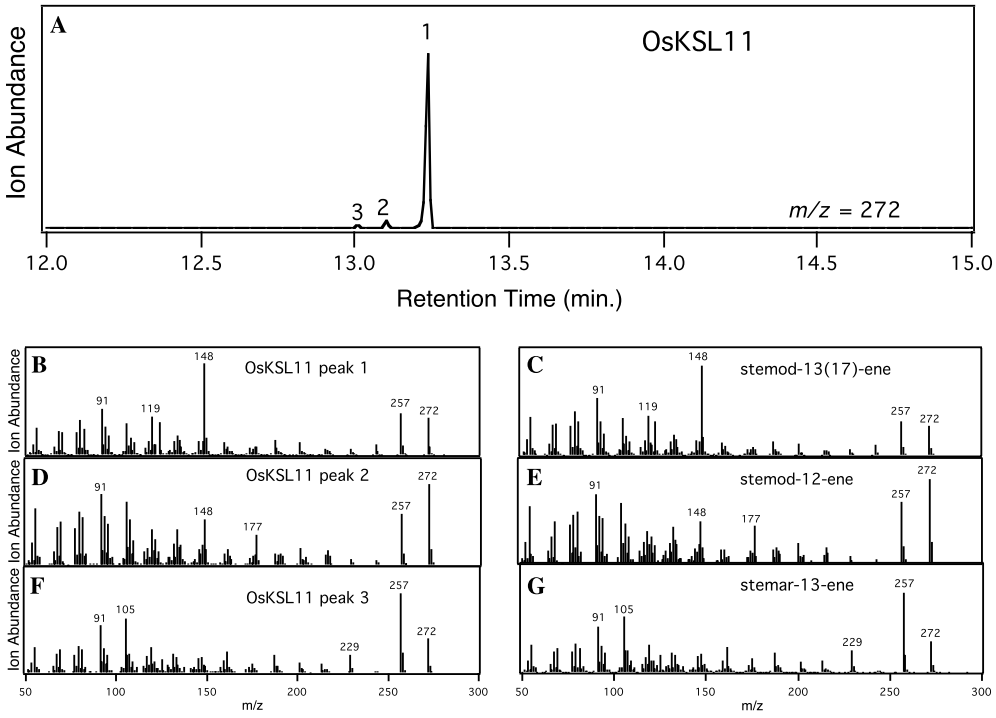


Fig. 3. Enzymatic products of OsKSL11 from *syn*-CPP. (A) GC–MS chromatograph (272 *m/z* extracted ion trace). (B) Mass spectrum of OsKSL11 product peak 1 (RT = 13.23 min). (C) Mass spectrum of authentic stemod-13(17)-ene (RT = 13.23 min). (D) Mass spectrum of OsKSL11 product peak 2 (RT = 13.10 min). (E) Mass spectrum of authentic stemod-12-ene (RT = 13.10 min). (F) Mass spectrum of OsKSL11 product peak 3 (RT = 13.01 min). (G) Mass spectrum of authentic stemar-13-ene (RT = 13.01 min).

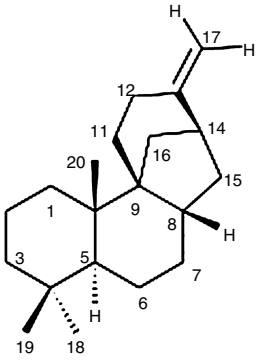
that the major enzymatic product resulting from reactions with *syn*-CPP did not correspond to authentic standards for any of the known rice diterpenes (Fig. 3). To produce sufficient quantities of enzymatic product for NMR analysis, we chose to use a coupled assay with purified GST-tagged and truncated versions of OsCPS4 (i.e., *syn*-CPP synthase [27]) and OsKSL11 (i.e., because GGPP is much more readily available than *syn*-CPP). In this way, it was possible to produce ~150 μg of the unknown diterpene product. Comparison of the GC–MS fragmentation pattern and NMR proton data with literature values initially suggested that OsKSL11 was producing aphidicol-16-ene [37]. However, given the close phylogenetic relationship with *syn*-stemarene synthase (OsKSL8) and fact that OsKSL11 also produces small amounts (~3%) of stemar-13-ene (*endo* double bond), we strongly suspected that this product was actually the mechanistically related stemod-13(17)-ene (see Fig. 5). Synthetic (±)-stemodene, as a mixture of the *exo* (13(17)-ene) and *endo* (12-ene) double bond isomers, was obtained as a kind gift from Dr. James White [34]. The enzymatic products of OsKSL11 with *syn*-CPP were then shown to be ~92% *exo*-stemodene, ~5% *endo*-stemodene, and the aforementioned ~3% *endo*-stemarene, by GC analyses, including GC–MS comparison to authentic samples (Fig. 3). The identification of the major product as *exo*-stemodene was confirmed by high field proton NMR spectra of the enzymatic product mixture and comparisons with spectra and data of the (±)-*exo*-stemodene standard, for which proton and carbon NMR assignments (Table 1) were made with the aid of COSY, NOE, HMQC, and APT spectral analyses.

Discussion

Due to our interest in the CPP stereospecific class I labdane-related diterpene synthases as model systems for investigating the enzymatic determinants of substrate and product specificity in terpene synthases, we have undertaken a functional genomics approach towards elucidating the enzymatic function of the corresponding gene family in rice. As part of this work, we identified a putative kaurene synthase-like gene that was predicted from the rice genome sequence (GenBank Accession AK108710). However, rather than the targeted gene sequence we found a novel class I diterpene synthase (OsKSL11) that is not present in either the genome or extensive cDNA sequence data available for rice [35,36]. Nevertheless, OsKSL11 clearly falls within the rice kaurene synthase-like family (Fig. 4). Further, even in its current 'finished' form the rice genome sequence still contains a number of gaps representing ~5% of the total sequence [35]. Given the size of the rice class I diterpene synthase family (now over 10 members), it is perhaps not surprising that an unexpected/'orphan' family member was found.

Functional characterization of OsKSL11 demonstrated that this is a *syn*-CPP specific *exo*-stemodene synthase (Fig. 3). Rice has not previously been demonstrated to produce stemodene or any derived natural products despite

Table 1
500 MHz ^{1}H and 126 MHz ^{13}C NMR data and assignments for synthetic (±)-stemod-13(17)-ene in C_6D_6



C#	δ_C	δ_H	m	*J*(Hz)
1	42.5	Hα 1.14	td	12.3, 3.2
		Hβ 1.30–1.46	m	
2	37.2	Hα 1.52	br d	12.7
		Hβ 1.43	qt	12.5, 3.0
3	36.7	Hα 1.07	td	12.8, 3.3
		Hβ 1.33	dt	13.1, 3.6
4	33.7			
5	47.8	1.17	d	12.7
6	19.5	Hα 1.30–1.46	m	
		Hβ 1.11	qd	12.8, 3.3
7	23.0	Hα 1.40	ddd	12.2, 7.4, 2.5
		Hβ 1.74–1.81	m	
8	39.6	1.63–1.69	m	
9	51.7			
10	39.1			
11	32.7	Hα 1.42	dd	10.1, 5.2
		Hβ 1.40–1.42	m	
12	28.9	Hα 2.10	ddd	15.0, 4.4, 3.3
		Hβ 2.23–2.31	m	
13	155.5			
14	44.3	2.73	t	6.7
15	40.4	Hα 1.27	ddd	13.8, 6.1, 2.6
		Hβ 1.82	dd	13.5, 8.1
16	38.2	Hα 1.97	ddd	11.2, 5.6, 1.7
		Hβ 1.24	d	11.3
17	102.8	H*E* 4.58	t	2.2
		H*Z* 4.67	t	2.4
18	19.2	0.90	s	
19	23.3	0.89	s	
20	35.1	0.91	s	

δ: chemical shift in ppm (C_6H_6 reference 7.16 ppm); m: apparent multiplicity; *J*: apparent coupling constant.

intensive phytochemical investigations. However, these studies have been almost exclusively targeted at identification of leaf phytoalexins [5–12], and it is possible that stemodane diterpenoids are produced in other tissues and/or in response to other conditions, such as viral rather than microbial infection. Interestingly, the stemodane and stemarene diterpene skeletal types were both first defined as natural products from *Stemodia maritime*, a medicinal plant used in traditional Caribbean medicine to treat venereal disease [38,39]. Stemodin, the major diterpenoid natural product in *Stemodia*, possesses antiviral activity [40], which may help account for the use of this plant as an herbal medicine and, more generally, for the function of this natural product in plants. Nevertheless, nothing is known about the enzymatic genes involved in biosynthesis of the *Stemodia* diterpenoid natural products, and OsKSL11 seems to be the first identified stemodene synthase.

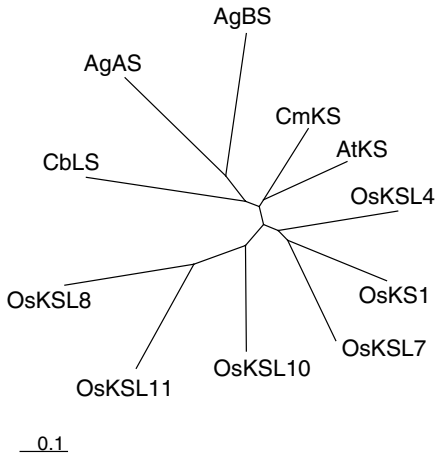


Fig. 4. Phylogenetic tree illustrating clustering of OsKSL11 with the other rice kaurene synthase-like (OsKSL) genes. Depicted are: kaurene synthases from *Arabidopsis* (AtKS), pumpkin (CmKS), and rice (OsKS), as well as all the functionally identified OsKSL family members. Also shown are other disparate, but 'insertional' element containing class I enzymes, the diterpene abietadiene synthase from *Abies grandis* (AgAS), the sesquiterpene (*E*)−α-bisabolene synthase from *A. grandis* (AgBS) and the monoterpenoid linalool synthase from *Clarkia breweri* (CbLS). The tree was derived from alignment of cDNA coding regions and visualized with TreeView [42].

Formation of the stemodene and stemarene backbones can be envisioned as arising from largely overlapping cyclization mechanisms. In particular, initial cyclization of *syn*-CPP to an isopimarenyl$^+$ intermediate that undergoes a 1,2-hydride shift from C9 to C8, followed by further cyclization to an *abeo*-stachanyl$^+$ intermediate. Alternative ring rearrangements can then form either stemodenyl$^+$ or stemarenyl$^+$ intermediates that undergo deprotonation to yield the final tetracyclic diterpenes (Fig. 5). The highly homologous OsKSL11 and OsKSL8 presumably catalyze these alternative, yet mechanistically related, cyclization reactions. Hence, structure–function analysis of this pair of cyclases should facilitate identification of the underlying enzymatic determinants for this relatively subtle change in product outcomes. In fact, OsKSL11 and OsKSL8 seem to be the most closely related pair of rice class I diterpene synthases (89% amino acid identity). Thus, identification of OsKSL11 measurably increases the utility of the rice class I diterpene synthase family for examining enzymatic specificity.

The existence of OsKSL11 indicates that rice has the capacity to produce stemodene. However, rice has not been shown to produce stemodane type diterpenoid natural products. Nevertheless, while even latent, currently untapped potential may offer some advantage in plasticity of natural products biosynthesis, the production of stemodanes by rice cannot be ruled out. It has recently been

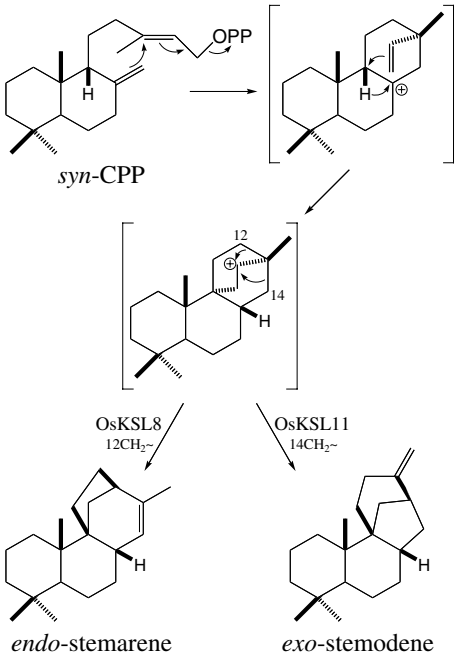


Fig. 5. Proposed overlapping cyclization mechanisms for stemarene (OsKSL8) and stemodene (OsKSL11) synthases (shown are their major products).

observed that *Arabidopsis thaliana* is capable of producing a much wider range of natural products than was previously appreciated [41]. The unexpected production of stemodene by OsKSL11 extends such observations to rice, and we suggest here that extensive, currently unrecognized biosynthetic capacity will be common in many, if not all, plant species.

Acknowledgments

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