Following evolution's lead to a single residue switch for diterpene synthase product outcome

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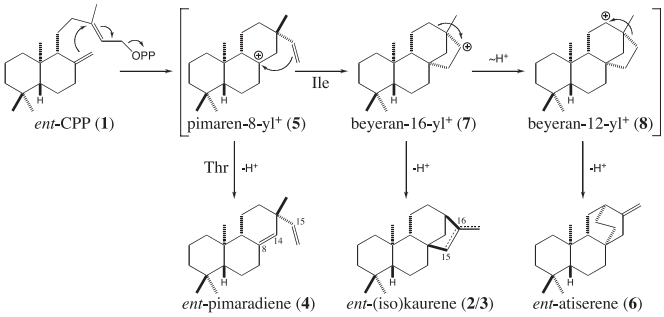
There have been few insights into the biochemical origins of natural product biosynthesis from primary metabolism. Of particular interest are terpene synthases, which often mediate the committed step in particular biosynthetic pathways so that alteration of their product outcome is a key step in the derivation of novel natural products. These enzymes also catalyze complex reactions of significant mechanistic interest. Following an evolutionary lead from two recently diverged, functionally distinct diterpene synthase orthologs from different subspecies of rice, we have identified a single residue that can switch product outcome. Specifically, the mutation of a conserved isoleucine to threonine that acts to convert not only the originally targeted isokaurene synthase into a specific pimaradiene synthase but also has a much broader effect, which includes conversion of the *ent*-kaurene synthases found in all higher plants for gibberellin phytohormone biosynthesis to the production of pimaradiene. This surprisingly facile switch for diterpene synthase catalytic specificity indicates the ease with which primary (gibberellin) metabolism can be subverted to secondary biosynthesis and may underlie the widespread occurrence of pimaradiene-derived natural products. In addition, because this isoleucine is required for the mechanistically more complex cyclization to tetracyclic kaurene, whereas substitution with threonine ''short-circuits'' this mechanism to produce the ''simpler'' tricyclic pimaradiene, our results have some implications regarding the means by which terpene synthases specify product outcome.

enzyme specificity | natural product biosynthesis | terpene synthase | biochemical evolution

The evolution of secondary metabolism presumably originates via changes in the catalytic specificity of enzymes recruited from primary metabolism. Although a recent report demonstrates that small numbers of changes in a sesquiterpene synthase can dramatically shift product outcome (i.e., plasticity), the target enzyme was already involved in secondary metabolism, and the parent wild-type enzyme was quite promiscuous (i.e., produced many different products) (1). Thus, how readily the typically specific enzymes involved in primary metabolism can be subverted into secondary metabolism remains a matter of conjecture.

Terpene synthases carry out complex electrophilic cyclization/rearrangement reactions, creating diverse hydrocarbon skeletal structures from simpler isoprenoid precursors, which often represents the committed step in particular biosynthetic pathways. Hence, altering the function of these enzymes is expected to represent a key step in the evolution of secondary metabolism. In addition, terpene synthases have attracted a great deal of interest because of their complex reaction mechanisms and wide variety of resulting products. Of particular interest is how these enzymes specify product outcome (2). Recent reports have demonstrated that specificity can be dramatically shifted by changes in a small number of amino acid residues (1, 3, 4).

Kaurene synthase (KS) catalyzes the cyclization of *ent*-copalyl diphosphate (*ent*-CPP, **1**) to *ent*-kaur-16-ene (**2**) through a multiple-step reaction mechanism (Scheme 1) (5). This enzyme is found in all higher plants because kaurene is an intermediate en route to the diterpenoid gibberellin phytohormones required for normal growth and development (6). Hence, KS participates in primary metabolism.



Scheme 1. Cyclization mechanism for pimaradienes, kaurenes, and atiserene. Diphosphate ionization-initiated cyclization of *ent*-CPP (**2**) to a pimaren-8-yl$^+$ (**5**) intermediate may be followed by secondary cyclization to a beyeran-16-yl$^+$ (**7**) intermediate that can either undergo ring rearrangement to the kauranyl ring structure, or a 1,3-hydride shift to a beyeran-12-yl$^+$ (**8**) intermediate that undergoes ring rearrangement to the atiseranyl ring structure. In each case, the final carbocation intermediate is quenched by deprotonation [dotted bonds indicate alternative double-bond placement in *ent*-kaur-16-ene (**2**) versus *ent*-isokaur-15-ene (**3**)].

The rice (*Oryza sativa*) genome contains an expanded family of KS-like genes with differing function (7). In the course of our biochemical characterization of the rice KS-like gene family, OsKSL, we found that one such enzyme from subspecies *indica*, OsKSL5i, cyclized *ent*-CPP (**1**) specifically (>90%) to *ent*-isokaur-15-ene (**3**) (8). However, another group had previously reported that the orthologous OsKSL5j from subspecies *japonica* rice specifically produced *ent*-pimara-8(14),15-diene (**4**), representing deprotonation of the putative pimaren-8-yl carbocation (**5**) intermediate in the cyclization of *ent*-CPP (**1**) to *ent*-(iso)kaurene (Scheme 1) instead (9). Here, we demonstrate that alternation of a single residue is sufficient for the change in product outcome between these two orthologous diterpene synthases, and the extension of this single residue switch in product outcome to disparate kaurene synthases involved in gibberellin biosynthesis, with significant implications for not only how terpene synthases control their product outcome, but the evolution of secondary metabolism as well.

Results

The functionally distinct rice subspecies-associated orthologs OsKSL5i and OsKSL5j are 98% identical at the amino acid level,

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Abbreviations: AtKS, KS from *Arabidopsis thaliana*; CPP, copalyl diphosphate; FID, flame ionization detection; GGPP, (*E*,*E*,*E*)-geranylgeranyl diphosphate; KS, kaurene synthase.

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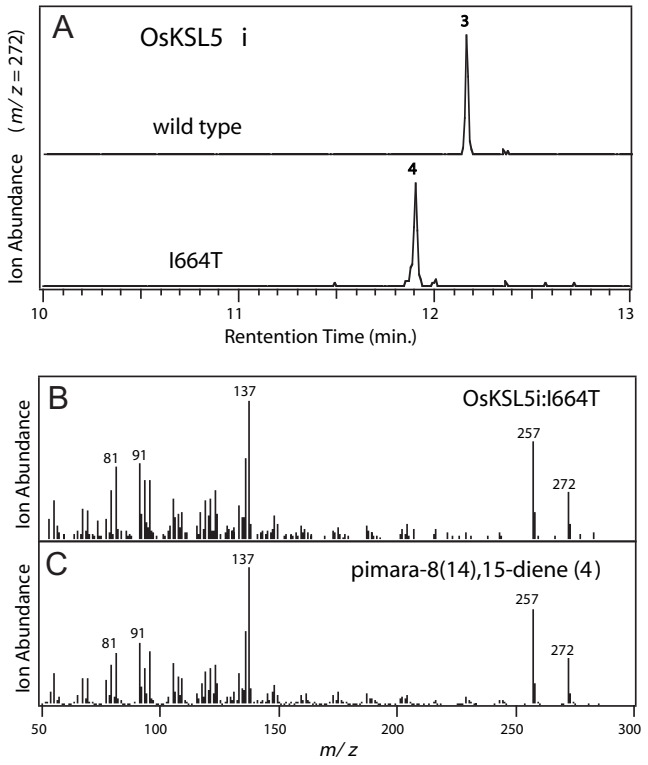

Fig. 1. Effect of I664T mutation on OsKSL5i product outcome. (*A*) Chromatograms from GC–MS analysis of wild-type and I664T mutant of OsKSL5i (as indicated). Numbers correspond to the chemical structures defined in the text and Scheme 1. (*B*) Mass spectra from the OsKSL5i:I664T product peak (retention time = 11.91 min). (*C*) Mass spectra from an authentic sample of pimara-8(14),15-diene (**4**) (retention time = 11.92 min).

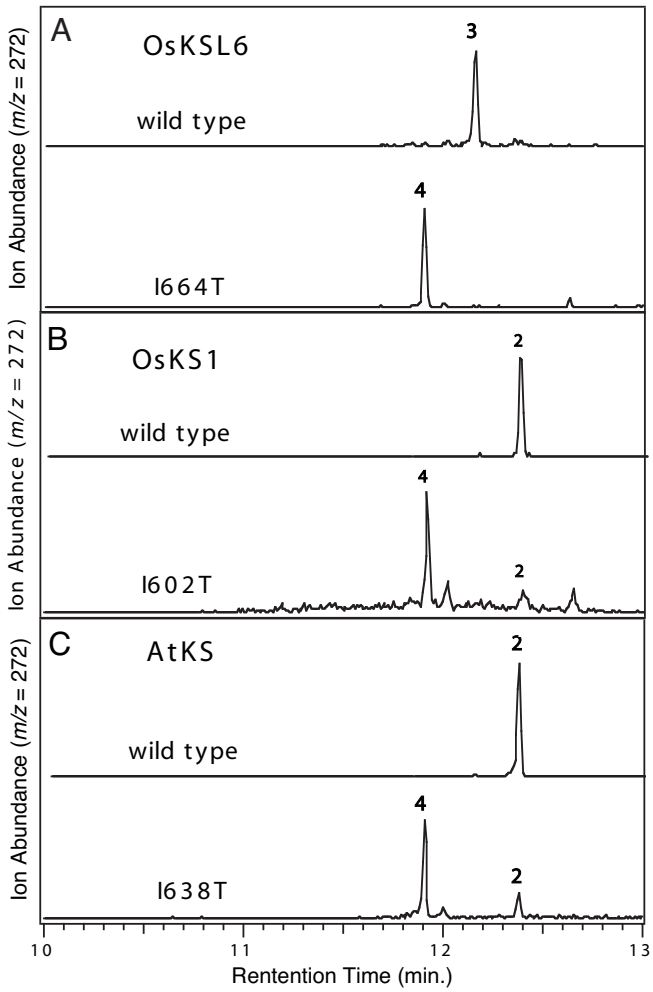

Fig. 2. Effect of isoleucine-to-threonine mutation on (iso)kaurene synthase product outcome. Chromatograms from GC–MS analysis of the indicated diterpene synthases (wild-type or indicated mutant). (*A*) OsKSL6. (*B*) OsKS1. (*C*) AtKS. Numbers correspond to the chemical structures defined in the text and Scheme 1. Enzymatic products were identified by comparison of retention time and mass spectra to authentic standards (i.e., see Fig. 1).

and modeled enzyme structures indicate that there are only three differences in the active site (8). To determine which differences, if any, were responsible for the observed change in function, each of these three divergent active-site residues in the *ent*-isokaurene synthase OsKSL5i was mutated to the corresponding aa found in the *ent*-pimaradiene synthase OsKSL5j. The product profile of the resulting OsKSL5i:V661L, OsKSL5i:I664T, and OsKSL5i:I718V mutants was characterized by GC–MS analysis and comparison of the enzymatic product to authentic standards. Whereas OsKSL5i:V661L and OsKSL5i:I718V continued to specifically produce *ent*-isokaur-15-ene (**3**), OsKSL5i:I664T specifically produced *ent*-pimara-8(14),15-diene (**4**) (Fig. 1). Thus, this single isoleucine-to-threonine change was sufficient to convert the specific isokaurene synthase OsKSL5i into a specific pimaradiene synthase.

Rice contains another *ent*-isokaurene synthase (OsKSL6) that shares 89% aa identity with OsKSL5i (8, 9), and the isoleucine residue identified above is conserved in OsKSL6 (also at position 664). To determine whether the same change would similarly convert OsKSL6 into a *ent*-pimaradiene synthase, the analogous OsKSL6:I664T mutant was constructed and its product profile characterized by GC-MS. Notably, this isoleucine-to-threonine change also was sufficient to convert OsKSL6 from a specific *ent*-isokaurene to specific *ent*-pimaradiene synthase (Fig. 2*A*).

Although kaurene synthases are much less well conserved with OsKSL5i (41–52% aa identity), a corresponding isoleucine residue is further found in all of the known KS (Fig. 3). The KS most similar to OsKSL5i is the rice paralog OsKS1, sharing 52% aa identity (8). To ascertain whether this isoleucine residue plays a similar role in KS as it does in OsKSL5i and OsKSL6, the analogous OsKS1:I602T mutant was constructed and characterized. Strikingly, this same isoleucine-to-threonine change also switched OsKS1 from a specific kaurene synthase to a pimaradiene synthase, albeit one that still produces some [≈30% by GC-flame ionization detection (FID) analysis] of *ent*-kaur-16-ene (**2**) (Fig. 2*B*). In addition, although the KS from the dicot *Arabidopsis thaliana* (AtKS) was the most distantly related, sharing only 41% aa identity with OsKSL5i, the corresponding AtKS:I638T mutant also largely produced (≈80%) *ent*-pimara-8(14),15-diene (**4**) along with smaller amounts (≈20%) of *ent*-kaur-16-ene (**2**) (Fig. 2*C*). Thus, this single isoleucine-to-threonine change, requiring substitution of only a single nucleotide, was sufficient to essentially switch the product outcome of a wide range of (iso)kaurene synthases to the production of pimaradiene (**4**).

To further probe the role of the residue at this position in determining product outcome, particularly for secondary (tetra)cyclization within the initially formed tricyclic pimaren-8-yl$^+$ (**5**) intermediate (Scheme 1), OsKSL5j was cloned and the converse threonine-to-isoleucine mutant was constructed and characterized. Intriguingly, OsKSL5j:T664I no longer produced *ent*-pimara-8(14),15-diene but neither did it exclusively produce isokaur-15-ene (**3**). Instead, this mutant produced a mixture of

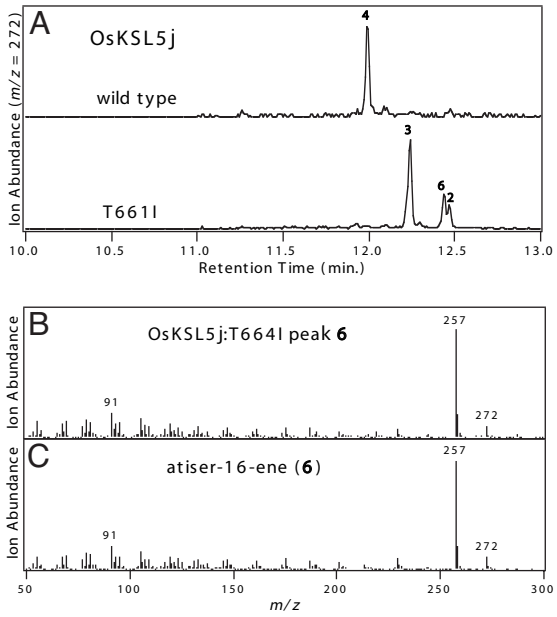

Fig. 3. Effect of threonine-to-isoleucine mutation on OsKSL5j product outcome. (*A*) Chromatograms from GC–MS analysis of wild-type and I664T mutant of OsKSL5j (as indicated). Numbers correspond to the chemical structures defined in the text and Scheme 1. (*B*) Mass spectrum from the OsKSL5j:T664I product peak labeled ''6'' (retention time = 12.43 min). (*C*) Mass spectrum from an authentic sample of atiser-16-ene (**6**) (retention time = 12.43 min).

tetracyclic diterpenes (Fig. 3), exhibiting a 50:37:13 ratio of isokaur-15-ene (**3**) to atiser-16-ene (**6**) to kaur-16-ene (**2**). Although OsKSL5i did produce small amounts of atiser-16-ene (**6**) and kaur-16-ene (**2**) (≈10% altogether), the product profile of the OsKSL5j:T664I mutant demonstrated decreased product specificity resulting from one or more of the other differences between OsKSL5i and OsKSL5j. Regardless, the specific production of tetracyclic diterpenes by OsKSL5j:T664I indicated the importance of an isoleucine at this position for secondary cyclization of the pimaren-8-yl$^+$ intermediate (**5**) to a beyeran-16-yl$^+$ intermediate (Scheme 1).

For the observed single residue switch of diterpene synthase product outcome to be relevant in biological settings and play a role in biochemical evolution, the effect of such a change on enzymatic activity must be minimal, such that effective metabolic flux toward the new product can be observed. To ascertain the effect of the reported mutations, each of the wild-type and mutant diterpene synthases was coexpressed in recombinant *Escherichia coli* with a (*E*,*E*,*E*)-geranylgeranyl diphosphate (GGPP) and *ent*-CPP synthase. In every case, production of the expected diterpene(s) was readily observed, demonstrating that these mutants are biologically functional, mediating observable metabolic flux at least in this heterologous setting. In addition, it has been reported that terpene biosynthesis in metabolically engineered *E. coli* is limited, at least in part, by terpene synthase activity (10), suggesting that terpene production levels can serve as a proxy for relative enzymatic activity. Quantitative product analysis (by GC-FID analysis) demonstrated that the isoleucine-to-threonine mutants reported above reduced the amount of diterpene(s) produced by 3- to 11-fold, indicating that these mutations had small but observable deleterious effects on catalytic activity. Furthermore, the OsKSL5j:T664I mutant actually increased diterpene production ≈15-fold over that mediated by wild-type OsKSL5j, also indicating that the change from isoleucine to threonine at this position reduces catalytic activity. Finally, consistent with our hypothesis that the final amount of diterpene from these metabolically engineered *E. coli* is proportional to enzymatic activity, kinetic analysis of AtKS (apparent k_{cat} = 0.06 ± 0.04 s^{-1} and K_M = 0.3 ± 0.1 μM) and the AtKS:I638T mutant (apparent k_{cat} = 0.02 ± 0.01 s^{-1} and K_M = 0.4 ± 0.2 μM) demonstrated a ≈4-fold decrease in catalytic efficiency [(2 versus 0.5) × 10^5 M^{-1} s^{-1}], similar to the ≈3-fold reduction suggested by quantification of the amount of diterpene produced by metabolically engineered *E. coli*.

Table 1. Sequence comparison of selected diterpene synthases

Synthase	Position	F helix*	Product
AtKS	634	LGP**I**VLPATYLI	Kaurene
CmKS	639	LGP**I**ILPMLFFV	Kaurene
OsKS1	654	LGP**I**VLPTLYFV	Kaurene
OsKSL6	660	VGP**I**ITSAALFV	Isokaurene
OsKSL5i	660	VGP**I**ITSAALFV	Isokaurene
OsKSL5j	617	LGP**T**ITSAALFV	Pimaradiene
OsKSL4	688	LGP**T**ILIALYFM	Pimaradiene
OsKSL10	655	FAS**F**VCPPLYFL	Pimaradiene

OsKSL nomenclature has been previously defined and followed here (8). CmKS refers to the KS from pumpkin (*Cucurbita maxima*) (22).

*The portion of the F helix containing the targeted residue position (indicated in bold) from selected diterpene synthases, whose product structures are indicated on the right.

Discussion

Given the complex catalytic mechanisms and the wide variety of resulting products arising from terpene synthases, as well as their critical role in determining metabolic fate, how these enzymes specify product outcome has been a subject of great biochemical interest (2). Recent results indicate that small numbers of aa substitutions are sufficient to dramatically alter the product profile of sesquiterpene synthases (1, 3, 4). However, there have been no previous reports targeting the catalytic specificity of diterpene synthases. In addition, previous reports have largely focused on interconversion of pairs of terpene synthases sharing significant similarity (>70% aa identity), although one recent study more generally investigated the plasticity of terpene synthase activity (1). Here, following a lead from two recently diverged rice subspecies-associated diterpene synthase orthologs, a specific isoleucine-to-threonine change was found to be sufficient to essentially switch the product outcome of not only the originally targeted OsKSL5i *ent*-isokaurene synthase ortholog and a very similar OsKSL6 paralog, but also kaurene synthases exhibiting much less similarity (41–52% aa identity). In addition, OsKS1 and AtKS are among the most disparate pair of kaurene synthases, sharing only 47% aa identity, which is close to the low end of the 46–55% range of aa identity shared by the known KS.

The extension of these mutational results to disparate kaurene synthases highlights the importance of the targeted isoleucine in enzymatic cyclization of *ent*-CPP to diterpene tetracyclic structures and also may be evolutionarily relevant. In particular, the absolute requirement for gibberellin biosynthesis in all flowering plants (i.e., angiosperms) provides a reservoir of biosynthetic genes, duplication of which (i.e., as a result of the whole-genome duplications that are prevalent in plant evolutionary history) has enabled the derivation of alternative diterpenoid metabolism. Accordingly, there are >1,000 known (iso)kaurene-derived natural products, as well as ≈500 known to be derived from pimaradienes (11). Although only a few pimaradiene synthases have been identified (7), none contains an isoleucine at the position identified here, instead containing either threonine or phenylalanine (Table 1). Hence, it seems likely that the surprisingly facile ability of changes to this particular isoleucine residue to switch the product outcome of a duplicated KS enzyme to

pimaradiene at least partially underlies the observed large numbers of pimaradiene-derived natural products. Nevertheless, this primary change in product outcome may then be followed by secondary changes to restore some of the catalytic activity that seems to be lost with this particular mutation and increase specificity for pimaradiene production.

Production of *ent*-pimara-8(14),15-diene (**4**) by mutant (iso)kaurene synthases represents an interruption of the normal cyclization of bicyclic *ent*-CPP to tetracyclic (iso)kaurene at the tricyclic pimarenyl stage (Scheme 1). Although there has been a previous report of a single tyrosine-to-phenylalanine mutation in the structurally defined sesquiterpene 5-epi-aristolochene synthase that interrupts a hydrogen bond network and results in the specific production of a similarly abortive, partially cyclized product (12), this effect appears to be specific to that particular enzyme, revealing an expected stable hydrocarbon intermediate, and is not more broadly applicable (13). There is no evidence that a stable pimaradiene hydrocarbon is formed in (iso)kaurene synthase reactions, and the production of pimaradiene by these mutant diterpene synthases presumably represents deprotonation of the initially formed pimaren-8-yl carbocation (**5**) intermediate.

The production of pimara-8(14),15-diene (**4**) seems to readily occur upon mutation of this particular isoleucine to threonine with the isokaurene synthases but also seems to be less favorable in KS. This almost certainly arises from the fact that production of pimara-8(14),15-diene (**4**) results from deprotonation of the same carbon that is deprotonated in formation of isokaur-15-ene (**3**). Thus, I664T mutants of the isokaurene synthases OsKSL5i and OsKSL6 can readily quench pimaren-8-yl$^+$ (**5**) by deprotonation to pimara-8(14),15-diene (**4**). By contrast, kaurene synthases deprotonate an extracyclic methyl group, which would not quench the pimaren-8-yl$^+$ (**5**) intermediate, and the corresponding isoleucine-to-threonine mutation is less specific in this context, producing a mixture of pimaradiene with smaller amounts of kaurene. This presumably reflects the lack of an appropriate group for ready deprotonation of the pimaren-8-yl$^+$ (**5**) intermediate, setting up a kinetic competition between such immediate deprotonation to pimara-8(14),15-diene (**4**) and further cyclization that enables deprotonation to kaur-16-ene (**2**) as specified by the original wild-type KS.

Given the nature of the mutations studied here, we hypothesize that the polarity introduced by the threonine hydroxyl group may act to stabilize the pimaren-8-yl$^+$ (**5**) intermediate long enough for deprotonation to occur. Consistent with this hypothesis, in modeled enzyme structures the relevant residue is located in the F helix on the same side of the active site as the DDXXD Mg^{2+} binding motif, which is involved in pyrophosphate positioning, but toward the bottom of the cavity, which can be readily pictured as near the location of the pimaren-8-yl carbocation (Fig. 4). The observation of a phenylalanine residue at this position in a pimaradiene synthase (Table 1) also is consistent with this hypothesis, as the ability of aromatic residues to stabilize carbocations has long been recognized (14). In addition, the ability of the converse threonine-to-isoleucine mutation of the pimaradiene synthase OsKSL5j to drive secondary cyclization to tetracyclic diterpenes is further consistent with the lack of stabilization of the pimaren-8-yl$^+$ (**5**) intermediate, leading to such secondary (tetra)cyclization.

Our hypothesis regarding the role of this particular isoleucine in the secondary (tetra)cyclization mediated by diterpene synthases such as KS is consistent with structural and mechanistic studies indicating that terpene synthase active sites present an overall hydrophobic environment and largely control product outcome through the conformation/fold of the substrate (i.e., relative positioning of the electrophilic double bonds) (2). However, although selected carbocation intermediates appear to be stabilized within terpene synthase active sites [e.g., ion pairing between the released pyrophosphate anion and beyeran-16-yl carbocation (**6**) intermediate in KS (A. Roy, F. G. Roberts, P.R.W., R.J.P., and R. M. Coates; unpublished data)], our hypothesis further implies that product outcome is also directed by the lack of stabilization of other carbocation intermediates [e.g., the pimaren-8-yl$^+$ (**5**) intermediate in KS-catalyzed cyclization]. These observations may be coupled because the presence of the ionized pyrophosphate group in the active site should exert a significant electrostatic effect. In particular, driving terpene synthase reactions toward intermediates wherein the carbocation is localized proximal to the multiple counterion charges on the pyrophosphate [i.e., in KS from pimaren-8-yl$^+$ (**5**) to beyeran-16-yl$^+$ (**7**)]. This would be consistent with the observation that aza-analogs are generally observed to bind in orientations that provide counterion pairing between the aza group and pyrophosphate in tertiary crystal structures, regardless of the expected catalytically productive binding mode (15). Notably, the exertion of such an electrostatic effect on product outcome by the released pyrophosphate moiety also would provide an example of substrate-assisted catalytic specificity.

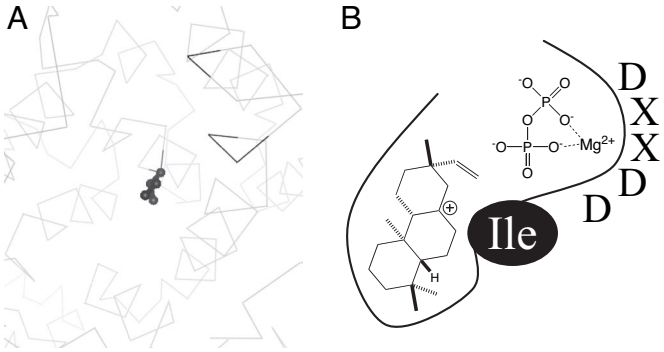

Fig. 4. Location of the targeted isoleucine residue. (*A*) In the active site of the modeled structure for AtKS (with the isoleucine side chain depicted in ball-and-stick rendering). Also indicated by darker coloring are the Cα backbone positions of the aspartates from the DDXXD Mg^{2+} binding motif. (*B*) Schematic depiction of the isoleucine (Ile) residue and DDXXD motif relative to the ionized diphosphate and pimaren-8-yl carbocation (**5**) intermediate.

Regardless of the mechanism by which the isoleucine-to-threonine mutation alters diterpene synthase product outcome, the ability of this single residue change to make such a dramatic difference in product outcome is remarkable. Furthermore, the demonstrated effect of this single residue switch in the widely disparate, albeit functionally conserved, kaurene synthases found in all plants for gibberellin biosynthesis, coupled to sequence analysis of the currently known pimaradiene synthases, suggests that changes to this particular isoleucine are likely to be at least partially responsible for the observed large numbers of pimaradiene-derived natural products, potentially providing an example of how secondary metabolism may have been derived from primary (gibberellin) metabolism. Thus, we have followed evolution's lead to a single residue switch for diterpene synthase product outcome that also may have wider evolutionary implications.

Materials and Methods

General Procedures. Authentic standards for the diterpenes identified here were kindly provided by Robert Coates (University of Illinois at Urbana–Champaign, Urbana, IL). Unless otherwise noted, all other chemicals were purchased from Fisher Scientific (Loughborough, Leicestershire, U.K.), and molecular biology reagents were from Invitrogen (Carlsbad, CA). GC was performed with Agilent (Palo Alto, CA) 6890N GC instruments by using an HP-1 column with FID or an HP-5 column with MS detection using a 5973N mass-selective detector in electron

ionization (70 eV) mode located in the W. M. Keck Metabolomics Research Laboratory at Iowa State University. Samples (5 μl) were injected at 40°C in splitless mode; the oven temperature was held at 40°C 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 and final hold.

Recombinant Constructs. The OsKS1, OsKSL5i, and OsKSL6 genes have been previously described (8), whereas AtKS was previously cloned and kindly provided by Shinjiro Yamaguchi (Institute of Physical and Chemical Research, Wako, Japan) (16). OsKSL5j was cloned from subspecies *japonica* cultivar Nipponbare rice by using the same primers and methods reported for isolation of OsKSL5i (8). All genes were transferred into the Gateway vector system via PCR amplification and directional topoisomerase-mediated insertion into pENTR/SD/D-TOPO and were verified by complete sequencing. Site-directed mutagenesis was carried out via PCR amplification of the pENTR constructs with overlapping mutagenic primers, and the mutant genes were verified by complete sequencing. The resulting wild-type and mutant genes were then transferred via directional recombination to the T7-promoter N-terminal GST fusion expression vector pDEST15.

Enzymatic Analysis. All constructs were expressed, purified, and the product profile of the resulting recombinant proteins was characterized in coupled assays by using the *ent*-CPP synthase from maize (An2) to convert GGPP (Sigma–Aldrich, St. Louis, MO) to *ent*-CPP (**1**) as previously described (8). In brief, 1-ml reactions in assay buffer (50 mM Hepes, pH 7.2, 0.1 mM $MgCl_2$, 10% glycerol, and 5 mM fresh DTT) containing 50 μM GGPP and 100 nM An2 were run for $\approx$2 h at room temperature, which is sufficient to completely convert the GGPP to *ent*-CPP (**1**). Then equivalent volumes of individual purified GST-tagged diterpene synthases were added, along with $MgCl_2$ to a final concentration of 10 mM, and the reactions incubated overnight at room temperature before extraction with hexanes (3 $\times$ 1 ml), partial purification over short silica gel columns, and subsequent GC–MS analysis. In addition, each construct was coexpressed in C41 *E. coli* with a GGPP and *ent*-CPP synthase carried together on a pACYCDuet vector (Novagen, San Diego, CA) and the resulting product isolated as recently described (17). In brief, the cotransformed *E. coli* were grown and recombinant protein expression induced in mixed-phase cultures, specifically, liquid media growth cultures containing 2% (wt/vol) adsorbent HP20 Diaion resin (Supleco, Bellefonte, PA) (i.e., liquid and solid phases). The production of diterpene(s) under these conditions is easily assessed by their elution from the HP20 beads with organic solvent. The resulting diterpene products were identified by GC–MS-based comparison to authentic standards, and relative amounts were quantified by GC-FID analysis.

Kinetic analysis was carried out by using An2 to convert [1-^{3}H]GGPP (American Radiolabeled Chemicals, St. Louis, MO) to *ent*-CPP (**1**), as described above. Complete conversion was verified by enzymatic dephosphorylation and GC-FID analysis, as previously described (18). This provided 50-μM substrate stock solutions for dilution into subsequent KS reactions. The kinetic assays were performed and analyzed similar to the manner described in ref. 19. In brief, duplicate 1-ml reactions in assay buffer with 7.5 mM $MgCl_2$ containing 10 nM purified GST-AtKS and 0.1 mg/ml α-casein were initiated by the addition of labeled *ent*-CPP (**1**), run for 1 min at room temperature, and stopped by the addition of KOH to 0.2 M and EDTA to 15 mM. However, for analysis of the GST-AtKS:I664T mutant, the enzymatic concentration was increased to 40 nM and incubation times increased to 2 min. The produced diterpenes were then extracted, the pooled extract passed over a short silica gel column, production formation assessed by scintillation count, and the resulting data analyzed by using Kaleidagraph (Synergy, Reading, PA).

Bioinformatics. All alignments and protein structure manipulation/visualization were performed with the VectorNTI software package (Invitrogen). Modeled protein structures were obtained through the Swiss-Model service (20) by using alignments of the relevant portions of the target diterpene synthases with 5-epi-aristolochene synthase, whose known structure (21) served as the template.

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