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Functional characterization of the rice kaurene synthase-like gene family

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Abstract

The rice (*Oryza sativa*) genome contains a family of kaurene synthase-like genes (*OsKSL*) presumably involved in diterpenoid biosynthesis. While a number of OsKSL enzymes have been functionally characterized, several have not been previously investigated, and the gene family has not been broadly analyzed. Here we report cloning of several *OsKSL* genes and functional characterization of the encoded enzymes. In particular, we have verified the expected production of *ent*-kaur-16-ene by the gibberellin phytohormone biosynthesis associated OsKS1 and demonstrated that OsKSL3 is a pseudo-gene, while OsKSL5 and OsKSL6 produce *ent*-(iso)kaur-15-ene. Similar to previous reports, we found that our sub-species variant of OsKSL7 produces *ent*-cassa-12,15-diene, OsKSL10 produces *ent*-(sandaraco)pimar-8(14),15-diene, and OsKSL8 largely *syn*-stemar-13-ene, although we also identified *syn*-stemod-12-ene as an alternative product formed in ~20% of the reactions catalyzed by OsKSL8. Along with our previous reports identifying OsKSL4 as a *syn*-pimara-7,15-diene synthase and OsKSL11 as a *syn*-stemod-13(17)-ene synthase, this essentially completes biochemical characterization of the *OsKSL* gene family, enabling broader analyses. For example, because several OsKSL enzymes are involved in phytoalexin biosynthesis and their gene transcription is inducible, promoter analysis was used to identify a pair of specifically conserved motifs that may be involved in transcriptional up-regulation during the rice plant defense response. Also examined is the continuing process of gene evolution in the *OsKSL* gene family, which is particularly interesting in the context of very recently reported data indicating that a *japonica* sub-species variant of OsKSL5 produces *ent*-pimara-8(14),15-diene, rather than the *ent*-(iso)kaur-15-ene produced by the *indica* sub-species variant analyzed here.

Keywords: *Orzya sativa*; Poaceae; Terpene synthase; *ent*-kaurene synthase; *ent*-isokaurene synthase; *ent*-sandaracopimaradiene synthase; *ent*-cassadiene synthase; *syn*-stemarene synthase; *syn*-stemodene synthase; *syn*-pimaradiene synthase; Labdane-related diterpenoids; Phytoalexin; Natural products biosynthesis; Plant defense; Gene family evolution

Abbreviations: AtKS, *Arabidopsis thaliana* kaurene synthase; CPP, copalyl diphosphate; CPS, copalyl diphosphate synthase; cv, cultivar; GGPP, (*E*,*E*,*E*)-geranygeranyl diphosphate; GST, glutathione-*S*-transferase; KO, kaurene oxidase; KOL, kaurene oxidase-like; KS, kaurene synthase; KSL, kaurene synthase-like; ORF, open reading frame; OsCPS, rice (*Oryza sativa*) copalyl diphosphate synthase; OsKS(L), rice (*Oryza sativa*) kaurene synthase(-like); ssp, sub-species.

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1. Introduction

Rice is an important food crop, and has become a model plant for the cereal plant family with the recent availability of draft genome sequences (Goff et al., 2002; Yu et al., 2002), as well as large numbers of defined full-length cDNAs (Kikuchi et al., 2003). This extensive sequence information has enabled functional genomics based approaches towards elucidating the biosynthetic machinery underlying rice metabolism. One specific area of interest is the production of natural products with antimicrobial activity, which are termed phytoalexins if their biosynthesis is induced by microbial infection and phytoanticipins if their biosynthesis is constitutive (VanEtten et al., 1994), as the production of these small organic compounds is associated with resistance to microbial diseases such as that caused by the agronomically devastating rice blast pathogen *Magneporthe grisea*.

Extensive phytochemical investigation has demonstrated that rice produces a number of phytoalexins in response to *M. grisea* infection (Peters, 2006). Intriguingly, the rice phytoalexins, with the exception of the flavonoid sakuranetin, all fall into the large family of labdane-related diterpenoid natural products characterized by minimally containing a labdane type bicyclic core structure. Thus, along with the ubiquitous gibberellin phytohormone, rice produces more than 10 other labdane-related diterpenoids as phytoalexins. These are momilactones A & B (Cartwright et al., 1977, 1981), oryzalexins A–F (Akatsuka et al., 1985; Kato et al., 1993, 1994; Sekido et al., 1986), oryzalexin S (Kodama et al., 1992), and phytocassanes A–E (Koga et al., 1997; Koga et al., 1995). In addition, momilactone B is constitutively secreted from rice roots and acts as an allelochemical in suppressing germination in nearby seeds (Kato-Noguchi and Ino, 2003; Kato-Noguchi et al., 2002). The identified rice labdane-related diterpenoid natural products fall into five structurally related groups (Fig. 1), with the gibberellins being elaborated from *ent*-kaurene, oryzalexins A–F from *ent*-sandaracopimaradiene, phytocassanes A–E from *ent*-cassadiene, oryzalexin S from *syn*-stemodene, and momilactones A and B from *syn*-pimaradiene (Mohan et al., 1996; Wickham and West, 1992; Yajima et al., 2004).

Labdane-related diterpenoids share an unusual biogenetic origin, as their biosynthesis is uniquely initiated by a consecutive pair of terpene synthase catalyzed reactions. In the first reaction, the characteristic bicyclic core structure is formed by class II labdane-related diterpene cyclases, which catalyze protonation-initiated cyclization of the universal diterpenoid precursor (*E*,*E*,*E*)-geranylgeranyl diphosphate (GGPP, **1**) to produce a specific stereoisomer of labdadienyl/copalyl diphosphate (e.g. **2** and **3**) or rearranged derivative structure such as clerodanyl diphosphate (MacMillan and Beale, 1999). The resulting cyclized diphosphate compounds can then be further cyclized and/or rearranged by more typical class I terpene synthases, which initiate catalysis via ionization of the allylic pyrophosphate group (Davis and Croteau, 2000). Notably, class I labdane-related diterpene synthases exhibit stereospecificity, e.g. all of the identified copalyl diphosphate (CPP) specific enzymes only react with single stereoisomers of CPP (Cho et al., 2004; Nemoto et al., 2004; Otomo et al., 2004a; Peters et al., 2000; Wilderman et al., 2004).

Prototypical plant terpene synthases are similar in size and consist of two structurally defined domains that have been simply termed the N- and C-terminal domains because these are associated with the corresponding region of their polypeptide sequence (Starks et al., 1997; Whittington et al., 2002). While both class II and class I labdane-related diterpene synthases are phylogenetically related to

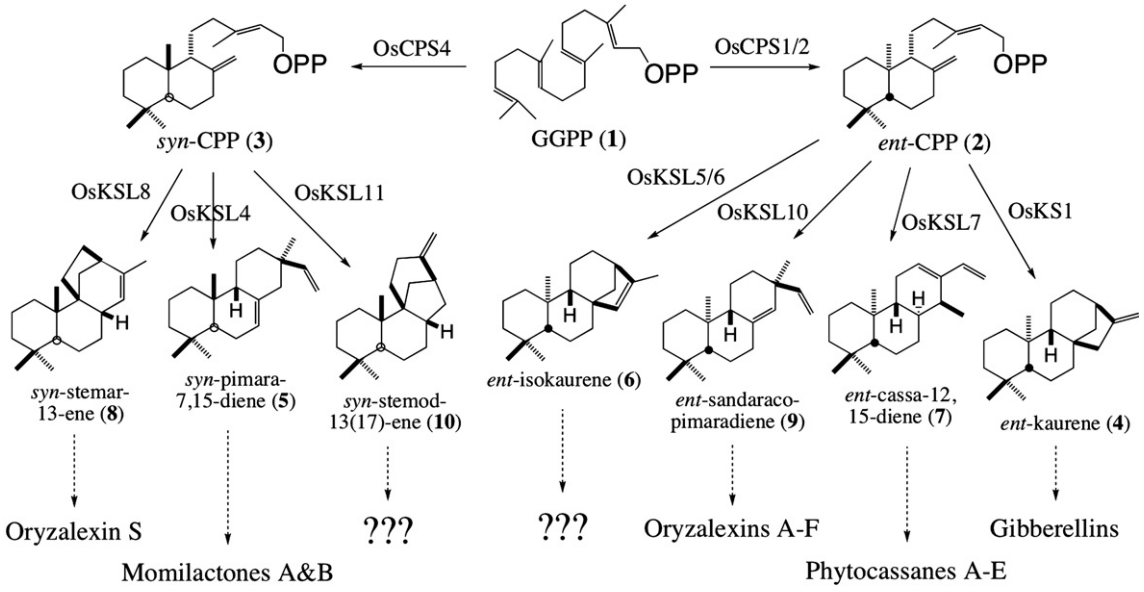

Fig. 1. Known labdane-related diterpene cyclization reactions in rice. The corresponding cyclases are indicated, along with their products and, where known, the derived natural products (dashed arrows indicate multiple biosynthetic steps).

other plant terpene synthases (Bohlmann et al., 1998; Martin et al., 2004), these also invariably contain additional N-terminal sequence (~210–240 amino acids) that has been termed the 'insertional' element. However, this sequence element was almost certainly present in the ancestral terpene synthase (Bohlmann et al., 1998; Martin et al., 2004; Trapp and Croteau, 2001), and is particularly well conserved, along with the central region that corresponds to the prototypical N-terminal domain, in class II diterpene cyclases (Xu et al., 2004). Class I terpene synthases contain a DDXXD motif in their C-terminal domain that is involved in ligation of the divalent metal ion co-factors required for their diphosphate ionization-initiated reaction mechanism (Davis and Croteau, 2000). By contrast, class II diterpene cyclases contain a DXDD motif in their central domain that is required for their protonation-initiated cyclization reactions (Peters and Croteau, 2002a; Peters et al., 2001). Indeed, the C-terminal domain has been termed the class I domain, with the prototypical N-terminal or central domain termed the class II domain (Christianson, 2006; Wendt and Schulz, 1998). Given the equally conserved nature of the 'insertional' element and central domain in class II diterpene cyclases, we suggest the use of class IIa and class IIb domains for these regions, respectively. Hence, the domain structure of plant labdane-related diterpene synthases consists of the class IIa, class IIb, and class I domains, although these all seem to be structurally interdependent and can not be divided into separate polypeptides that exhibit the associated activity (Peters et al., 2003).

Based on this characteristic domain structure and the class specific aspartate rich motifs, four class II and 11 class I labdane-related diterpene synthases have been found in the extensive sequence information available for rice. All four class II genes have been characterized, demonstrated to produce CPP, and were termed CPP synthases (CPS); specifically OsCPS1-4, with OsCPS1 and OsCPS2 producing *ent*-CPP (**2**) for gibberellin and phytoalexin biosynthesis, respectively, OsCPS3 being a pseduo-gene, and OsCPS4 producing *syn*-CPP (**3**) (Otomo et al., 2004b; Prisic et al., 2004; Sakamoto et al., 2004; Xu et al., 2004). The class I labdane-related diterpene synthases have been termed kaurene synthases (KS), specifically OsKS1–10 (Margis-Pinheiro et al., 2005; Otomo et al., 2004a; Sakamoto et al., 2004). However, mutational analysis has demonstrated that only OsKS1 is involved in gibberellin biosynthesis and, presumably, produces kaurene (Margis-Pinheiro et al., 2005; Sakamoto et al., 2004). The other characterized family members do not produce kaurene (**4**) and some also have been given alternative names (e.g. OsDTC1, OsDTC2, and OsDTS2). Furthermore, the same OsKS nomenclature has been assigned to different genes by Sakamoto et al. (2004) and Margis-Pinheiro et al. (2005). Thus, to avoid confusion we have suggested use of OsKSL (rice kaurene synthase-like), with the numbering scheme used by Sakamoto et al. (2004) where appropriate, for the non-kaurene producing class I labdane-related diterpene synthases from rice (Morrone et al., 2006). Accordingly, OsKS1 retains its original designation, but the *syn*-pimaradiene synthase originally termed OsDTS2 (Wilderman et al., 2004) or OsKS4 (Otomo et al., 2004a) should be referred to as OsKSL4, the *ent*-cassadiene synthase originally referred to as OsDTC1 (Cho et al., 2004) is OsKSL7, the *syn*-stemarene synthase originally termed OsDTC2 (Nemoto et al., 2004) is OsKSL8, and the *ent*-sandaracopimaradiene synthase originally referred to as OsKS10 (Otomo et al., 2004a) is OsKSL10 (see Table 1). As indicated by this listing, a number of the OsKSL family members were uncharacterized. Here we report cloning and characterization of many of the OsKSL enzymes, along with more general analysis of this gene family, and comparison to a similar report that appeared during the preparation of this manuscript (Kanno et al., 2006).

Table 1
Rice kaurene synthase-like gene family

Name[a]	Product	Inducible	Accession	Locus	Alternate	Reference
OsKS1	*ent*-Kaurene	No	NR[b]	Os04g52230	–	Sakamoto et al. (2004)
			AY347876			Margis-Pinheiro et al. (2005)
OsKSL2	unknown	unknown	DQ823350	Os04g52240	–	This study
OsKSL3	⟨pseudo-gene⟩	No	DQ823351	Os04g52210	OsKS2[c]	This study
OsKSL4	*syn*-Pimaradiene	Yes	AY616862	Os04g10060	OsDTS2	Wilderman et al. (2004)
			AB126934		–	Otomo et al. (2004)
OsKSL5	*ent*-iso-Kaurene	No	DQ823352	Os02g36220	OsKS6[c]	This study
OsKSL6	*ent*-iso-Kaurene	No	DQ823353	Os02g36264	OsKS5[c]	This study
OsKSL7	*ent*-Cassadiene	Yes	DQ823354[d]	Os02g36140	OsDTC1	Cho et al. (2004)
OsKSL8	*syn*-Stemarene[e]	Yes	AB118056	Os11g28530	OsDTC2	Nemoto et al. (2004)
OsKSL9	⟨pseudo-gene⟩	–	NR[b]	Os11g28500	–	Sakamoto et al. (2004)
OsKSL10	*ent*-Pimaradiene	Yes	DQ823355[d]	Os12g30824	–	Otomo et al. (2004)
OsKSL11	*syn*-Stemodene	No?	DQ100373	unknown	–	Morrone et al. (2006)

[a] Gene numbering based on Sakamoto et al. (2004), with kaurene synthase-like (KSL) nomenclature for the nonkaurene producing family members, as previously suggested (Morrone et al., 2006).
[b] NR, not reported, Sakamoto et al. (2004) did not report any sequence information.
[c] Partial gene sequences reported by Margis-Pinheiro et al. (2005).
[d] Novel sequence with verified biochemical activity matching that from the original reference given on the right.
[e] As identified in this study, OsKSL8 makes significant amounts of stemodene in addition to stemarene.

2. Results

2.1. Identification of rice kaurene synthase-like genes

Because of our interest in using CPP stereospecific diterpene synthases as model systems for investigating substrate and product specificity, we undertook a functional genomics based approach towards identifying the enzymatic activity of the corresponding class I labdane-related diterpene synthases from rice. Putative synthases were identified in silico using the sequence of the kaurene synthase from *Arabidopsis thaliana* in BLAST searches probing the extensive sequence information available for rice. The *OsKSL* genes were largely cloned on the basis of this information and assigned numbers by mapping onto the genome and comparison to the locations given by Sakamoto et al. (2004).

OsKS1 (AY347876) was obtained after identification of its role in GA biosynthesis via transposon insertional mutagenesis, as previously reported (Margis-Pinheiro et al., 2005). This partial cDNA clone was 99.9% identical to a recently released sequence (AB126933), and the one observed difference presumably reflects allelic variation, as both clones are from the *japonica* subspecies (ssp.) of rice.

OsKSL2 was initially found as a predicted gene from genomic sequencing (CAE05199), and we were only able to obtain partial cDNA clones that do not cover the full open reading frame (ORF), despite extensive efforts in both ssp. *indica* and *japonica*. We have deposited the largest such partial cDNA from ssp. japonica (DQ823350).

OsKSL3 has previously been reported as a partial cDNA sequence (Margis-Pinheiro et al., 2005). However, sequencing of multiple cDNA clones from both ssp. *indica* and *japonica*, as well as re-sequencing of the original partial cDNA clone (AY347879), conclusively demonstrated that this gene contains a single base insertion after nucleotide 1215 (DQ823351). The resulting frame-shift should result in a severely truncated protein (441 instead of 764 amino acid residues). Notably, whereas none of our other OsKSL clones have obvious splicing errors, all three of our OsKSL3 clones from ssp. *indica* cultivar (cv.) IR24 are mis-spliced at the border between exons 10 and 11 that joins nucleotides 1702 and 1703, suggesting degradation of this downstream splice site in ssp. *indica*.

OsKSL4 is the clone (AY616862) originally reported as OsDTS2 (Wilderman et al., 2004), which is 99.3% identical to another cDNA (AB126934) that was separately reported as OsKS4 (Otomo et al., 2004a). Presumably the few observed differences are due to inter-subspecies variation, as our OsKSL4/OsDTS2 clone is from ssp. *indica* cv. IR24 and the other is from ssp. *japonica* cv. Nipponbare.

OsKSL5 was initially found in the rice full-length cDNA sequencing project, although only as a single clone containing a two-base deletion that should result in a truncated protein, although there are two single-base insertions that occur later in the sequence (AK121446). While we obtained this clone from the Rice Genome Resource Center (www.rgrc.dna.affrc.go.jp) and verified the frame-shift mutations, partial cDNA sequences reported later (AB126935 and AY347882) indicated OsKSL5 was a functional gene, i.e. at least some alleles did not contain these frame-shift mutations. Encouraged by this, we proceeded to clone a cDNA covering the full ORF that also did not have the frame-shift mutations. This OsKSL5 cDNA was 99.0% identical to those previously reported, and the few observed differences are presumably due to the inter-subspecies variation between the ssp. *japonica* cv. Nipponbare used by others and the ssp. *indica* cv. IR24 that was the source of the clone reported here (DQ823352).

OsKSL6 was originally found among the genes predicted from the rice genome (Sakamoto et al., 2004). Two overlapping partial cDNA sequences (AB126936 and AY347881) that together span the entire predicted ORF were later reported. We cloned a cDNA covering the full ORF (DQ823353). This was 99.9% identical to the previously reported partial cDNAs, and the one observed difference is presumably an allelic variant, as all sequences were from ssp. *japonica* cv. Nipponbare.

OsKSL7 was originally identified as OsDTC1 (AB089272), which was cloned from cv. BL-1 rice (Cho et al., 2004). Here we report cloning a full-length ORF for OsKSL7 from ssp. *indica* cv. IR24 (DQ823354). This clone was 99.0% identical to the BL-1 derived ORF. Again, the few observed differences presumably are a result of inter-subspecies variation.

OsKSL8 was originally identified as OsDTC2 (AB118056), which was cloned from ssp. *japonica* cv. Nipponbare rice (Nemoto et al., 2004). We cloned two overlapping partial cDNAs, also from ssp. *japonica* cv. Nipponbare, which together covered the corresponding ORF. These were spliced together to (re)create an identical copy of the full-length ORF for OsKSL8.

OsKSL9 was originally reported to be a pseudogene (Sakamoto et al., 2004). In particular, the genomic sequence data indicates that the corresponding locus (Os11g28500) only encodes a partial ORF, which was not pursued.

OsKSL10 was initially found in the rice full-length cDNA sequencing project, although only as a single clone reported as containing two neighboring single-base insertions that would result in a truncated protein (AK072461). We also obtained this clone from the Rice Genome Resource Center. However, re-sequencing of this full-length cDNA demonstrated that the frame-shift insertions were a result of sequencing errors, along with two mis-called bases in the same region. This verified sequence (DQ823355) was 99.8% identical to a partial cDNA (AB126937) reported as OsKS10 (Otomo et al., 2004a). Both clones were from ssp. *japonica* cv. Nipponbare, and the very few observed differences are presumably a result of allelic variation.

OsKSL11 (DQ100373) is a full-length cDNA that has been previously reported from both ssp. *indica* and *japonica* (Morrone et al., 2006). Notably, corresponding sequence still cannot be located in the currently available rice genome (e.g. at www.gramene.org).

2.2. Functional characterization of the rice kaurene synthase-like gene products

All of the OsKS(L) proteins were expressed as fusions to the C-terminus of gluthathione-*S*-transferase (GST-OsKSL), which provided a convenient affinity tag for single step purification. When activity was not detected in the context of GST fusion proteins, it was necessary to turn to a thioredoxin fusion construct to obtain active recombinant preparations (i.e. for OsKSL8). The resulting recombinant fusion proteins were assayed with GGPP (**1**), *ent*-CPP (**2**), or *syn*-CPP (**3**) as substrate, and their enzymatic activity assessed by GC–MS analysis of organic extracts of these reactions. Enzymatic products were identified by GC–MS based comparison to authentic samples (Fig. 2). All of the active enzymes were specific for either *ent*- (**2**) or *syn*-CPP (**3**), and did not react with the alternative stereoisomer, nor with GGPP (**1**).

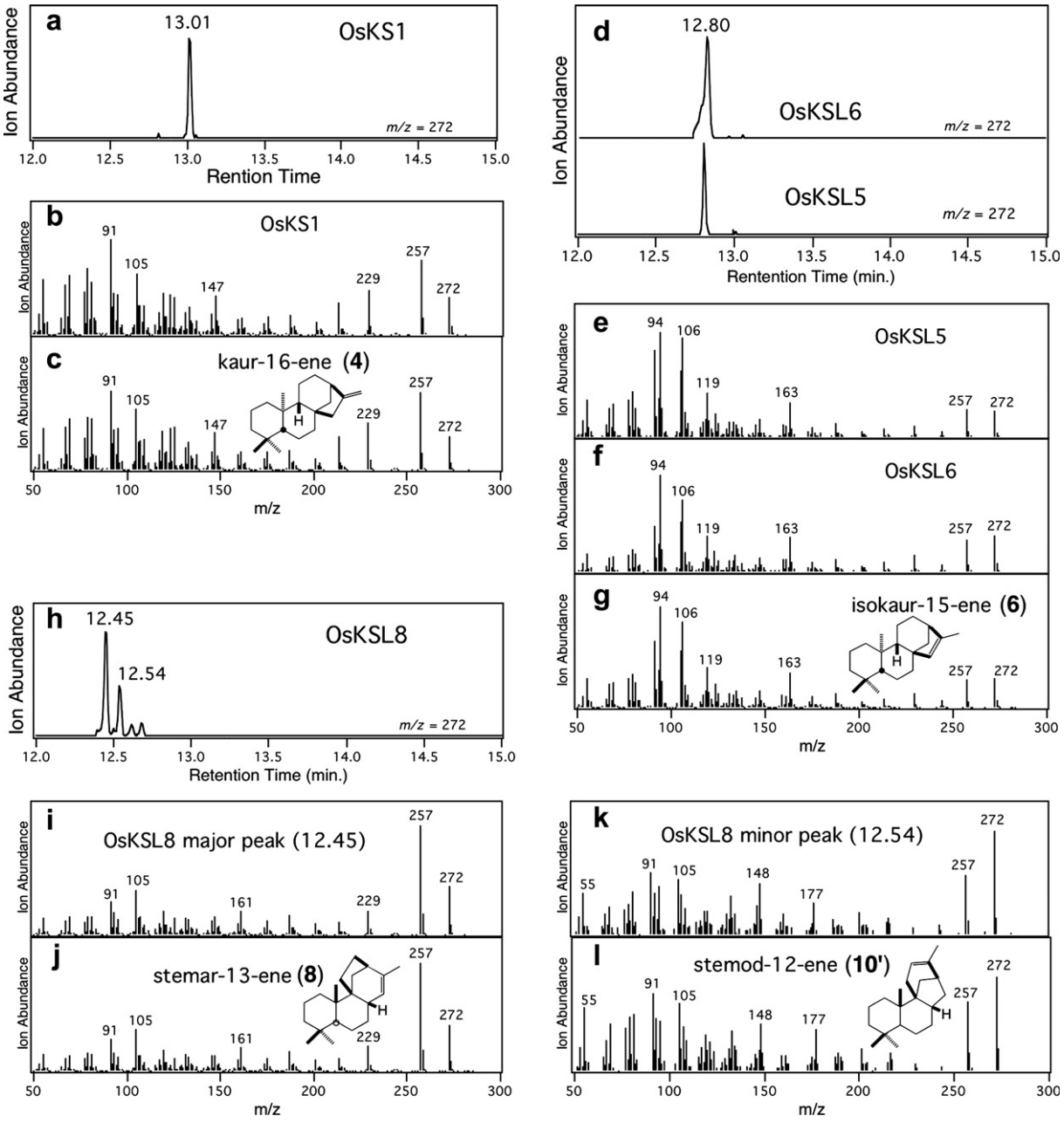

Fig. 2. GC–MS analysis of products formed by selected OsKS(L). (a) Chromatograph of the product formed by OsKS1 from *ent*-CPP. (b) Mass spectrum of OsKS1 product peak (RT = 13.01 min). (c) Mass spectrum of authentic *ent*-kaur-16-ene (**4**) (RT = 13.01 min). (d) Chromatograph of the product formed by OsKSL5 and OsKSL6 (as indicated) from *ent*-CPP. (e) Mass spectrum of OsKSL5 product peak (RT = 12.80 min). (f) Mass spectrum of OsKSL6 product peak (RT = 12.80). (g) Mass spectrum of authentic *ent*-(iso)kaur-15-ene (**6**) (RT = 12.81 min). (h) Chromatograph of the products formed by OsKSL8 from *syn*-CPP. (i) Mass spectrum of the major OsKSL8 product peak (RT = 12.45 min). (j) Mass spectrum of authentic *syn*-stemar-13-ene (**8**) (RT = 12.45). (k) Mass spectrum of the minor OsKSL product peak (RT = 12.54). (l) Mass spectrum of authentic *syn*-stemod-12-ene (**10′**) (RT = 12.55).

As expected from its known role in gibberellin biosynthesis (Margis-Pinheiro et al., 2005; Sakamoto et al., 2004), OsKS1 specifically reacts with *ent*-CPP (**2**) to produce kaur-16-ene (**4**) (Fig. 2A–C). OsKSL3 contains a frame-shift mutation and appears to be a pseudo-gene. Consistent with this interpretation, simply correcting the frame-shift does not restore activity, suggesting that other deleterious mutations have arisen. As previously demonstrated, OsKSL4 specifically utilizes *syn*-CPP (**3**) to produce pimara-7,15-diene (**5**) (Otomo et al., 2004a; Wilderman et al., 2004). Interestingly, both OsKSL5 and OsKSL6 specifically react with *ent*-CPP (**2**) to produce (iso)kaur-15-ene (**6**) (Fig. 2D–G). As previously reported for OsKSL7 (Cho et al., 2004), our ssp. *indica* variant specifically utilizes *ent*-CPP (**2**) to produce cassa-12,15-diene (**7**) (data not shown). Unlike all the other OsKS(L), which each essentially produce only a single diterpene, it has been previously reported that OsKSL8 specifically utilizes *syn*-CPP (**3**) to produce stemar-13-ene (**8**) as its major product (~70% of the total), along with significant (~20%) production of another, unidentified diterpene (Nemoto et al., 2004). Using our clone we observed the same product mix and were able to identify the unknown diterpene as stemod-12-ene (**10′**) (Fig. 2H–L). As previously reported for OsKSL10 (Otomo et al., 2004a), our allelic clone specifically reacts with *ent*-CPP to produce (sandaraco/iso)pimara-8(14),15-diene (**9**) (data not shown). Finally, as we have previously demonstrated, OsKSL11 produces stemod-13(17)-ene (**10**) from *syn*-CPP (**3**) (Morrone et al., 2006).

2.3. Analysis of inducible rice labdane-related diterpene synthase gene promoters

A number of OsKSL enzymes are involved in phytoalexin biosynthesis (Fig. 1), and it has been reported that transcription of that specific subset of the OsKSL family, i.e. OsKSL4, OsKSL7, OsKSL8, and OsKSL10, is induced by either fungal elicitor or UV-irradiation (Cho et al., 2004; Nemoto et al., 2004; Otomo et al., 2004a; Sakamoto et al., 2004; Wilderman et al., 2004). Also reported was similarly induced transcription of the phytoalexin biosynthesis associated *ent*- and *syn*-CPP synthases, OsCPS2 and OsCPS4, respectively (Otomo et al., 2004b; Prisic et al., 2004; Sakamoto et al., 2004; Xu et al., 2004). Given the observed differences in transcriptional regulation of the various rice diterpene synthase genes and their common evolutionary origin, we expected that comparison of their promoter regions might identify *cis*-acting regulatory elements, specifically potential binding sites for transcription factors involved in the observed induction of OsKSL4, OsKSL7, OsKSL8, and OsKSL10, as well as OsCPS2 and OsCPS4. Because *cis*-acting elements are generally located within a kilobase (kb) of the initial exon, our analyses focused on the 1.0 kb of sequence upstream of the *OsKS(L)* and *OsCPS* genes (except for *OsCPS4* where only 776 bases could be analyzed due to the presence of a gap in the genome sequence at this position). Due to the large number of potential binding sites that were identified, the results from searches for known transcription factor binding sites were difficult to interpret. To find sites conserved specifically within the inducible rice diterpene synthase promoters, we searched for sequence elements, on either strand, common to all the inducible *OsCPS* and *OsKSL* genes, but not found in either of the gibberellin biosynthesis associated and non-inducible *OsCPS1* and *OsKS1*, promoter regions. From this analysis we identified four 6-mer motifs: GTTTAT, GAAATT, TGCAAT, and ATATGG.

In addition to the known involvement of labdane-related diterpene synthases, evidence has been reported suggesting that two other genes may be involved in rice diterpenoid phytoalexin biosynthesis. Specifically, while rice contains five genes homologous to kaurene oxidase (KO), the first cytochrome P450 involved in gibberellin biosynthesis (Olszewski et al., 2002), only one of these (i.e. *OsKO2*) is actually involved in phytohormone metabolism (Sakamoto et al., 2004). Transcription of the two most divergent paralogs, termed KO-like (i.e. *OsKOL4* and *OsKOL5*), has been shown to be induced by either fungal elicitor or UV-irradiation, leading to the suggestion that OsKOL4 and OsKOL5 are involved in rice diterpenoid phytoalexin biosynthesis (Itoh et al., 2004). Notably, two of the specifically conserved motifs identified above, GTTTAT and GAAATT, are also found in the promoter regions of the similarly regulated OsKOL4 and OsKOL5 genes, while not being found in that of the GA biosynthesis specific and non-inducible OsKO2 paralog. By contrast, the TGCAAT motif was not found in the promoter regions of the inducible OsKOL genes, while the ATATGG motif was found in the promoter region of the gibberellin biosynthesis associated and non-inducible OsKO2, as well as that of OsKOL4 (although not OsKOL5). Thus, it seems likely that the two somewhat related GTTTAT and GAAATT motifs are regulatory elements involved in the observed similar transcription induction profiles of OsCPS2, OsCPS4, OsKSL4, OsKSL7, OsKSL8, OsKSL10, OsKOL4, and OsKOL5. These motifs also may be useful for identification of other enzymatic genes involved in rice phytoalexin biosynthesis, diterpenoid or otherwise (i.e. the flavonoid sakuranetin).

2.4. Sequence analysis of the rice kaurene synthase-like genes/enzymes

As selected for by the in silico search strategy, all of the *OsKS(L)* genes share significant homology, 50–57% identity, to the kaurene synthase from *A. thaliana* (AtKS). The encoded proteins also share significant homology with AtKS, exhibiting 39–47% identity. AtKS is most closely related to OsKS1, sharing 47% identity at the amino acid level. By contrast, the OsKSL enzymes are only 39–43% identical to AtKS at the amino acid level. This is consistent with conservation of kaurene synthase activity for AtKS and OsKS1, and the release of such selective pressure for the *OsKSL* genes, enabling the observed evolution of divergent function.

Alignment of the OsKS(L) family revealed 53–93% identity at the nucleotide level and 42–89% identity at the amino acid level (Fig. 3). Examining the resulting cDNA sequence based phylogenetic relationships demonstrates that the *OsKSL* genes are not broadly conserved by function (Fig. 4). For example, *syn*-CPP (**3**) and *ent*-CPP (**2**)

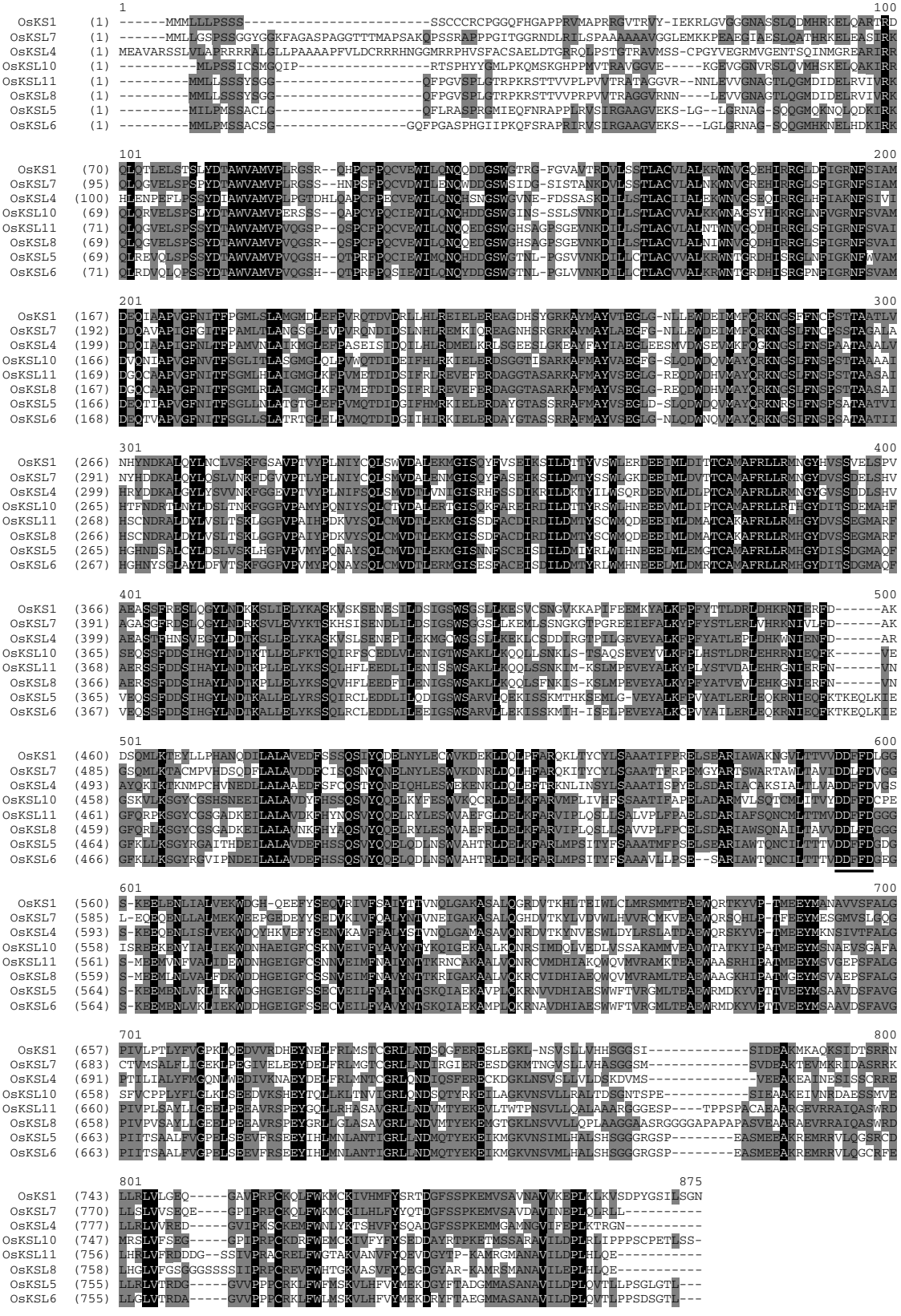

Fig. 3. Sequence comparison of the rice kaurene synthase-like enzymatic family. Shown are all the active enzymes, with the DDXXD motif underlined.

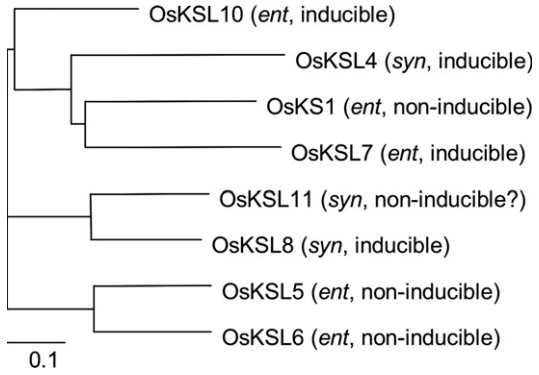

Fig. 4. Phylogenetic tree illustrating the relationship of the OsKSL gene family based on alignment of the corresponding cDNA (ORF only) sequences. Indicated is the enzymatic stereospecificity and inducible or non-inducible nature of the corresponding gene transcription.

specific OsKSL enzymes do not group together, as the *syn*-CPP (**3**) specific OsKSL4 is more closely related to the *ent*-CPP (**2**) specific OsKS1, OsKSL7, and OsKSL10 than the similarly *syn*-CPP (**3**) specific OsKSL8 and OsKSL11. There also is no clear phylogenetic relationship among the inducible versus non-inducible *OsKSL* genes. Although the inducible *OsKSL4*, *OsKSL7*, and *OsKSL10* may share a common ancestor, these are only distantly related to the similarly regulated *OsKSL8*, being much more closely related to the un-inducible *OsKS1* instead.

Nevertheless, there are two closely related pairs of *OsKSL* genes that do have some functional characteristics in common. OsKSL5 and OsKSL6 are the two most closely related OsKSL family members, exhibiting 93.0% identity at the nucleotide level and 89.0% identity at the amino acid level, and both are *ent*-CPP (**2**) specific and produce isokaurene (**6**). Further, both are similarly regulated, as their transcription is not inducible by UV-irradiation or fungal elicitor (Otomo et al., 2004a; Sakamoto et al., 2004). OsKSL8 and OsKSL11 are similarly closely related, exhibiting 92.0% identity at the nucleotide level and 88.6% identity at the amino acid level, and both are *syn*-CPP (**3**) specific and produce biogenetically related stemarane (e.g. **8**) and stemodane (e.g. **10** and **10′**) type diterpenes, whose cyclization mechanisms can be envisioned as largely overlapping (Fig. 5). Indeed, as demonstrated here, OsKSL8 produces significant amounts of both skeletal types (Fig. 2H–L). However, whereas OsKSL8 is associated with phytoalexin biosynthesis and exhibits inducible gene transcription (Nemoto et al., 2004), the physiological role of OsKSL11 remains unclear as its transcription does not appear to be inducible (Morrone et al., 2006).

Consistent with their observed enzymatic activity, all the OsKS(L) enzymes contain the class I activity associated DDXXD divalent metal binding motif, and not the class II activity associated DXDD motif. Comparison of the OsKS(L) with KS from other plant species revealed that, unlike the class II labdane-related diterpene synthases (e.g. CPS) that are highly conserved across the class IIa and class IIb regions, but not the class I domain (Xu et al., 2004), class I labdane-related diterpene synthases [e.g. KS(L)] are conserved across the entire mature protein sequence (Fig. 6), i.e. excluding the poorly conserved plastid-directing transit sequence found in all mono- and di-terpene synthases (Davis and Croteau, 2000). The observed conservation of the class I domain is consistent with previous suggestions that class I activity in labdane-related diterpene synthases is carried out in an active site that is essentially entirely located in this DDXXD containing structural domain (Peters et al., 2003; Peters and Croteau, 2002b; Peters et al., 2001), just as found in other terpene synthases (Christianson, 2006).

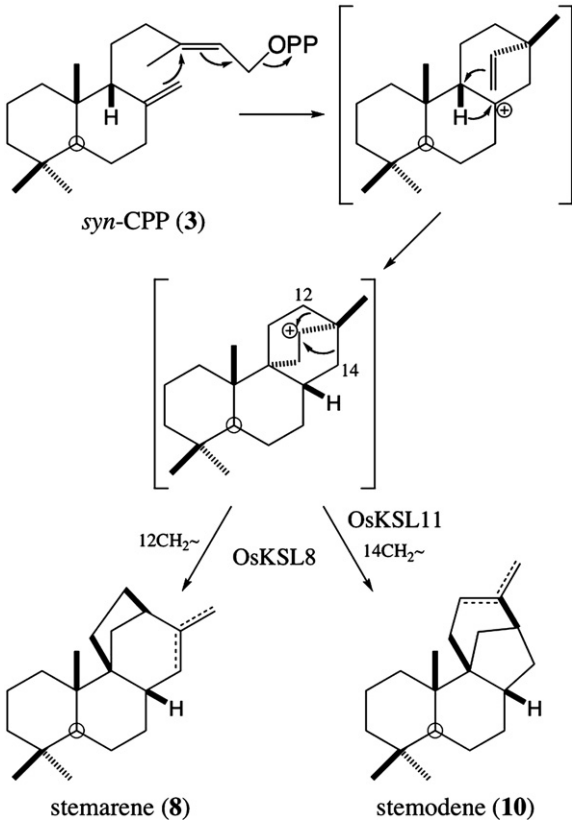

Fig. 5. Proposed biogenetic cyclization mechanisms leading to formation of stemarene (**8**) and stemodene (**10**) with the product outcomes catalyzed by OsKSL8 and OsKSL11 indicated (dotted bonds indicate alternative locations for the carbon–carbon double bond).

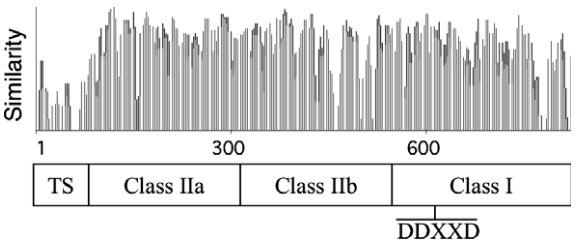

Fig. 6. Histograph depicting amino acid sequence similarity over all KS(L), i.e. class I labdane-related diterpene synthases (numbering based on the consensus sequence). Also depicted is the approximate corresponding domain structure defined in the text, along with approximate location of the class I associated DDXXD motif (T indicates transit peptide region).

Finally, it seems worth mentioning that the observed difference between allelic copies of the various *OsKSL* genes is limited to $\geqslant$99.8% identity, while the difference between inter-subspecies orthologs is 99.0–99.3% identity. The corresponding 0.7–1.0% inter-subspecies variation in the *OsKSL* gene family approximates the 0.5% rate of occurrence for single nucleotide polymorphisms/differences observed between the non-repetitive regions of the genomic sequences of ssp. *indica* and *japonica* (Yu et al., 2002).

3. Discussion

The specificity of class I labdane-related diterpene synthases for particular stereoisomers of CPP provides a potential model system for investigating the underlying steric constraints in the active sites of these and, by extension, other terpene synthases. Such studies would be greatly assisted by the identification of closely related yet functionally distinct class I labdane-related diterpene synthases. Notably, terpene synthases are generally conserved by taxonomic origin rather than enzymatic activity (Bohlmann et al., 1998; Martin et al., 2004). Thus, in order to provide a model system for investigation of class I labdane-related diterpene synthases, we have been engaged in a functional genomics based effort to elucidate the individual biochemical functions of each member of the rice kaurene synthase-like (*OsKSL*) gene family. While the biochemical function of some of the corresponding enzymes have been previously reported, a number remained uncharacterized. Here we report the completion of functional characterization of essentially all the individual family members (Table 1), which further enabled analysis of this gene family more generally.

One notable finding from our earlier studies was the functional pairing of the gene encoding the *syn*-CPP synthase *OsCPS4* near that for the *syn*-pimaradiene synthase (*OsKSL4*) in the rice genome (Wilderman et al., 2004). We also noted at that time that the genes encoding phytoalexin associated *ent*-CPP synthase *OsCPS2* and the *ent*-cassadiene synthase *OsKSL7* were similarly clustered together, along with *OsKSL5* and *OsKSL6*, and hypothesized that the enzymes encoded by these latter two gene also would prove to be specific for *ent*-CPP (**2**), which has now been verified (Fig. 2D–G). Thus, the two gene clusters containing both class I and II labdane-related diterpene synthase are grouped by biochemical function and not co-regulation. In particular, each cluster represents a functional biosynthetic module encoding enzymes that act consecutively to produce and then use a specific stereoisomer of CPP (**2, 3**), but the genes are not necessarily regulated in the same manner (i.e. while transcription of *OsCPS2* and *OsKSL7* is inducible, that of *OsKSL5* and *OsKSL6* in the same gene cluster is not).

In addition to these two class I and II labdane-related diterpene synthase containing gene clusters, the other mapped *OsKSL* genes cluster together. *OsKS1*, *OsKSL2*, and *OsKSL3* are group together in a tandem array, and *OsKSL8* is found next to the partial pseudo-gene *OsKSL9* (Sakamoto et al., 2004). Further, a similar partial gene sequence for another class I labdane-related diterpene synthase pseudo-gene is located near *OsKSL10* (rice gene locus Os12g30800). Hence, *OsKSL4* appears to be the only *OsKSL* not located near another such gene in the rice genome. While *OsKSL11* still can not be found in the currently available rice genome, there is a 'gap' in the genome sequence near *OsCPS4* and *OsKSL4*, and it is tempting to speculate that *OsKSL11* resides in this 'gap', which would be consistent with clustering of the *OsKSL* genes, as well as functional clustering by enzymatic stereospecificity (i.e. the shared *syn*-CPP (**3**) specific nature of OsCPS4, OsKSL4, and OsKSL11). In any case, it has been noted that these gene clusters all contain retrotransposon-like elements, which has been suggested to underlie the observed extensive duplication of *CPS* and *KSL* genes in rice (Sakamoto et al., 2004), and presumably also enabled their assembly into the functional biosynthetic modules noted above.

Intriguingly, it has been recently reported that barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) similarly contain multiple copies of *CPS*- and *KS*-like genes (Spielmeyer et al., 2004). This was based on chromosomal mapping using barley gene probes, which mapped to regions of the barley and wheat genomes that are syngenic with the rice labdane-related diterpene synthase gene clusters, indicating that these gene clusters were assembled prior to the evolutionary divergence between barley, wheat, and rice. Furthermore, a number of the barley genes mapped onto the rice genome most strongly to rice genes now known to be involved in phytoalexin biosynthesis (e.g. *OsCPS4* and *OsKSL4*), additionally suggesting that labdane-related diterpenoid secondary metabolism is widespread in the grass/cereal family (Poaceae). Consistent with this hypothesis, maize (*Zea mays*) has been shown to produce labdane-related diterpenes in response to fungal infection (Mellon and West, 1979), and phylogenetic analysis of the *CPS* gene family within the Poaceae has been used to suggest early *CPS* gene duplication and evolution of labdane-related diterpenoid biosynthesis in cereal crop plants (Harris et al., 2005; Prisic et al., 2004).

Given the number of genes examined here it is perhaps not surprising that evidence can be found for the on-going process of evolution in the rice KS(L) gene family. For example, *OsKSL3* appears to be a pseudo-gene undergoing gene 'decay'. The corresponding cDNA clearly contains a frame-shifting single base insertion that severely truncates the encoded polypeptide. Furthermore, this sequence appears to have accumulated a number of other deleterious mutations, as simply restoring the 'correct' reading frame does not result in the production of an active class I labdane-related diterpene synthase, and the observed downstream mis-splicing of the corresponding cDNA sequences from ssp. *indica* is a further indication of gene 'decay', at least in this sub-species.

The physiological role(s) of the isokaurene (**6**) produced by OsKSL5 and OsKSL6, as well as the stemodene (**10**) produced by OsKSL11, is not clear. Nevertheless, it seems likely that these diterpene hydrocarbons will be elaborated to more complex diterpenoid natural products that may have significant biological function(s). Given the important role in plant defense that the other non-gibberellin labdane-related diterpenoids play in rice and the non-inducible nature of the associated cyclases, one plausible role for isokaurene- and stemodene-derived diterpenoids would be to act as phytoanticipins and/or allelochemicals. An interesting alternative is that one or more such labdane-related diterpenoid could act as distinct (i.e. non-gibberellin) signaling molecule(s), analogous to the recently reported role of labda-11,13-diene-8α,15-diol in tobacco (Seo et al., 2003).

As a final note, while this manuscript was in preparation a similar study was published (Kanno et al., 2006), which reported identical findings regarding *OsKSL3* (i.e. this is a pseudo-gene containing a frame-shifting single base insertion) and *OsKSL6* (i.e. this encodes an *ent*-(iso)kaur-15-ene synthase). However, Kanno et al. (2006) do not report gene promoter analysis, nor do they discuss KS(L) gene evolution. There also is one significant difference. Whereas our ssp. *indica* variant of *OsKSL5* (*OsKSL5i*) encodes an *ent*-isokaurene synthase (Fig. 2D–G), their ssp. *japonica* variant of *OsKSL5* (*OsKSL5j*; AB126935) encodes an *ent*-pimara-8(14),15-diene synthase, which represents deprotonation of the probable pimarenyl^{+} intermediate in cyclization of *ent*-CPP (**2**) to kaurane type diterpenes (Fig. 7). This difference in product outcome represents an intriguing example of sub-species specific difference in natural products metabolism, although the corresponding physiological significance is not clear as there are no currently known rice metabolites derived from either diterpene. Regardless, such emerging functional divergence in product outcome, at least in ssp. *japonica*, between the closely related and, therefore, presumably recently duplicated *OsKSL5* and *OsKSL6* is consistent with the hypothesis that there is selective pressure to differentiate similarly regulated genes, and serves as a further example of the continuing process of gene evolution in the KS(L) gene family.

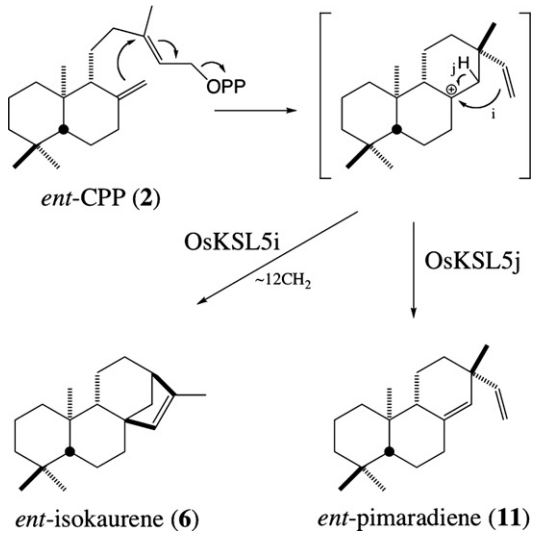

Fig. 7. Proposed biogenetic cyclization mechanism catalyzed by OsKSL5i to produce iso-kaurene (**6**), along with the abortive production of pimara-8(14),15-diene (**11**) catalyzed by OsKSL5j. The further cyclization catalyzed by OsKSL5i is indicated by the arrow marked 'i', the deprotonation catalyzed by OsKSL5j is indicated by the arrow marked 'j'.

OsKSL5i and OsKSL5j share 98.1% identity at the amino acid level, and of the fifteen differences between their sequences, only three appear in or near the active site in modeled structures, and only two of these are conserved between OsKSL5i and OsKSL6, which both produce isokaurene. Because it has been demonstrated that very small numbers of substitutions in the active site of class I terpene synthases are sufficient to alter product profile (Greenhagen et al., 2006; Kollner et al., 2004; Yoshikuni et al., 2006), it seems likely that these two or three changes in active site residues between OsKSL5i and OsKSL5j (i.e. Val661, Ile664, and Ile718 in OsKSL5i, which are Leu, Thr, and Val, respectively, in OsKSL5j) may be responsible for the observed difference in product outcome (i.e. abortive production of pimara-8(14),15-diene (**11**) from *ent*-CPP (**2**) by OsKSL5j, rather than the more complex cyclization to isokaurene (**6**) catalyzed by OsKSL5i), which will be used to guide future structure-function investigations.

4. Conclusions

In summary, we report here cloning and/or biochemical characterization of seven members of the rice kaurene synthase-like gene family (Table 1). While some of these previously had been analyzed either in vitro or by in planta mutagenesis, we verified here the expected production of *ent*-kaurene (**4**) by the gibberellin biosynthesis associated OsKS1, and have clarified the product composition resulting from OsKSL8 catalyzed cyclization (Fig. 2). In addition, novel data are reported demonstrating that *OsKSL3* is a pseudo-gene, while *OsKSL5i* and *OsKSL6* encode active enzymes that produce isokaurene (**6**) from *ent*-CPP (**2**). With essentially all of the rice kaurene synthase-like genes characterized, it also was possible to carry out analysis of this gene family more broadly, revealing a pair of potential defense-related inducible promoter motifs, and enabling more detailed examination of the labdane-related diterpene synthase gene clusters found in the rice genome. This particularly included examination of related gene clusters, and associated diterpene metabolism, in other species from the grass/cereal family, suggesting the widespread occurrence, and continuing evolution, of defensive labdane-related diterpenoid secondary metabolism throughout this important crop plant family. Notably, such widespread retention of this type of defensive metabolism indicates that labdane-related diterpenes are particularly good scaffolds for assembling antibiotic compounds and further suggests that these natural products will prove to

be important components of the defense response in many, if not all, cereal crop plants.

5. Experimental

5.1. General chemicals

Unless otherwise noted, all chemicals were purchased from Fisher Scientific (Loughborough, Leicestershire, UK) and molecular biology reagents from Invitrogen (Carlsbad, CA, USA). Prior to use CH_3CN and DMF were distilled from P_2O_5 and stored over 3 Å molecular sieves.

5.2. Instrumentation

All NMR spectroscopy was carried out in the School of Chemical Sciences NMR facility at the University of Illinois. ^{1}H NMR spectra in $CDCl_3$ and CD_3OD were referenced internally with $CHCl_3$ (7.26 ppm) or with CD_3OH (4.78 ppm). ^{31}P NMR spectra was carried out in CD_3OD with 85% H_3PO_4 as external reference (0.00 ppm). Gas chromatography–mass spectrometry (GC–MS) analyses were performed using an HP1-MS column on an Agilent (Palo Alto, CA, USA) 6890N GC instrument with a 5973N mass selective detector located in the W.M. Keck Metabolomics Research Laboratory at Iowa State University, as previously described (Xu et al., 2004). Briefly, 5 μL samples were injected at 40 °C in splitless mode, the oven temperature held for 3 min, then raised at 20 °C/min to 300 °C, and held there for 3 min. MS data were collected from 50 to 500 *m*/*z* during the temperature ramp.

5.3. Diterpene substrates and standards

GGPP (**1**) was purchased from Sigma–Aldrich. The acquisition of reference samples of sandaracopimaradiene, *ent*-kaurene (**4**), *syn*-pimaradiene (**5**), and *syn*-stemarene (**8**), along with the preparation of *syn*-CPP (**3**), have been previously described (Mohan et al., 1996). A reference sample of *ent*-isokaurene (**6**) was prepared by separation of a 1:1 mixture of *endo*- and *exo*-cyclic isomers (**6** and **4**, respectively) obtained by I_2 equilibration of *ent*-kaurene (**4**) (Bell et al., 1966) using $AgNO_3$-silica gel chromatography (Williams and Mander, 2001). The *exo*- and *endo*-isomers of *syn*-stemodene (**10** and **10′**, respectively) have also been previously described (White and Somers, 1994), and later separated and characterized (Morrone et al., 2006). A reference sample of *ent*-cassadiene (**7**) was kindly provided by Drs. Arata Yajima and Goro Yabuta (Yajima et al., 2004).

5.4. Synthesis of ent-CPP

While the preparation of *ent*-CPP (**2**) has been previously described (Mohan et al., 1996), it was prepared for this study using a modified procedure. For this process *ent*-copalol (0.73 g) was obtained from Brazilian copal resin as previously outlined (Mohan et al., 1996). The modified procedures for conversion of *ent*-copalol to the diphosphate is presented below.

5.4.1. Ent-copalyl chloride

The preparation of *ent*-copalyl chloride followed the method of Collington and Meyers (Meyers and Collington, 1971). A solution of *ent*-copalol (0.050 g, 0.171 mmol) and 2,4,6-collidine (0.208 g, 1.71 mmol) under N_2 was stirred and cooled at 0 °C as a suspension of LiCl (0.073 g, 1.712 mmol) partially dissolved in dry DMF (3.0 mL) was added. CH_3SO_2Cl (0.059 g, 0.514 mmol) was added dropwise. After 1.5 h at 0 °C, the pale yellow reaction mixture was poured into ice-water (10 mL), and the product was extracted with cold ether (4 × 20 mL). The combined organic phases were washed with saturated aqueous $Cu(NO_3)_2$ (2 × 10 mL), 5% $NaHCO_3$ (2 × 10 mL) and brine (2 × 10 mL), dried ($MgSO_4$), and evaporated to yield a colorless oil (0.050 g; 95% yield), which was used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$)δ 5.40 (*tm*, $J = 8.1$ Hz, 1H, =C*H*CH$_2$), 4.81 (*br s*, 1H, =CH$_2$), 4.48 (*br s*, 1H, =CH$_2$), 4.09 (*d*, $J = 7.9$ Hz, 2H, CH$_2$OPP), 2.37 (*ddd*, $J = 12.7$, 4.0, 2.4 Hz, 1H), 2.16 (*tt*, $J = 9.9$, 4.3 Hz, 1H), 1.95 (*td*, $J = 13.0$, 4.9 Hz, 1H), 1.80–1.88 (*m*, 1H), 1.73–1.75 (*m*, 1H), 1.71 (*s*, 3H, CH$_3$C=), 1.53–1.57 (*m*, 2H), 1.44–1.52 (*m*, 1H), 1.38–1.40 (*m*, 1H), 1.35–1.37 (*m*, 1H), 1.27–1.33 (*m*, 1H), 1.24–1.26 (*m*, 1H), 1.15–1.20 (*m*, 1H), 1.06 (*dd*, $J = 12.5$, 2.6 Hz, 1H), 0.95–1.02 (*m*, 1H), 0.85 (*s*, 3H), 0.78 (*s*, 3H), 0.66 (*s*, 3H) ppm; ^{13}C NMR (100 MHz, CD_3OD) δ 148.3, 143.5, 124.3, 106.1, 55.8, 55.3, 41.9, 41.1, 38.8, 38.0, 33.4, 30.1, 24.2, 21.7, 21.5, 19.9, 16.0, 14.3 ppm.

5.4.2. Ent-CPP

The conversion of *ent*-copalyl chloride to *ent*-CPP (**2**) was modeled after two methods already in the literature (Mohan et al., 1996; Woodside et al., 1993) using previously described ion exchange and purification methods (Zhao, 2005). In a dry vial equipped with a stirring bar were placed *ent*-copalyl chloride (0.050 g, 0.162 mmol) and dry 4 Å molecular sieves (0.70 g). Dry CH_3CN (2.0 mL) and HOPP (NBu_4)$_3$ (0.296 g, 0.324 mmol) were added quickly. The vial was sealed with a septum and purged with N_2, and the suspension was stirred at room temperature for 12 h. The solids were removed by filtration and washed with CH_3CN (70 mL). The CH_3CN filtrate was washed with pentane (3 × 10 mL), and CH_3CN was removed with a rotary evaporator. The remaining tetrabutylammonium salt was dissolved in 18 mL of 0.2% (w/v) NH_4HCO_3 and 2% (v/v) isopropyl alcohol in H_2O (solvent A) and applied to a Bio-Rad AG50W-X8 ion exchange column (*d* 1.2 cm, *h* 25 cm, volume 30 mL) that had previously been freshly washed with saturated NH_4OH and deionized water until pH 7.0, and the column was eluted with solvent A (42 mL). The total eluate (~60 mL) was lyophilized three times for 3 h each. The crude white powder was suspended in dry MeOH

(8 mL), vortexed, and centrifuged, then the supernatant removed and concentrated to 0.5–0.6 mL in vacuo. This extraction/vortexing/centrifugation/concentration procedure was repeated three times. ^{31}P NMR analyses of the four supernatant fractions were conducted to determine the ratio of product diphosphate (2*d*, δ_P −8 to −9 ppm) to PPi (*s*, δ_P + 3 ppm). Supernatant fractions 1 and 2 were combined and concentrated to give *ent*-CPP (**2**) NH_4 salt as a white powder, which was not further purified (yield: 17.6 mg, purity 82%), in 19% overall yield for the two steps from *ent*-copalol. ^{31}P NMR (376 MHz, CD_3OD) δ −8.25 (*d*, $J = 16.6$ Hz, 1P), −8.85 (*d*, $J = 19.3$ Hz, 1P) ppm; ^{1}H NMR (400 MHz, CD_3OD) δ 5.37 (*tm*, $J = 8.0$ Hz, 1H, $=CHCH_2$), 4.83 (*s*, 1H, $=CH_2$), 4.52 (*s*, 1H, $=CH_2$), 3.35 (*br s*, 2H, CH_2OPP), 2.39 (*ddd*, $J = 12.5$, 4.0, 2.4 Hz, 1H), 2.11–2.18 (*m*, 1H), 1.95–2.01 (*m*, 1H), 1.82–1.87 (*m*, 1H), 1.73–1.80(*m*, 1H), 1.69 (*s*, 3 H, $CH_3C=$), 1.57–1.64 (*m*, 2H), 1.46–1.51 (*m*, 1H), 1.40–1.44 (*m*, 1H), 1.36–1.40 (*m*, 1H), 1.33 (dd, $J = 13.0$, 4.3 Hz, 1H), 1.26–1.30 (*m*, 1H), 1.17–1.25 (*m*, 1H), 1.12 (dd, $J = 12.7$, 2.6 Hz, 1H), 1.00–1.07 (*m*, 1H) ppm.

5.5. Plant material

Rice plants (*Orzya sativa* L. ssp. *indica* cv. IR24), and seedlings (ssp. *japonica* cv. Nipponbare) were those previously described (Xu et al., 2004). Briefly, leaves were detached from 4-week-old greenhouse grown plants and UV-irradiated for 25 min from 15 cm with 254 nm wavelength light. These were then incubated for 24 h in dark humid conditions at 30 °C. Seedlings were germinated from surface sterilized seeds on filter paper on top of 1.2% agar plates (eight seeds per plate) incubated in the dark at 30 °C for one week. The seedlings were then sprayed with approximately 2 mL 0.1% Tween 20 containing 0.5 mM methyl jasmonate per plate and incubated under the same conditions for 24 h. Total RNA was extracted using the Concert Plant RNA Reagent, and mRNA purified using Dynabeads Oligo$(dT)_{25}$ (Dynal Biotech, Oslo, Norway).

5.6. Cloning

The OsKSL genes were largely obtained via RT-PCR using the primer pairs shown in Table 2. All of the genes were initially cloned into pCR-BluntII-TOPO and completely sequenced. The corresponding ORFs were then transferred to the Gateway vector system via PCR amplification and directional topoisomerization based insertion into pENTR/SD/D-TOPO and verified by complete sequencing. The resulting clones were subsequently transferred via directional recombination to the T7-based N-terminal GST fusion expression vector pDEST15 and, for OsKSL8, the T7-based N-terminal thioredoxin fusion expression vector pTH8 (Hammarström et al., 2002).

Table 2
OsKSL cloning primers

OsKS1-F	*CACC*ATGAGGGACCAGCTCCAGACATTGGA
OsKS1-R	TCAATTGCCCGACAAAATAGAGCCATATGGAT
OsKSL2frag-F	CAGCTCGGCTCCTCGCCGGAGA
OsKSL2frag-R	ACATACCGGCTCTGCCTCCACTC
OsKSL3-F	*CACC*ATGTTTCAGTTAGAATTAGTGAACGTCGTC
OsKSL3-R	TCACGAAGCAGGAATGATATATATGGGTTC
OsKSL5-F	*CACC*ATGATACTTCCTATGAGTTCAGCATGCTT
OsKSL5-R	TCACAGCGTTCCCAAACCAGATGGAAG
OsKSL6-F	*CACC*ATGATGCTTCCTATGAGTTCAGCATGC
OsKSL6-R	TCACAGTGTTCCCGAATCAGATGGAG
OsKSL7-F	*CACC*ATGATGCTGCTAGGTTCCCCT
OsKSL7-R	CTACAATAATCTGAGTTGAAG
OsKSL8-3′RACE-F	*CACC*ATGATGCTGCTGAGTTCCTC
OsKSL8-3′frag-F	TCTGCTGTTGTCCCCTTGTTCCCCTGC
OsKSL8-3′frag-R	TTACTCTTGCAGGTGCAGTGGCTCCAGA
OsKSL10-F	*CACC*ATGGTAAGAAAACAGTTGCAGAGAG
OsKSL10-R	TCATGAGGACAACGTTTCTGG

Italicized *CACC* indicates introduced sequence required for directional topoisomerization mediated cloning.

For OsKS1 we used the partial cDNA (AY347876) previously reported as OsKS1A (Margis-Pinheiro et al., 2005). For OsKSL2, despite repeated attempts with both RT-PCR and RACE reactions from both ssp. *japonica* cv. Nipponbare and ssp. *indica* cv. IR24, it was only possible to generate fragmentary sequence information. The largest such partial cDNA (1545 nucleotides), from ssp. *japonica* cv. Nipponbare, was deposited (DQ823350). For OsKSL3 three independent full-length cDNAs were obtained from both ssp. *japonica* cv. Nipponbare and ssp. *indica* cv. IR24 rice. All six clones contained an 'extra' adenosine base after nucleotide 1215 of the originally predicted sequence. This was further confirmed by re-sequencing of the originally reported partial cDNA clone (AY347879), which also proved to contain this frame-shift insertion. The three clones from ssp. *indica* cv. IR24 were additionally mis-spliced between exons 10 and 11 (joining nucleotides 1702 and 1703), with one being un-spliced (i.e. containing the corresponding intron) and the other two missing nucleotides 1703–1709 (i.e. as a result of being spliced at the wrong nucleotide). Outside of these splicing differences the six full-length clones were approximately 99% identical, with almost all of the differences arising from inter-subspecies variation between the ssp. *indica* and ssp. *japonica* clones. A representative cDNA clone from ssp. *japonica* cv. Nipponbare has been deposited (DQ823351). The frame-shift insertion in this particular cDNA was also 'removed' by PCR-based mutagenesis to generate a 'corrected' OsKSL3. Cloning of OsKSL4 from ssp. *indica* cv. IR24 has been previously reported (Wilderman et al., 2004). For OsKSL5 two independent clones were obtained from ssp. *indica* cv. IR24

and fully sequenced, demonstrating complete identity, to verify the deposited sequence (DQ823352). For OsKSL6 two independent clones were obtained from ssp. *japonica* cv. Nipponbare and fully sequenced, demonstrating complete identity, to verify the deposited sequence (DQ823353). For OsKSL7 two independent clones were obtained from ssp. *indica* cv. IR24 and fully sequenced, demonstrating complete identity, to verify the deposited sequence (DQ823354). For OsKSL8 we were not able to clone a full-length cDNA, instead consistently finding the closely related OsKSL11 in both ssp. *indica* and *japonica* (Morrone et al., 2006). It was possible to clone partial cDNA fragments separately corresponding to the first 1715 and last ~900 nucleotides of the reported ORF of OsKSL8 (AB118056; 2463 nucleotides total). However, sequencing of the 3′ end fragment revealed a 21 nucleotide deletion in the last exon (i.e. nucleotides 2171–2192 were missing), which is extremely GC-rich and contains quite repetitive sequence elements in this region. Attempts to clone a 3′ end fragment with the correct sequence were unsuccessful, and ultimately the missing nucleotides were inserted via PCR extension, and the full-length ORF subsequently re-created using the 5′ end and corrected 3′ end fragments in a mega-primed PCR approach. For OsKSL10 a full-length cDNA was obtained from the Rice Genome Resource Center (ssp. *japonica* cv. Nipponbare), re-sequenced, and the corrected sequence deposited (DQ823355). Cloning of OsKSL11 from both ssp. *indica* and *japonica* has been previously reported (Morrone et al., 2006).

5.7. Recombinant expression

Heterologous expression was performed using the Over-Express C41 strain of *E. coli* (Avidis, France), as previously described (Xu et al., 2004). Briefly, 50-mL NZY cultures inoculated from 5 to 7 individual transformants/colonies were grown at 37 °C to mid-log phase ($A_{600} \sim 0.6$), then transferred to 16 °C for 1–2 h prior to induction with 1 mM IPTG for overnight (14–18 h) expression. Cells were harvested by centrifugation, resuspended in 1 mL cold lysis buffer (50 mM Bis–Tris, pH 6.8, 1 mM DTT), lysed by mild sonication (15 s, continuous output, half-maximum power), and clarified by centrifugation (40,0000*g*, 20 min). Recombinant GST-tagged proteins were purified using GST-agarose beads (Sigma–Alrich, St. Louis, MO, USA) in batch mode. The thioredoxin-OsKSL8 fusion protein was only partially purified using ceramic hydroxyapetite type II (BioRad, Hercules, CA, USA) in batch mode. The batch purification was carried out at 4 °C during which clarified extract was incubated 5–10 min with ~0.3 mL beads, washed 5 times with 1 mL lysis buffer, then eluted in 0.5 mL (either 10 mM glutathione in lysis buffer for GST-OsKSL bound to glutathione beads, or 0.2 M sodium phosphate, pH 6.8, for thioredoxin-OsKSL8 bound to ceramic hydroxyapetite), the resulting eluate was filtered (0.2 μm) and immediately used in enzymatic assays.

5.8. Functional characterization

Assays were generally carried out with GGPP (5 μg) as substrate in coupled reactions with either the *syn*-CPP synthase OsCPS4 or *ent*-CPP synthase ZmCPS2/An2 (Harris et al., 2005), both expressed and purified as GST-fusion proteins. For every gene product individual enzyme assays also were run with 5 μg GGPP (**1**), *ent*-CPP (**2**), or *syn*-CPP (**3**) directly. Reactions were run in 0.5 mL assay buffer (50 mM Hepes, pH 7.2, 10 mM $MgCl_2$, 10% glycerol, and 5 mM DTT) using 25 μL recombinant CPS and/or 25 μL recombinant OsKS(L) for 3–16 h at room temperature. These assays were extracted three times with an equal volume of hexanes, which were pooled, dried under nitrogen, and re-dissolved in 100 μL of hexanes prior to GC–MS analysis.

5.9. Sequence analysis

BLAST searches were carried out on-line at both GenBank (www.ncbi.nih.gov) and TIGR (www.tigr.org). The cloned cDNAs were mapped back onto the rice genome by web-based alignment at Gramene (www.gramene.org), and the corresponding up-stream sequences also obtained here. Notably, 776 bases upstream of the OsCPS4 gene there seems to be a gap in the genome sequence, as represented by a stretch of several hundred unidentified bases (i.e. 'N's). Promoter analysis was done on-line at PlantCare (bioinformatics.psb.ugent.be/webtools/plantcare/html) and TAIR (www.arabidopsis.org/tools/bulk/motiffinder/index.jsp). All other sequence analysis was carried out with the VectorNTI software package (Invitrogen) using standard parameters.

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