



Functional characterization of wheat *ent*-kaurene(-like) synthases indicates continuing evolution of labdane-related diterpenoid metabolism in the cereals

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ABSTRACT

Wheat (*Triticum aestivum*) and rice (*Oryza sativa*) are two of the most agriculturally important cereal crop plants. Rice is known to produce numerous diterpenoid natural products that serve as phytoalexins and/or allelochemicals. Specifically, these are labdane-related diterpenoids, derived from a characteristic labdadienyl/copalyl diphosphate (CPP), whose biosynthetic relationship to gibberellin biosynthesis is evident from the relevant expanded and functionally diverse family of *ent*-kaurene synthase-like (KSL) genes found in rice (the OsKSLs). Herein reported is the biochemical characterization of a similarly expansive family of KSL from wheat (the TaKSLs). In particular, beyond *ent*-kaurene synthases (KS), wheat also contains several biochemically diversified KSLs. These react either with the *ent*-CPP intermediate common to gibberellin biosynthesis or with the normal stereoisomer of CPP that also is found in wheat (as demonstrated by the accompanying paper describing the wheat CPP synthases). Comparison with a barley (*Hordeum vulgare*) KS indicates conservation of monocot KS, with early and continued expansion and functional diversification of KSLs in at least the small grain cereals. In addition, some of the TaKSLs that utilize normal CPP also will react with *syn*-CPP, echoing previous findings with the OsKSL family, with such enzymatic promiscuity/elasticity providing insight into the continuing evolution of diterpenoid metabolism in the cereal crop plant family, as well as more generally, which is discussed here.

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1. Introduction

Cereal crop plants provide the bulk of the world's caloric intake, with wheat and rice representing the two most important for direct human consumption (Dyson, 1996). Rice has served as a model for the cereal crop plant family, as its agricultural importance and relatively small genome size led to early and thorough sequencing (Goff et al., 2002; International Rice Genome Sequencing Project, 2005; Yu et al., 2002), which was complemented by a large scale cDNA sequencing effort (Kikuchi et al., 2003). The resulting

complete gene list has enabled comprehensive investigation of various aspects of rice physiology and metabolism. Such work then provides the basis for similar investigations in other cereal crops such as wheat.

Rice is a particularly prolific producer of labdane-related diterpenoids, which have been suggested to act as phytoalexins in defense against microbial pathogens and as allelochemicals suppressing the growth of neighboring weed plants (Peters, 2006; Toyomasu, 2008). The biosynthesis of this super-family of natural products is derived from that of the gibberellin phytohormones, at least in plants, and is characterized by a similar pair of sequentially catalyzed cyclization reactions (Peters, 2010). In particular, bicyclization of the general diterpenoid precursor (*E,E,E*)-geranylgeranyl diphosphate (GGPP), typically to a labdadienyl/copalyl diphosphate (CPP) intermediate, which is then often further cyclized to an olefin. The corresponding enzymes have been termed CPP synthases (CPSs) and *ent*-kaurene synthase-like (KSL), respectively, for their relationship to those found in all plants for gibberellin biosynthesis (Peters, 2006). Specifically, these

Abbreviations: KS, *ent*-kaurene synthase; KSL, *ent*-kaurene synthase-like; TaKS(L), wheat (*Triticum aestivum*) KSL; HvKS, barley (*Hordeum vulgare*) kaurene synthase; OsKS(L), rice (*Oryza sativa*) KSL; AtKS, *Arabidopsis thaliana* KS; CPP, copalyl diphosphate; CPS, CPP synthase; TaCPS, wheat (*Triticum aestivum*) CPS; OsCPS, rice (*Oryza sativa*) copalyl diphosphate synthase; GC-MS, gas chromatography with mass spectrometric detection; GGPP, (*E,E,E*)-geranylgeranyl diphosphate; RACE, rapid amplification of cDNA ends; ORF, open reading frame.

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fall into the same phylogenetically defined sub-families – i.e., TPS-c and TPS-e/f, respectively (Chen et al., 2011).

Rice contains expanded gene families encoding CPS (OsCPSs) and KSL (OsKSLs), and these have been extensively investigated, with biochemical function assigned to each (Cho et al., 2004; Kanno et al., 2006; Nemoto et al., 2004; Otomo et al., 2004a,b; Pristic et al., 2004; Wilderman et al., 2004; Xu et al., 2004, 2007). In addition, consistent with a role in phytoalexin biosynthesis, many of the OsKSLs exhibit inducible gene transcription (Peters, 2006; Toyomasu, 2008). Previous work with maize (*Zea mays*) demonstrated the inducible production of labdane-related diterpenes in this distantly related cereal crop plant (Mellon and West, 1979), and recently it has been shown that maize produces such diterpenoid phytoalexins (Schmelz et al., 2011). In addition, it has been suggested that CPS gene expansion and functional diversion to secondary/more specialized metabolism occurred early in the cereal crop plant family – i.e., the *Poaceae* (Pristic et al., 2004). Thus, it seems likely that more specialized labdane-related diterpenoid metabolism will be widespread throughout the *Poaceae*. Indeed, gene probing/mapping experiments using CPS and KS homologs from barley suggests that at least barley and wheat contain expanded CPS and KSL gene families (Spielmeyer et al., 2004). Such expansion and diversification of diterpene synthases is not confined to the *Poaceae* – e.g., having been shown in gymnosperms as well (Keeling et al., 2011; Martin et al., 2004).

Some preliminary analysis of wheat CPSs (TaCPSs) has been previously reported (Toyomasu et al., 2009). However, only the *ent*-kaurene synthase (KS) activity expected for the requisite gibberellin biosynthesis has been previously reported from wheat (Aach et al., 1995). Herein reported is the cloning and functional characterization of seven members of the wheat KSL family (TaKSLs), along with demonstrating KS activity for the previously isolated barley KSL (HvKS), also discussed are the implications of these findings for the evolution of diterpenoid metabolism in the cereal crop family, as well as plants more generally.

2. Results

2.1. Identification of kaurene synthase-like genes from wheat and barley

Isolation of HvKS has been previously reported (Spielmeyer et al., 2004). TaKSL genes were initially identified by homology searches against the available EST data with the known OsKSL. The corresponding full-length cDNAs were then cloned by RT-PCR and, where necessary, by RACE. Through this effort, five clearly full-length KSLs were found; *TaKSL1*, 2, 3, 4, and 6, which encode proteins of 837, 853, 856, 837, and 852 amino acid (aa) residues (GenBank Accessions AB597957–AB597960 and AB597962), respectively. In addition, two closely related genes (94% identical at the nucleotide sequence level) were cloned and assigned as *TaKSL5-1* and *TaKSL5-2* under the assumption that these are polyploidy derived homoeologs (e.g., the most notable difference is an in-frame 63 nucleotide deletion in *TaKSL5-2* relative to *TaKSL5-1*). Notably, as previously reported (Hillwig et al., 2011), *TaKSL5-1* and *TaKSL5-2* are substantially shorter than other KSLs, resembling plant mono- and sesqui- terpene synthases in length. Specifically, these encode proteins of 663 and 641 aa long, respectively, and are missing the large N-terminal domain usually associated with diterpene synthases (Cao et al., 2010), although they otherwise closely align with the other TaKSLs (Fig. 1). While many of these KSLs have a Gly in place of a position generally conserved as Ser/Thr within the terpene synthase secondary divalent metal binding motif, such substitution has been observed before and found not to be deleterious (Zhou and Peters, 2009), encouraging further analysis.

2.2. Induction of transcription of wheat labdane-related diterpene synthase genes

In rice, mRNA levels of the OsKSLs, as well as OsCPSs, involved in phytoalexin biosynthesis were dramatically increased by UV-irradiation (Peters, 2006). Thus, the possibility that mRNA levels of some of the *TaKSLs* would be induced in response to UV-irradiation was analyzed by qRT-PCR. Indeed, mRNA levels of *TaKSL1*, *TaKSL2*, *TaKSL3*, and *TaKSL5*, were found to be higher in UV-irradiated leaves, although those of *TaKSL4* and *TaKSL6* were not (Fig. 2).

2.3. Biochemical characterization

The TaKSLs and HvKS were functionally characterized via use of a previously developed metabolic engineering system that enables co-expression of both GGPP synthase and CPS producing the three commonly found stereoisomers of CPP, along with downstream KSL heterologously expressed as GST fusion proteins, which does not affect product outcome (Cyr et al., 2007). This enabled analysis of their activity with CPP of *ent*- (**2**), normal (**3**), or *syn*- (**4**) stereochemistry (Fig. 3). Using this approach, HvKS was demonstrated to selectively react with *ent*-CPP (**2**) and produce only *ent*-kaurene (**5**) – i.e., HvKS does not react with normal (**3**) or *syn*-CPP (**4**). Similarly, the closely related TaKSL6 specifically reacts with **2** to produce **5**. In addition, the putative homoeologs TaKSL5-1 and TaKSL5-2 also selectively react with **2** to chiefly produce **5**, although both also produce *ent*-beyerene (**6**), in a ~3:1 ratio. However, TaKSL5-1 and TaKSL5-2 may be sesquiterpene synthases, as they more readily react with (*E,E*)-farnesyl diphosphate to produce (*E*)-nerolidol, as previously reported (Hillwig et al., 2011). TaKSL4 reacts with normal CPP (**3**) to produce pimara-8(9),15-diene (**7**), and also will react with *syn*-CPP (**4**) to produce a number of diterpenes, chiefly *syn*-pimara-9(11),15-diene (**8**), along with at least five other relatively minor unidentified products, but does not react with *ent*-CPP (**2**). TaKSL3 exhibits relatively low activity, although it does selectively react with *ent*-CPP (**2**) to generate two diterpene products. Unfortunately, due to the low yield of those products, it was not possible to obtain enough of these compounds for structural characterization. No activity was detectable with TaKSL2 using a construct in which only the usual pseudo-mature truncation were made (i.e., to simply remove the N-terminal plastid targeting sequence). In an attempt to find an active enzyme an extensively truncated construct, resembling TaKSL5-1 and TaKSL5-2 [i.e., similar to plant mono- and sesqui- terpene synthases in missing the N-terminal domain generally associated with plant diterpene synthases (Cao et al., 2010)], was made and found to react with not only *ent*-CPP (**2**) to produce the known *ent*-pimara-8(14),15-diene (**9**), but also with normal CPP (**3**) to make abietadiene (**10**), although not with *syn*-CPP (**4**). Finally, TaKSL1 also reacts with normal CPP (**3**) to produce iso-pimara-7,15-diene (**11**), as well as with *syn*-CPP (**4**) to produce *syn*-iso-pimara-7,15-diene (**12**), but not with *ent*-CPP (**2**).

2.4. Molecular phylogenetic analysis of cereal KS(L)

Previous molecular phylogenetic analysis of cereal CPSs indicates that expansion and functional divergence of at least two copies of such an enzyme encoding gene to more specialized metabolism occurred prior to the speciation event separating the wheat and rice lineages (Toyomasu et al., 2009). To determine if similar early multiplication and functional divergence occurred with the KSLs, as well as to provide insight into potential physiological function of the various TaKSL family members, a molecular phylogenetic analysis of the cereal KS(L)s was carried out. Specifically, the full-length amino acid sequences of all the OsKSLs with HvKS and all the TaKSLs (but TaKSL5-1 and TaKSL5-2) were aligned. TaKSL5-1 and TaKSL5-2 were excluded on the basis of their significant difference

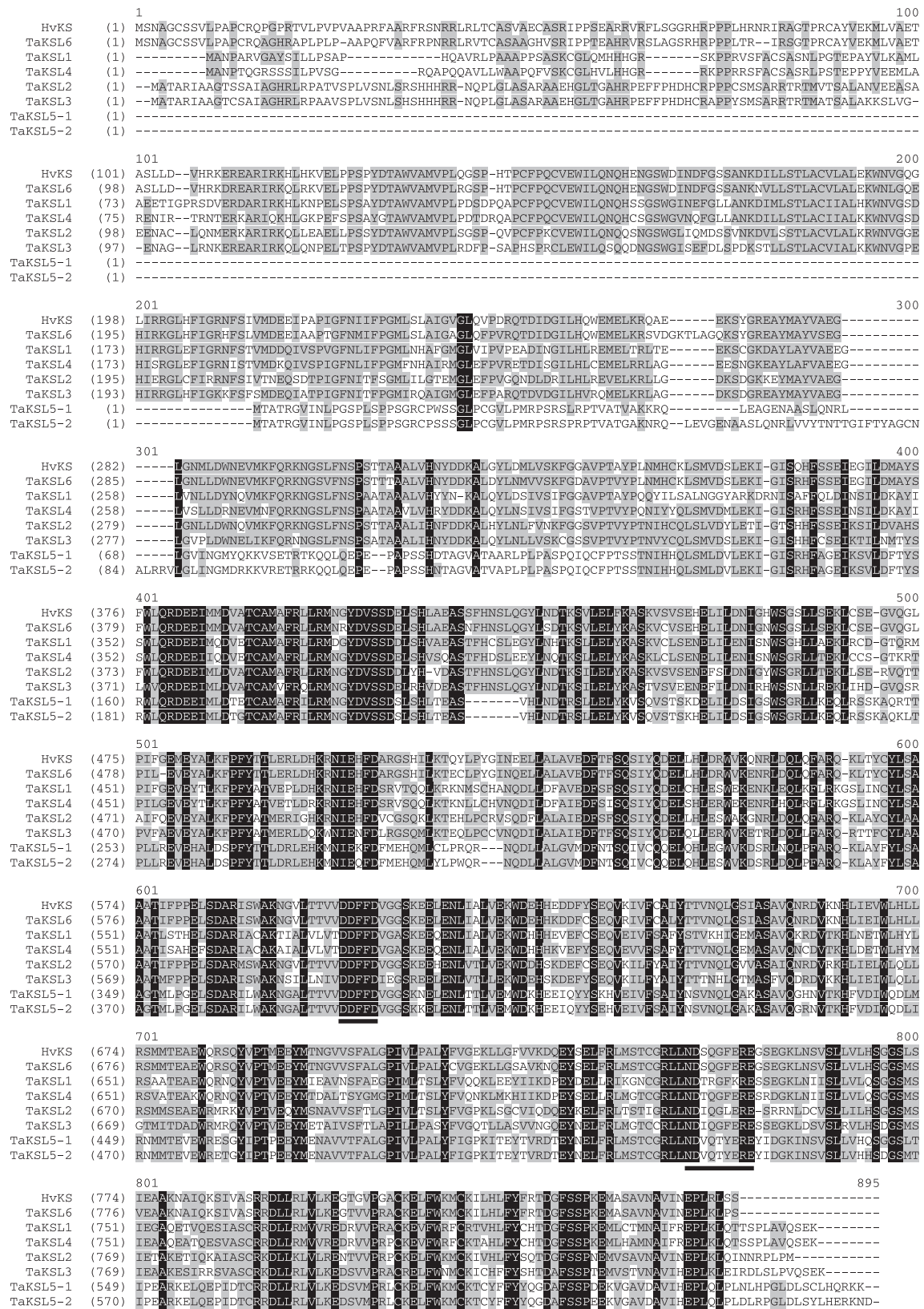


Fig. 1. HvKS and TaKSL1-6 amino acid sequence alignment (with the primary DDxxD and secondary Nxx(S/T)xxxE divalent metal binding motifs underlined).

in size as well as presumed function as sesquiterpene synthases rather than KSL. The KS from the dicot *Arabidopsis thaliana* (AtKS) also was included to provide a designated outgroup sequence for the resulting phylogenetic tree, which was constructed using the nearest neighbor joining method (Fig. 4).

While the TaKSLs and OsKSLs largely cluster independently, indicating continued expansion and divergence of this gene family after separation of the wheat/rice lineages, there is some overlap. In particular, OsKSL4 clusters with TaKSL1 and TaKSL4, suggesting early KSL gene duplication and functional divergence in the small

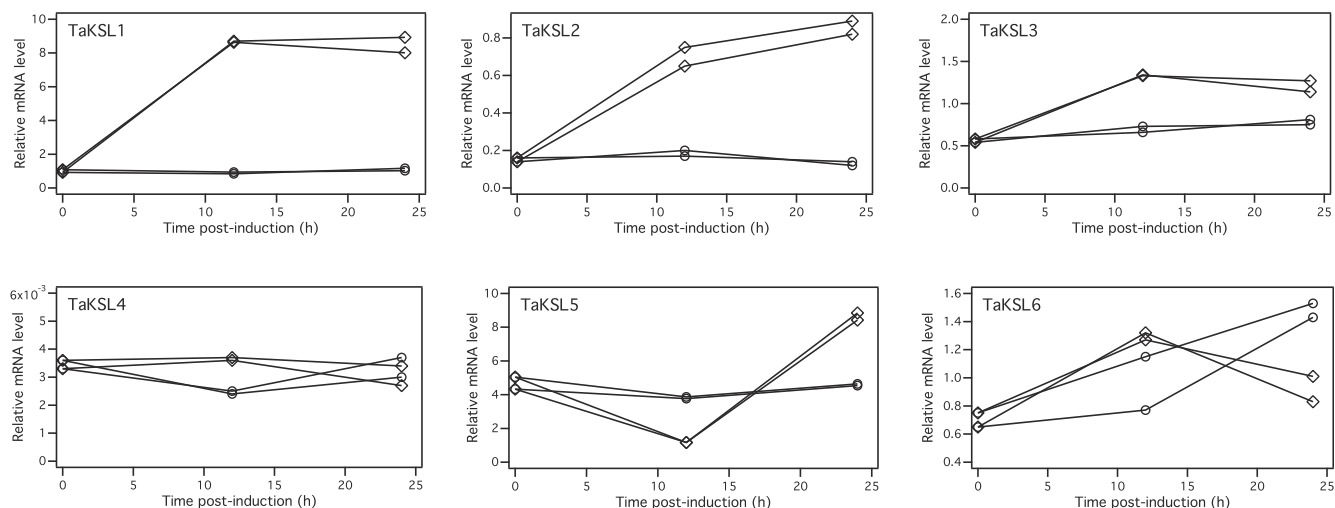


Fig. 2. Transcriptional induction of the *TaKSLs* by UV-irradiation. The mRNA levels for all the *TaKSLs* are normalized to that for *TaKSL1* pre-induction, with data from control plants indicated by circles and that from induced plants by diamonds (duplicate data points from technical replicates shown). Analysis of biological replicates demonstrates the same overall expression pattern.

grain cereal lineage. Given the inducible transcription of at least *TaKSL1* and *OsKSL4*, it seems likely that the ancestral enzymatic gene was similarly involved in more specialized metabolism. In addition, consistent with their shared conservation of KS activity, HvKS and *TaKSL6* are the most closely related KSLs (sharing 91% aa sequence identity), and both are quite similar to *OsKS1* as well (~68% aa identity), which is required for gibberellin biosynthesis in rice (Sakamoto et al., 2004). This conservation pattern reflects the underlying evolutionary separation of rice from wheat and barley, as these fall into the *Poaceae* subfamilies *Oryzoideae* and *Pooideae*, respectively (Kellogg, 1998). Thus, it seems likely that HvKS and *TaKSL6* are similarly involved in gibberellin biosynthesis, although this remains to be demonstrated. Consistent with this hypothesis, the highly divergent sequences, including overall length, as well as reduced product fidelity and inducible gene transcription, of *TaKSL5-1* and *TaKSL5-2* suggests that these are not involved in gibberellin metabolism, and these have been shown elsewhere to cluster with the rice pseudogenes *OsKSL2* and *OsKSL3*, rather than *OsKS1* (Hillwig et al., 2011).

Notably, while the rice KSLs have clearly undergone repeated evolutionary diversification of substrate specificity (e.g., the *syn*-CPP specific *OsKSL4* and *OsKSL11* fall into separate clusters), the *TaKSLs* characterized in this study do exhibit such functional conservation. In particular, the *ent*-CPP specific *TaKSLs* cluster together, with the normal/*syn*-CPP specific *TaKSL1* and *TaKSL4* falling into a separate cluster. This latter cluster further includes the *OsKSL4* that exhibits a similar substrate range (Morrone et al., 2011), suggesting an early origin for such dual substrate stereo-specificity, which is consistent with early diversification of the responsible CPS suggested in the accompanying report (Wu et al., in press). However, there is no clear phylogenetic relationship among the inducible versus non-inducible *TaKSLs*, which also was noted for the rice *OsKSLs* (Peters, 2006). For example, while *TaKSL1* and *TaKSL2* exhibit UV-inducible transcription, *TaKSL1* clusters with the non UV-inducible *TaKSL4* rather than similarly regulated *TaKSL2*.

3. Discussion

The results reported here demonstrate that the *KSL* gene family in wheat has undergone expansion and functional diversification. These findings resemble the results described for the

upstream *CPS* gene family in the accompanying report (Wu et al., in press). Together, these results suggest some, albeit as yet undefined, physiological role(s) for the derived labdane-related diterpenoids. In rice, which has undergone similar *CPS* and *KSL* gene family expansion, the resulting natural products are thought to serve as inducible phytoalexins against fungal pathogens, constitutive phytoanticipins against bacterial infection, and/or as allelochemicals (Peters, 2006; Toyomasu, 2008). Given the similar transcriptional induction of *TaKSL1*, *TaKSL2*, *TaKSL3* and *TaKSL5-1* and *TaKSL5-2* as observed with the phytoalexin relevant diterpene synthases from rice, it seems likely that the diterpenoids derived from these wheat KSLs will serve as phytoalexins in wheat. On the other hand, the conservation of HvKS and *TaKSL6* with *OsKS1*, both in biochemical function and lack of transcriptional induction as well as phylogenetically, strongly indicates that these are involved in gibberellin biosynthesis. Finally, it is possible that the non-inducible *TaKSL4* might serve in biosynthesis of phytoanticipins. Alternatively, *TaKSL4* may serve to produce allelochemicals, much as has been shown for the rice momilactones (Xu et al., 2012).

The dual normal/*syn*-CPP reactivity observed here with *TaKSL1* and *TaKSL4* provides some insight into the underlying enzymatic catalysis, as the products resulting from reaction with these alternative substrates exhibit certain similarities (Fig. 5). Specifically, *TaKSL1* produces a pimaradiene with β -methyl at C13 and C7,8-double bond resulting from deprotonation at C7 of the relevant isopimara-15-en-8-yl⁺ intermediate from both normal (**3**) and *syn*-CPP (**4**). *TaKSL4* produces a pimaradiene with α -methyl at C13 and double bond involving C9 from both substrates. However, when reacting with *syn*-CPP (**4**), this entails a hydride shift from C9 to C8, enabling deprotonation at C11 to form the observed C9,11-double bond, rather than the direct deprotonation at C9 of the initially formed pimara-15-en-8-yl⁺ intermediate observed upon reaction with normal CPP (**3**). The shared C13 configuration of the pimaradienes resulting from reaction with these alternative substrates further implies that both *TaKSL1* and *TaKSL4* bind normal (**3**) and *syn*-CPP (**4**) in a similar conformation (Fig. 6). On the other hand, the production of abietadiene (**10**) from normal CPP (**3**) by *TaKSL2* prevents insight into the configuration of C13 in the relevant pimarenyl⁺ intermediates, which then cannot be compared to the *ent*-CPP (**2**) conformation implied by the observed production of *ent*-pimara-8(14),15-diene (**9**).

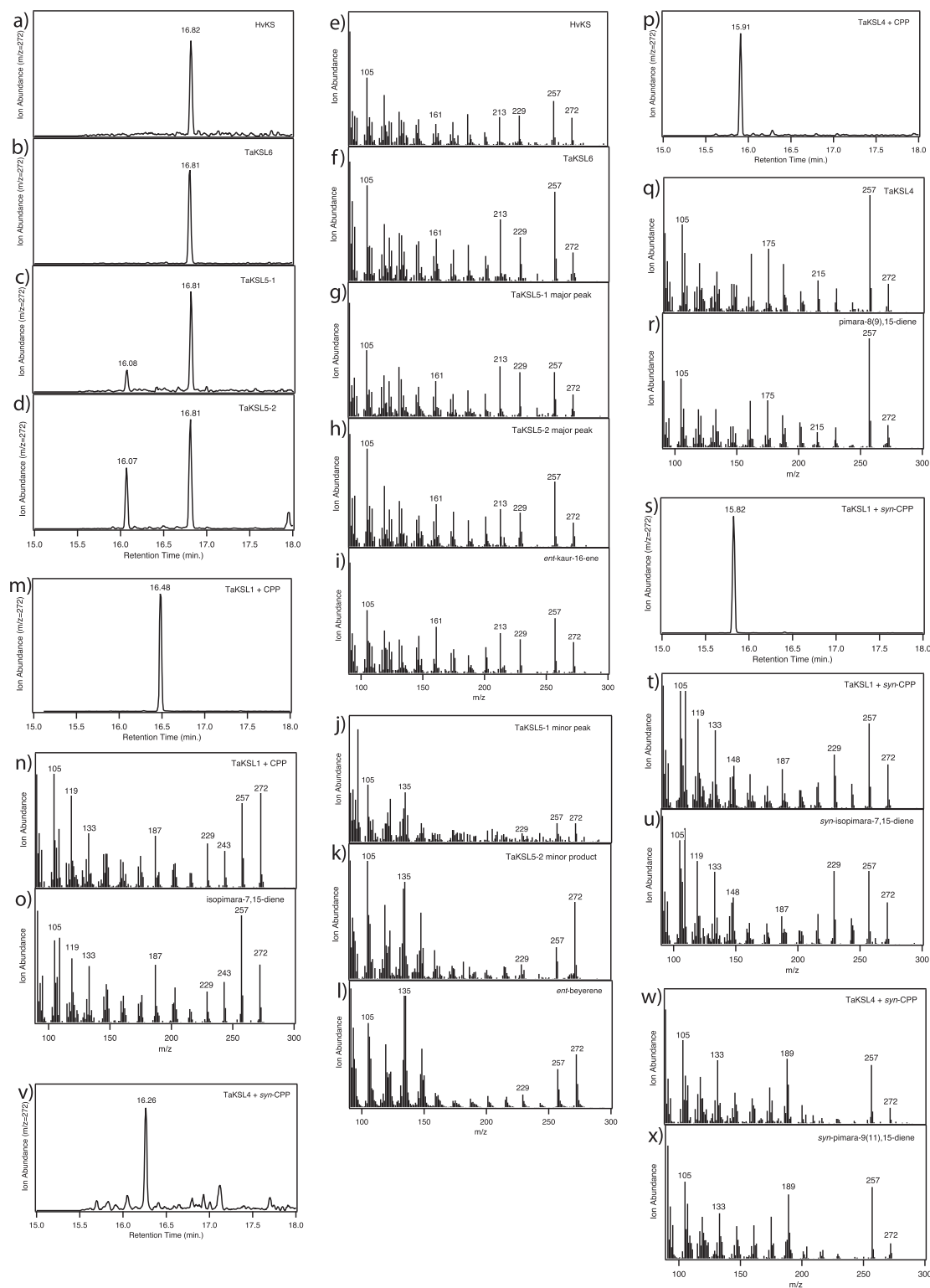


Fig. 3. Identification of products formed by TakS(L)s via GC-MS based comparison to authentic standards. (a–d) Chromatograph of the products formed by HvKS, TakSL6, TakSL5-1, and TakSL5-2, respectively, from *ent*-CPP (**2**). (e–i) Mass spectrum of HvKS and TakSL6 products, as well as the major product of TakSL5-1 and TakSL5-2, from **2**, with comparison to authentic *ent*-kaur-16-ene (**5**) (RT = 16.81 min). (j–l) Mass spectrum of the minor product of TakSL5-1 and TakSL5-2 from **2**, with comparison to authentic *ent*-beyer-15-ene (**6**) (RT = 16.05 min). (m) Chromatograph of the product formed by TakSL1 from normal CPP (**3**). (n,o) Mass spectrum of TakSL1 product from **3**, with comparison to authentic isopimara-7,15-diene (**11**) (RT = 16.49 min). (p) Chromatograph of the product formed by TakSL4 from **3**. (q,r) Mass spectrum of TakSL4 product from **3**, with comparison to authentic pimara-8(9),15-diene (**7**) (RT = 15.91 min). (s) Chromatograph of the product formed by TakSL1 from *syn*-CPP (**4**). (t,u) Mass spectrum of TakSL1 product from **4**, with comparison to authentic *syn*-isopimara-7,15-diene (**8**) (RT = 15.82 min). (v) Chromatograph of the products formed by TakSL4 from **4**. (w,x) Mass spectrum of TakSL4 product from **4**, with comparison to authentic *syn*-isopimara-9(11),15-diene (**12**) (RT = 16.26 min). In all cases, the retention time of the enzymatic product was ≥ 0.02 min. different from that of the matching authentic sample.

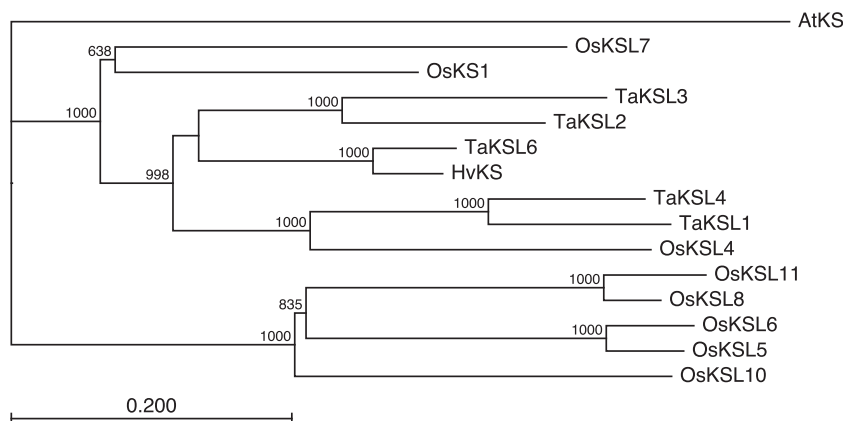


Fig. 4. Molecular phylogenetic tree for characterized cereal KS(L)s, constructed using the Nearest Neighbor Joining method, and with AtKS as the designated outgroup sequence.

The dual reactivity of TaKSL2 with the normal (**3**) and *ent*-CPP (**2**) found in wheat is similar to the ability of OsKSL10 to react with the *syn*- (**4**) and *ent*-CPP (**2**) found in rice. However, while the dual normal/*syn*-CPP reactivity of TaKSL1 and TaKSL4 found here is analogous to that reported for OsKSL4 and OsKSL11 (Morrone et al., 2011), these do not seem to be physiologically relevant.

Specifically, because rice does not produce normal CPP (**3**) and, as described in the accompanying report (Wu et al., in press), wheat does not seem to produce *syn*-CPP (**4**). Nevertheless, such latent plasticity presumably enables facile evolution of diterpenoid metabolism, as changes in CPP stereochemistry arising from functional diversification of the upstream CPSS would be readily

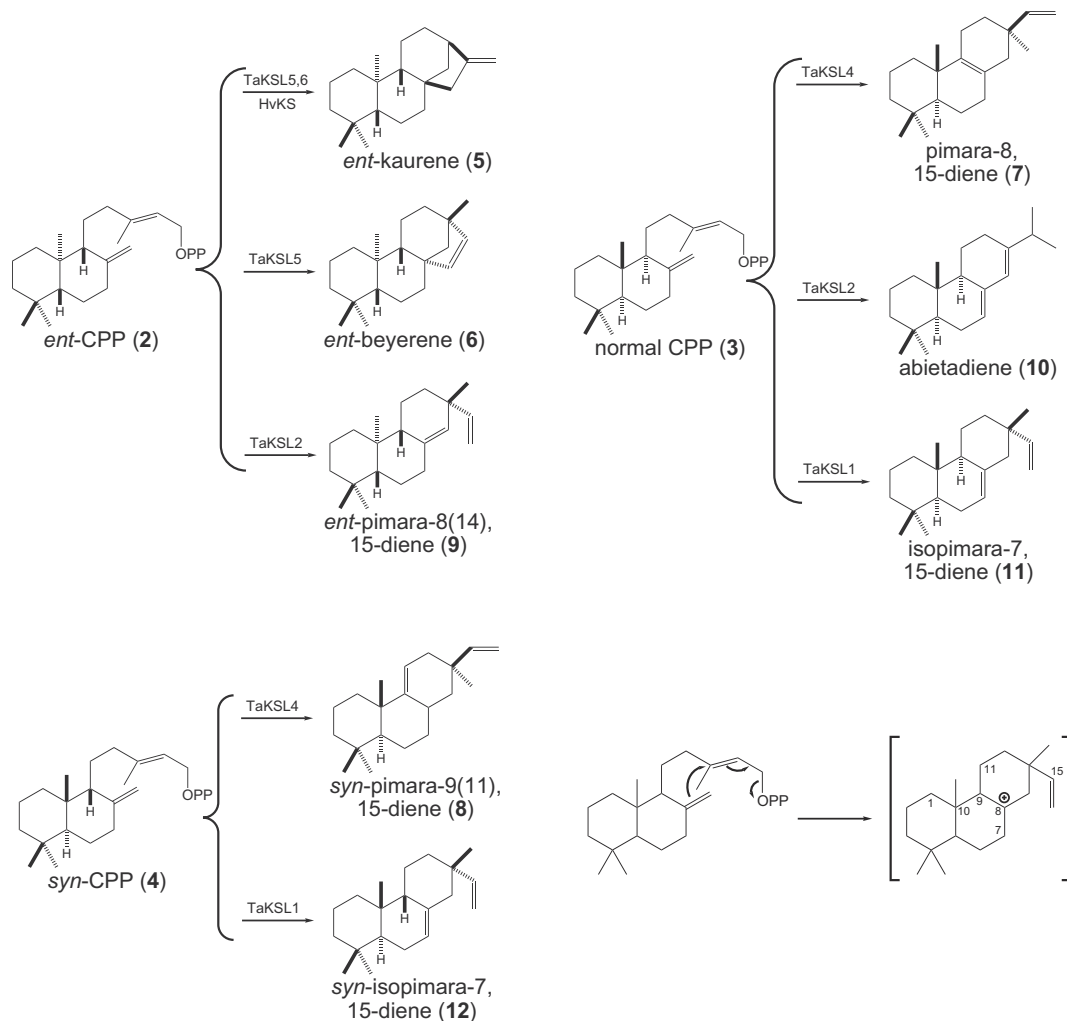


Fig. 5. Reactions catalyzed by HvKS and the various TaKSLs. Also shown is generic initially catalyzed cyclization to a pimar-15-en-8-yl carbocationic intermediate, with carbon numbering indicated as mentioned in the text.

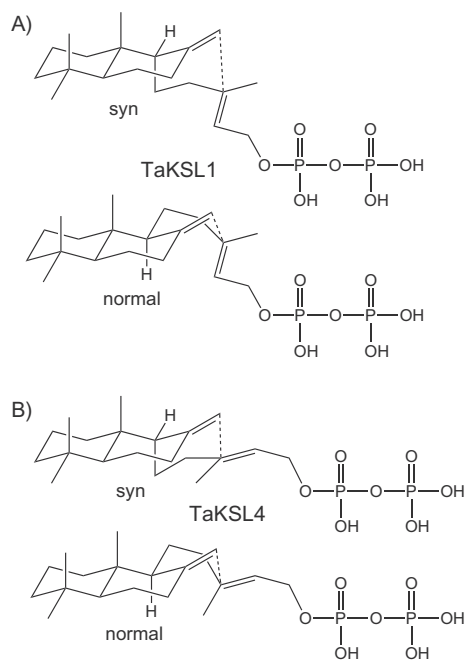


Fig. 6. Comparison of the proposed configuration of the alternative substrates in the active site of the dual normal/syn-CPP reactive TaKSL1 (A) and TaKSL4 (B).

accommodated by these promiscuous KSLs. In addition, downstream enzymes such as cytochrome P450 monooxygenases exhibit similar promiscuity (Wang et al., 2012). Such broader metabolic plasticity enables the immediate appearance of multi-step biosynthetic pathways from changes in an upstream enzyme. Indeed, such changes in CPS stereochemistry appear to have been directly relevant in the cereal crop plant family. As described in the accompanying report (Wu et al., in press), wheat and rice produce alternative stereoisomers of CPP (i.e., normal (**3**) or syn (**4**), respectively, in addition to the *ent*-CPP (**2**) required for GA biosynthesis). Such stereochemical variation is then readily accommodated by the presence of these promiscuous enzymes. In fact, it may be these evolutionary changes in CPS stereochemical outcome that led to this observed dual reactivity for some of the rice and wheat KSLs – i.e., this reflects promiscuous intermediate stages during the evolution of altered substrate specificity, which is a concept that has been more generally postulated (Tawfik, 2010).

4. Conclusion

The results presented here demonstrate that wheat contains an expanded and biochemically diverse family of KSLs. While the physiological roles of the ensuing labdane-related diterpenoids largely remain unclear, the KSL gene family seems to have undergone continued evolution in both wheat and rice since the separation of these related cereal crop plants, suggesting continuing relevance for these natural products. Given the similar inducible transcriptional regulation of unrelated KSLs from wheat and rice, it seems likely that wheat may use labdane-related diterpenoids in plant defense, analogous to their roles in rice. However, their independent evolutionary trajectories suggest that there will be differences in their exact function. Regardless of physiological role, the biochemical activity exhibited by the KSLs characterized here significantly expands our understanding of the diterpenoid metabolic repertoire of wheat. In addition, the dual substrate reactivity demonstrated here for some of the wheat KSLs echoes recently reported promiscuity of the rice KSLs (Morrone et al., 2011), and this flexibility in substrate recognition presumably enabled the

evolution of expanded labdane-related diterpenoid biosynthesis demonstrated here for the cereal crop plant family. In particular, the ability to produce alternative stereochemical variants of CPP may then lead to immediate appearance of more elaborated natural products (i.e., via promiscuity of the downstream KSLs and cytochromes P450). Similar latent substrate promiscuity may contribute to the evolution of more specialized terpenoid metabolism observed in all plants more generally.

5. Experimental

5.1. General

Unless otherwise noted, all chemical reagents were purchased from Fisher Scientific (Loughborough, Leicestershire, UK), and molecular biology reagents from Invitrogen (Carlsbad, CA, USA). All recombinant expression was carried out with the OverExpress C41 strain of *Escherichia coli* (Lucigen, Middleton, WI, USA). Gas chromatography with mass spectrometric detection (GC–MS) analyses were performed using a Varian (Palo Alto, CA, USA) 3900 instrument with HP-5 ms column and Saturn 2100 ion trap mass spectrometer in electron ionization (70 eV) mode. Samples (1 μ L) were injected in splitless mode at 50 $^{\circ}$ C and, after a 3 min. hold, the temperature raised at 14 $^{\circ}$ C/min. to 300 $^{\circ}$ C, where it was held for 3 min. MS data was collected from m/z from 90 to 600, starting 12 min. after the injection until the end of the run. GC with flame ionization detection (FID) was carried out using an Agilent (Santa Clara, CA, USA) 6890 GC with HP-5 ms column, and the same protocol utilized for GC–MS analyses. Bioinformatic sequence analyses were carried out either with the VectorNTI (Fig. 1) or CLC Sequence Viewer (Fig. 4) software packages.

5.2. Cloning

BLAST searches were carried out using the OsKSL as probes in DDBJ (<http://blast.ddbj.nig.ac.jp/top-e.html>). TaKSL1, 2, 3, 4, 5 and 6 correspond to the sequences of BE585476, CV775031, BE585481, CK205050/CA646242, DR740781/CJ593452, and BE419989, respectively. The corresponding full-length cDNAs were then cloned from *Triticum aestivum* cv. Nourin-61-gou by rapid amplification of cDNA ends (RACE) and end-to-end RT-PCR using gene specific primers based on these EST sequences, much as described previously (Toyomasu et al., 1998).

Each KSL was transferred to the Gateway vector system via PCR amplification and directional topoisomerization insertion into pENTR/SD/D-TOPO, with the ensuing constructs verified by complete gene sequencing. These clones were subsequently transferred via directional recombination to the T7-based N-terminal GST fusion expression vector pDEST15.

5.3. Functional characterization via metabolic engineering

Functional characterization of HvKS and the TaKSLs was accomplished by use of our previously described modular metabolic engineering system (Cyr et al., 2007). Briefly, class I labdane-related diterpene synthases such as the TaKSLs are co-expressed, from pDEST expression vectors, with pACYC-Duet (Novagen/EMD) derived plasmids that carry a GGPP synthase and CPS (pGGxC). These lead to production of the three most common stereoisomers of CPP, specifically pGGnC leads to production of normal CPP (**3**), pGGeC leads to production of *ent*-CPP (**2**), and pGGsC leads to production of *syn*-CPP (**4**). Accordingly, HvKS and the TaKSLs were separately co-expressed with each of the three pGGxC vectors (i.e., in all possible pairings). The resulting recombinant bacteria were then analyzed as previously described (Morrone et al., 2009). Briefly,

Table 1
Primers used in RT-PCR analysis of mRNA levels.

Gene	Forward primer	Reverse primer
TaKSL1	GCGGTTAACTCATTGCGAGA	CCTCACTTGACTCCCTCTTGA
TaKSL2	ATGTGGAGGAGGCATCTGC	GGCAACAACCTCAGCTTCAGG
TaKSL3	CTCTTGGCATCTGTTGTAATGG	GTTGAGGAGTCGGCAACAAG
TaKSL4	CGCTTACCTCATAACGGGATG	CGTCTCTGGATTCCCTCTCA
TaKSL5	GATCAAGAGTGTCTGGACTTCA	CAGATGAACGGAGGCTTCG
TaKSL6	ACTCACCCGGATTTCGCT	GCGGTAGTTCAACCTTGCG
18S	GGAGCGATTGTCTGGTTA	ATCTAAGGGCATCACAGACC

NZY liquid media cultures 50 mL were grown with shaking to $A_{600} \sim 0.6$ at 37 °C, the temperature reduced to 16 °C for 1 h prior to induction with IPTG (added to a final concentration of 0.5 mM), followed by continued fermentation at 16 °C for an additional ~72 h. These cultures were then extracted with an equal volume of hexanes, dried under N_2 , and resuspended in hexanes (100 μ L) for GC–MS analysis, with product identification accomplished by comparison to authentic standards.

5.4. Analysis of inducible wheat labdane-related diterpene synthase gene transcription

To measure *TaKSL* mRNA levels in response to UV irradiation, leaf sheaths were obtained from wheat plants that had been cultivated in a growth chamber for 2 weeks at 25 °C and exposed to UV light for 15 min according to a previously described method (Otomo et al., 2004b), and then harvested 20 h or 40 h after irradiation. Total RNA was extracted from frozen samples using an RNAqueous column with Plant RNA Isolation Aid (Ambion), and cDNA was synthesized from 1- μ g aliquots of total RNA by using a QuantiTect reverse transcription kit (Qiagen). Real-time QRT-PCR, using SYBR Green II, was carried out in a TP800 thermal cycler (Takara). This experiment was repeated three times (i.e., with separately grown plants), as previously described (Sawada et al., 2008), but with the primers listed in Table 1, and the data from a representative experiment (run in duplicate) shown in Fig. 2.

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