

Characterization of CYP76M5–8 Indicates Metabolic Plasticity within a Plant Biosynthetic Gene Cluster^{*,§}

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Background: Biosynthetic gene clusters are unusual in plants, yet may provide insight into the associated evolution of secondary metabolism.

Results: The cytochromes P450 CYP76M5–8, found in one such cluster, have multiple functions in rice diterpenoid metabolism.

Conclusion: Plant biosynthetic gene clusters can encode metabolic plasticity.

Significance: Such plasticity may enable further evolution, and be a broader feature of plant secondary metabolism.

Recent reports have revealed genomic clustering of enzymatic genes for particular biosynthetic pathways in plant specialized/secondary metabolism. Rice (*Oryza sativa*) carries two such clusters for production of antimicrobial diterpenoid phytoalexins, with the cluster on chromosome 2 containing four closely related/homologous members of the cytochrome P450 CYP76M subfamily (CYP76M5–8). Notably, the underlying evolutionary expansion of these CYP appears to have occurred after assembly of the ancestral biosynthetic gene cluster, suggesting separate roles. It has been demonstrated that CYP76M7 catalyzes C11 α -hydroxylation of *ent*-cassadiene, and presumably mediates an early step in biosynthesis of the derived phytocassane class of phytoalexins. Here we report biochemical characterization of CYP76M5, -6, and -8. Our results indicate that CYP76M8 is a multifunctional/promiscuous hydroxylase, with CYP76M5 and -7 seeming to provide only redundant activity, while CYP76M6 seems to provide both redundant and novel activity, relative to CYP76M8. RNAi-mediated double knock-down of CYP76M7 and -8 suppresses elicitor inducible phytocassane production, indicating a role for these monooxygenases in phytocassane biosynthesis. In addition, our data suggests that CYP76M5, -6, and -8 may play redundant roles in production of the oryzalexin class of phytoalexins as well. Intriguingly, the preceding diterpene synthase for oryzalexin biosynthesis, unlike that for the phytocassanes, is not found in the chromosome 2 diterpenoid biosynthetic gene cluster. Accordingly, our results not only uncover a complex evolutionary history, but also further suggest some intriguing differences between plant biosynthetic gene clusters and the seemingly similar microbial operons. The implications for the underlying metabolic evolution of plants are then discussed.

Traditionally, genes are considered to be essentially randomly distributed in the genomes of multicellular eukaryotic organisms, in contrast with microbes, where co-regulated genes with related functions (e.g. those involved a particular biosynthetic pathway) often are clustered together (1). Nevertheless, it has been demonstrated that nonhomologous enzymatic genes for some more specialized/secondary biosynthetic pathways also are physically clustered together in some plant genomes (2), for example, the cyclic hydroxamic acid pathway in maize (3), triterpenoid biosynthetic gene clusters in oats and *Arabidopsis* (4, 5), and diterpenoid biosynthetic gene clusters in rice (6–9). Although such clusters appear to be an emerging theme in plant secondary metabolism (2), these still represent exceptions rather than reflecting the general genomic organization pattern found in microbial genomes. Thus, it remains unclear how these clusters were assembled, and what the evolutionary implications are for the contained genes. Of particular interest here is whether the activity of the encoded enzymes limited to that required for the corresponding biosynthetic pathway. This is thought to be largely true in microbial biosynthetic gene clusters, and such specificity has been implied for the enzymes found in plant biosynthetic gene clusters as well, yet broader activity would have interesting implications for metabolic evolution.

Rice (*Oryza sativa*) produces a complex mixture of antimicrobial natural products in response to microbial infection (10, 11), wherein separate groups are produced in response to fungi (e.g. the blast pathogen *Magnaporthe oryzae*), relative to bacteria (e.g. the leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae*). In particular, the oryzalexins seem to be antibacterial phytoalexins (12), whereas the other groups (Fig. 1) are antifungal phytoalexins (10, 11). These phytoalexins are almost entirely all labdane-related diterpenoids, whose biosynthesis is characterized by an initial pair of sequential cyclization reactions (13). Specifically, bicyclization of the universal diterpenoid precursor (*E,E,E*)-geranylgeranyl diphosphate by copalyl/labdadienyl diphosphate (CPP)² synthases

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[§] This article contains supplemental Figs. S1–S14 and Tables S1–S9.

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² The abbreviations used are: CPP, copalyl diphosphate; CPS, CPP synthase; CYP, cytochrome P450; KSL, kaurene synthase like; CPR, cytochrome P450 reductase; GS, gas chromatography.

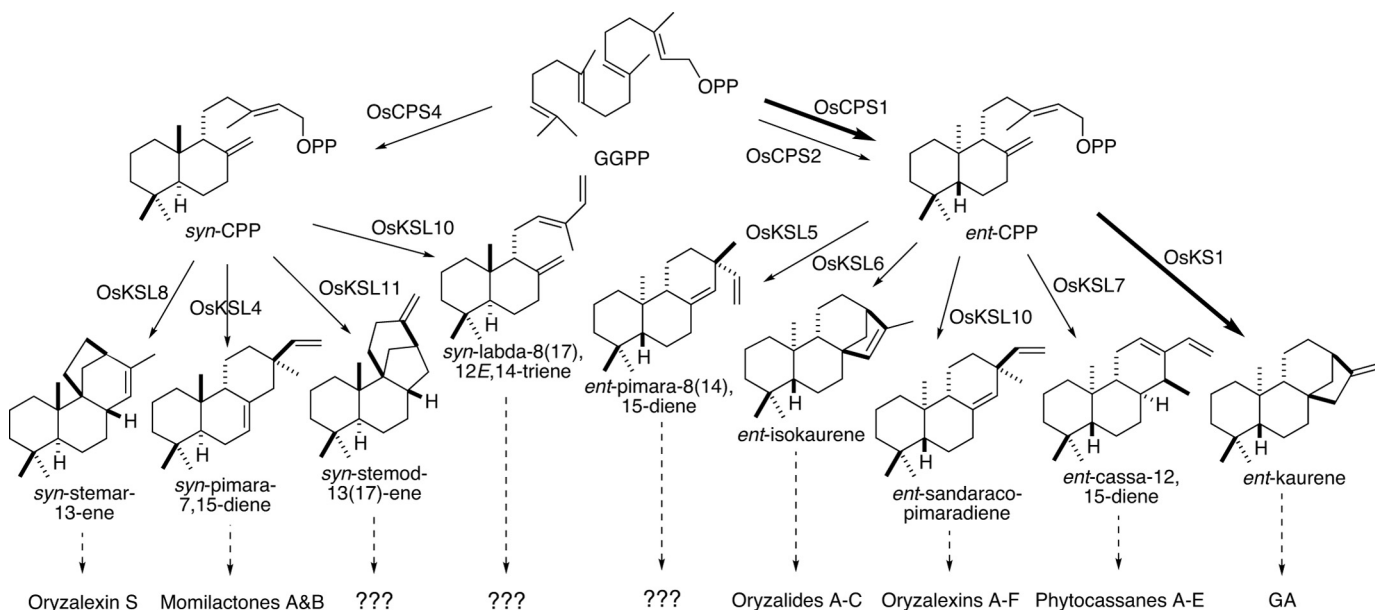


FIGURE 1. **Functional map of rice diterpenoid biosynthesis.** The cyclases and corresponding reactions that convert geranylgeranyl diphosphate (GGPP) to CPP and, subsequently, to cyclic olefins are indicated, along with the downstream natural products, where known. *Thicker arrows* indicate enzymatic reactions specifically involved in GA metabolism; *dashed arrows* indicate multiple enzymatic reactions.

(CPS), which is followed by further cyclization and/or rearrangement to a polycyclic olefin catalyzed by enzymes often termed kaurene synthase-like (KSL) for their relationship to the presumably ancestral *ent*-kaurene synthase from gibberellin phytohormone metabolism (Fig. 1). Production of the bioactive diterpenoid phytoalexins then further requires the action of at least cytochrome P450 (CYP) monooxygenases (14).

Two labdane-related diterpenoid phytoalexin gene clusters have been reported in rice, one located on chromosome 4 and the other on chromosome 2. The gene cluster on chromosome 4 is involved in producing momilactones, and contains the relevant *syn*-CPP synthase (OsCPS4) and *syn*-pimaradiene synthase (OsKSL4) (7). In addition, this cluster also contains a short chain alcohol dehydrogenase that catalyzes the final step in production of momilactone A, and two closely related P450s (CYP99A2 and -3), one or both of which are required for momilactone biosynthesis (8). Recently, we have demonstrated that CYP99A3 acts as a *syn*-pimaradiene oxidase to produce *syn*-pimaradien-19-oic acid, presumably as an intermediate en route to the 19,6- γ -lactone ring of the momilactones (15). However, we were unable to assign a separate biochemical function to CYP99A2. Thus, it remains unclear why this apparently momilactone biosynthesis dedicated gene cluster contains these two closely related CYP.

The other diterpenoid biosynthetic gene cluster, on chromosome 2 (Fig. 2), is uniquely multifunctional, being involved in the production of at least two classes of phytoalexins, the antifungal phytocassanes and antibacterial oryzalides (9). Both of these, along with the antifungal oryzalexins, share an *ent*-CPP intermediate in common with gibberellin hormone biosynthesis (Fig. 1). However, there appear to be separate *ent*-CPP synthases for gibberellin (OsCPS1) *versus* phytoalexin (OsCPS2) biosynthesis (6, 16, 17). The chromosome 2 gene cluster contains the phytoalexin-specific OsCPS2, along with subsequently acting *ent*-CPP-specific KSL (OsKSL5–7). Of these,

OsKSL7 produces the *ent*-cassadiene precursor to the phytocassanes, and OsKSL6 the *ent*-isokaurene precursor to the oryzalides, although the metabolic fate of the *ent*-pimaradiene produced by OsKSL5 remains unclear (18–20). In addition, this gene cluster further contains six CYP, which fall into two closely related/homologous groups, composed of CYP71Z6 and -7 and CYP76M5–8, respectively.

We have previously reported that the CYP76M7 found in the gene cluster on rice chromosome 2 catalyzes C11 α -hydroxylation of *ent*-cassadiene, presumably en route to the C11-keto functionality found in all the bioactive phytocassanes (9). Phylogenetic analysis carried out as part of that study indicated that the co-clustered and closely related CYP76M5–8 seem to be derived from a common ancestral CYP76M subfamily member that was incorporated in the original form of this biosynthetic gene cluster and subsequently underwent gene expansion. This raises the question of what metabolic function(s) are carried out by these other CYP76M subfamily members, *i.e.* what provided the selective pressure that enabled the expanded CYP76M5–8 version of the biosynthetic gene cluster to sweep through the population. Here, we report biochemical characterization of CYP76M5, -6, and -8, uncovering a range of activity. Although we provide strong support for a role for CYP76M7 and -8 in phytocassane biosynthesis via RNAi-mediated double knock-down lines, our biochemical data further suggests putative roles in “other” biosynthetic pathways for CYP76M5–8. In particular, ones for which the upstream enzymatic genes are not co-clustered (*e.g.* that for the oryzalexins). The implications of this for the interplay between biosynthetic gene clusters and metabolic evolution in plants are then discussed.

EXPERIMENTAL PROCEDURES

General—Unless otherwise noted, chemicals were purchased from Fisher Scientific and molecular biology reagents from Invitrogen. Sequence analyses were done with the CLC

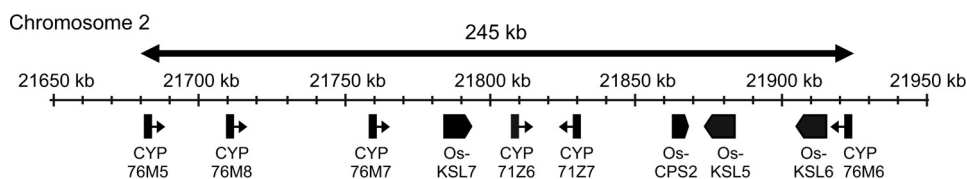


FIGURE 2. **Schematic of the rice chromosome 2 diterpenoid biosynthetic gene cluster.** Note that no other genes appear to be present in these regions (adapted from Swaminathan *et al.* (9)).

Sequence Viewer program (version 6.5; CLCbio), with the presented phylogenetic tree and bootstrap values calculated via the neighbor-joining algorithm (21), with 1000 replicates. Determination of the presented gene map, along with the CYP nomenclature used here, has been previously described (9). Gas chromatography (GC) was performed with a Varian (Palo Alto, CA) 3900 GC with Saturn 2100 ion trap mass spectrometer (MS) in electron ionization (70 eV) mode for GC-MS and GC-MS/MS analysis, or with an Agilent 6890N GC for flame ionization detection. Samples (1 μ l) were injected in splitless mode at 50 °C and, after holding for 3 min at 50 °C, the oven temperature was raised at a rate of 14 °C/min to 300 °C, where it was held for an additional 3 min. MS data from 90 to 600 m/z were collected starting 12 min after injection until the end of the run. GC-MS chromatograms and mass spectra for all the compounds identified here are presented in supplemental Figs. S2–S9.

Recombinant Constructs—Construction of the CYP76M5–8 Gateway expression system entry vectors (pENTR/SD/D-TOPO), using the native genes obtained from the KOME rice cDNA data bank (22), has been previously described (9). For functional bacterial expression, CYP76M5, -6, and -8 were modified at their N termini via a two-stage PCR process, first removing part of the 5' end of the open reading frame (39 codons for CYP76M5, 38 codons for CYP76M6, and 33 codons for CYP76M8), and then adding 10 new codons (encoding the amino acid sequence “MAKKTSSKGK”) in each case, which was based on the modifications used for bacterial expression of the mammalian CYP2B subfamily (23). The resulting constructs were cloned into pENTR/SD/D-TOPO via directional topoisomerization, and verified by complete gene sequencing. These were then transferred into a previously described pCDF-Duet (Novagen)-derived vector, specifically via directional recombination into a DEST cassette contained in the first multiple cloning site, whereas the vector also contains a rice CPR (OsCPR1) in the second multiple cloning site (9). Many of the CYP76M subfamily members (as described under “Results”) were completely recoded to optimize codon usage for *Escherichia coli* expression via gene synthesis (GenScript; see supplemental data for corresponding nucleotide sequences). These synthetic gene constructs also were then N-terminal modified as described above with all constructs, then inserted into the Gateway system as described above.

Recombinant Expression—Recombinant baculovirus-based expression of CYP76M5–8 has been previously described (9). Briefly, the recombinant baculoviruses were created using the Bac-to-Bac Baculovirus expression system with Gateway-derived pDEST8 constructs. These were then used to express targeted CYP76M subfamily members in Sf21 insect cells, with microsomes or lysates isolated from the resulting recombinant

cell cultures, and used for *in vitro* assays, again as previously described (9), but with substitution of the described diterpenes for the previously tested *ent*-cassadiene. Protein expression was confirmed by Western blot analysis using antibodies directed against the incorporated His₆ tag.

All native and recoded P450 genes were recombinantly expressed in the C41 Overexpress strain of *E. coli* (Lucigen, Middleton, WI) using our previously described modular diterpene metabolic engineering system (24). Specifically, we co-expressed these CYP from the OsCPR1 co-expression constructs described above, with a (*E,E,E*)-geranylgeranyl diphosphate synthase and CPS carried on co-compatible pGGXC vectors, and KSL was expressed from the additionally co-compatible pDEST14 or pDEST15 (*i.e.* use of pDEST15 enabled expression as a fusion to glutathione *S*-transferase). Enzymatic products were extracted from liquid cultures (media and cells), typically 50-ml volumes grown for 72 h at 16 °C after induction, with an equal volume of hexane and analyzed by GC-MS. In every case, the expected diterpene olefin product (*i.e.* given the co-expressed diterpene synthases) was observed, with hydroxylated diterpenoids detected as described above (Fig. 4).

Diterpenoid Production—To obtain novel enzymatic products in sufficient amounts for NMR analysis, flux was increased into isoprenoid metabolism and the cultures were also simply scaled up. To increase flux, the cultures were further transformed with pMBI vector encoding the bottom half of the mevalonate-dependent isoprenoid precursor pathway (25), which is co-compatible with those mentioned above, and 20 mM mevalonolactone fed to these recombinant cultures, as previously described (26). The resulting hydroxylated diterpenoids were extracted and purified as previously described (9). Briefly, these compounds were produced in 3 $\times$ 1-liter cultures, which were extracted with an equal volume of a 1:1 mixture of ethyl acetate and hexanes, and the organic extract was then dried by rotary evaporation. The resulting residue was dissolved in 5 ml of 45% methanol, 45% acetonitrile, 10% dH₂O, and the hydroxylated diterpenoids were purified by HPLC. This was carried out using an Agilent 1100 series instrument equipped with autosampler, fraction collector, and diode array UV detection, over a ZORBAX Eclipse XDB-C8 column (4.6 $\times$ 150 mm, 5 μ m) at a 0.5 ml/min flow rate. The column was pre-equilibrated with 20% acetonitrile/dH₂O, sample loaded, then the column was washed with 20% acetonitrile/dH₂O (0–2 min), and eluted with 20–100% acetonitrile (2–7 min), followed by a 100% acetonitrile wash (7–27 min). Following purification, each compound was dried under a gentle stream of N₂, and then dissolved in 0.5 ml of deuterated chloroform (CDCl₃; Sigma), with this evaporation-resuspension process repeated two more times to completely remove the protonated acetonitrile solvent,

resulting in a final estimated ~5–10 mg of each novel diterpenoid.

Chemical Structure Identification—NMR spectra for the diterpenoids were recorded at 25 °C on a Bruker Avance 500 spectrometer equipped with a cryogenic probe for ^1H and ^{13}C . Structural analysis was performed using one-dimensional ^1H , and two-dimensional DQF-COSY, HSQC, HMQC, HMBC, and NOESY experiment spectra acquired at 500 MHz, and one-dimensional ^{13}C and DEPT135 spectra (125.5 MHz) using standard experiments from the Bruker TopSpin version 1.3 software. All compounds were dissolved in 0.5-ml of chloroform- d and placed in NMR tubes for analyses, and chemical shifts were referenced using known chloroform- d (^{13}C 77.23, ^1H 7.24 ppm) signals offset from TMS. Correlations from the HMBC spectra were used to propose a partial structure, whereas connections between protonated carbons were obtained from DQF-COSY data to complete the partial structure and assign proton chemical shifts, which was particularly important for assigning the alcohol carbon to the C6 *versus* C7 position. The configuration of the A and B rings is predetermined by the configuration of the CPP intermediate, because the ring structure was not altered. Thus, nuclear Overhauser effect (NOE) signals between the C5 proton and the alcohol methine proton could be used to assign the α or β configuration of the C7 hydroxylation products. For the C6-hydroxylated products, all derived from *syn*-CPP, the β configuration of the hydroxyl group was based on the lack of NOE signals between the C6 methine proton and the C5 and/or C9 methine protons typically observed and the C20 chemical shift observed for *ent*-sandacopimaradiene and *ent*-pimaradiene (0.695 and 0.754 ppm). If the C6 hydroxyl group was in the α configuration the C20 methyl ^1H signal would be at least 1.0 ppm, as observed for the *ent*-kaurene and *ent*-isokaurene CYP76M6 and -8 produced alcohols due to slight de-shielding caused by the hydroxyl group.

Kinetic Analysis—Kinetic analysis of CYP76M6 and -8 was carried out much as previously described (15). Briefly, these were co-expressed with OsCPR1, using the constructs described above, in the C41 Overexpress strain of *E. coli*. These recombinant cultures were complemented with the addition of 1 mM thiamine, 5 mg/liter of riboflavin, and 75 mg/liter of δ -amino levulinic acid at the time of induction. *In vitro* assays were carried out with microsomal preparations from these cultures, with quantification of CYP by reduced CO-binding difference spectra using an extinction coefficient of $91\text{ mM}^{-1}\text{ cm}^{-1}$ (27). Kinetic assays were performed in 1-ml assays, with CYP76M6 (55 pmol), CYP76M7 (165 pmol), or CYP76M8 (220 pmol), and the relevant substrates added to final concentrations ranging from 0.5 to 240 μM , but otherwise carried out as previously described (9). After 30 min, 50 μl of 1 M HCl was added to stop the reaction, enzymatic products were extracted by ethyl acetate, confirmed by GC-MS, and quantified by GC with flame ionization detection, using an external standard curve of *ent*-kauren-19-ol.

RNAi-mediated Double Knockdown of CYP76M7 and -8—A CYP76M8-specific RNAi plasmid was constructed using a pANDA Gateway destination vector. The 618-bp trigger region, consisting of 123 bp from the 3'-UTR and 495 bp from

the 3'-ORF, was amplified by PCR and cloned into pENTR/SD/D-TOPO as described above. This was then transferred to pANDA via directional recombination, and the resulting pANDA-CYP76M8-RNAi vector was transformed, using a previously described *Agrobacterium*-mediated protocol (28), into rice (var. *Nipponbare*) cells. Double knockdown of CYP76M7 and -8 was confirmed by RT-PCR. For each sample, 1 μg of total RNA was used for reverse transcription (RT) to synthesize first strand cDNA using the PrimeScript RT reagent kit with gDNA Eraser (TAKARA, Shiga, Japan). cDNA samples diluted 10-fold were subjected to quantitative RT-PCR (CYP76M5–8 and CYP76M14) using SYBR Green technology on an ABI PRISM 7300 Real-time PCR System (Applied Biosystems). To calculate the mRNA level of genes, the copy numbers of mRNAs were determined by generating a standard curve using a series of known concentrations of the target sequence. The results were expressed as relative mRNA values normalized to the expression level of *UBQ* (ubiquitin fused to ribosomal protein L40, D12629) as the internal control. The primers used are listed in supplemental Table S8.

To analyze diterpenoid phytoalexin levels, media samples (0.5 ml) from rice cell cultures that had been treated with 1 ppm chitin elicitor or 2% cholic acid were extracted three times with ethyl acetate (0.5 ml each extraction). The combined ethyl acetate extracts were evaporated to dryness. The residues from each sample were dissolved in 1 ml of 79% ethanol, 7% acetonitrile, and 0.01% acetic acid. Phytocassane and momilactone levels were analyzed by HPLC-ESI-MS/MS, using extracts from chitin-elicited cultures (5 μl injections), as previously described (29).

RESULTS

Molecular Phylogenetic Relationships Among Rice CYP76M Subfamily Members—In our previous work (9), we investigated broader CYP71 clan relationships using phylogenetic analysis based on amino acid sequence alignments. However, this did not clearly resolve the higher level grouping within the rice CYP76M subfamily, *i.e.* these branch points had rather low bootstrap values; <410 of 1000 repeats. Given the focus here on the CYP76M subfamily, we aligned the relevant nucleotide sequences to more precisely evaluate the underlying phylogenetic relationships of these closely related enzymatic genes (Fig. 3). This analysis indicates an early divergence in the CYP76M subfamily, with CYP76M5–8 falling into a distinct clade (albeit one that also includes CYP76M14), whereas the remainder of the rice CYP76M subfamily members form a separate group. Transcription of CYP76M5–8, and not the other subfamily members, is elicited by induction of rice cell cultures with the fungal cell wall component chitin oligosaccharide (30). Such differential induction of CYP76M14, along with its deeply branched position, brings into question its true phylogenetic relationship with CYP76M5–8. In any case, along with their physical co-clustering, CYP76M5–8 appear to be most closely related to each other. Moreover, at least the closely related and physically adjacent CYP76M7 and -8 subfamily members seem to have arisen from a tandem gene duplication event that occurred after initial incorporation of CYP76M subfamily

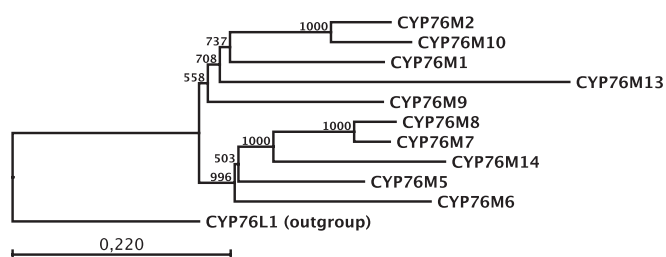


FIGURE 3. Molecular phylogenetic analysis of the rice CYP76M subfamily (based on alignment of mRNA open reading frames, with CYP76L1 as the outgroup sequence). Note that this differs from the previously reported amino acid-based phylogeny (9), in indicating an early divergence in the CYP76M subfamily, and more clearly demonstrating that the CYP76M5–8 found in the rice chromosome 2 diterpenoid biosynthetic gene cluster fall into a distinct clade (albeit one that also includes CYP76M14).

member(s) into the chromosome 2 diterpenoid biosynthetic gene cluster.

Interestingly, whereas many of the other CYP76M subfamily members are scattered across the genome, CYP76M2 and -10 are found close together, along with two other CYP76M subfamily pseudogenes (CYP76M4p and -11p), on chromosome 8. However, given the phylogenetic distance, as well as differences in relative orientation and intervening diterpene synthases in the case of the chromosome 2 cluster (Fig. 2), these clusters of CYP76M subfamily members appear to have separately undergone gene expansion.

Initial Functional Characterization of CYP76M5, -6, and -8—Our previous work demonstrated the ability of CYP76M7 to catalyze C11 α -hydroxylation of *ent*-cassadiene, presumably en route to the C11-keto functionality found in all the bioactive phytocassanes (9). However, the other co-clustered CYP76M subfamily members are similarly up-regulated during the rice plant defense response (30) (supplemental Fig. S1), suggesting that these also might play a role in rice diterpenoid phytoalexin biosynthesis, e.g. by analogous hydroxylation of diterpene precursors or later intermediates. This was initially examined by heterologous expression of these three CYP76M subfamily members (i.e. CYP76M5, -6, and -8) in Sf21 insect cell cultures, which were fed other *ent*-CPP-derived diterpenes found in rice. In particular, because of the presence of the relevant OsCPS2 in this chromosome 2 biosynthetic gene cluster, we hypothesized that these co-clustered CYP might react with other such *ent*-CPP-derived diterpenes. Accordingly, we assessed their ability to modify *ent*-sandaracopimaradiene and *ent*-pimaradiene, as well as *ent*-cassadiene. The only CYP that exhibited activity upon such heterologous expression was CYP76M6, which was able to hydroxylate *ent*-sandaracopimaradiene, based on GC-MS analysis detection of a resulting diterpenoid alcohol (molecular mass = 288 Da). However, whereas the corresponding proteins were expressed for CYP76M5 and -8, it was not determined if these CYP were functionally folded, as the CO difference binding spectra were not measured with these preparations.

For further characterization we turned to the modular metabolic engineering system we have previously developed (24). To functionally express plant CYP in bacteria we have found it generally useful to replace their N-terminal membrane anchor sequence with a solubilizing leader peptide (9, 15, 31). Hence,

CYP76M6 was N-terminal modified, as previously described for CYP76M7 (9). Co-expression of this recombinant CYP76M6 construct with the requisite NADPH-cytochrome P450 reductase (CPR) redox partner from rice (OsCPR1) and the relevant diterpene synthases (i.e. OsCPS2 and OsKSL10) led to production of the same hydroxylated *ent*-sandaracopimaradiene observed in the insect cell culture expression experiment described above (supplemental Fig. S2). To determine whether CYP76M6 will react with other endogenously found diterpenes, it was co-expressed with OsCPR1 and all other functional combinations of rice diterpene synthases (see Fig. 1), and we found that it will also hydroxylate *syn*-stemodene (supplemental Fig. S3).

Encouraged by this result, we similarly modified the other two co-clustered CYP76M subfamily members (i.e. CYP76M5 and -8), and co-expressed each of these with OsCPR1 and all functional pairings of rice diterpene synthases to test their ability to elaborate the labdane-related diterpenes found in rice. Although no activity was detected with the modified CYP76M5, the modified CYP76M8 was found to hydroxylate a wide range of diterpenes (i.e. *syn*-pimaradiene, *ent*-pimaradiene, *ent*-sandaracopimaradiene, *ent*-isokaurene, *ent*-kaurene, and *ent*-cassadiene; supplemental Figs. S2 and S4–S8). With *ent*-cassadiene, CYP76M8 catalyzes the same C11 α -hydroxylation as the previously characterized CYP76M7 (supplemental Fig. S8), whereas with *ent*-sandaracopimaradiene CYP76M8 produces the same hydroxylated diterpenoid alcohol as CYP76M6 (supplemental Fig. S2). However, it also should be noted that CYP76M8 does not react with all the rice diterpenes (i.e. not *syn*-stemodene, *syn*-stemarene, or *syn*-labdatriene).

As our results with CYP99A3 demonstrated that complete gene recoding to optimize codon usage for expression in *E. coli* can lead to activity when none was observed with the native gene sequence (15), we had such a construct synthesized for CYP76M5 (see supplemental data for sequence). This synthetic gene also was N-terminal modified as described above. When this CYP76M5 construct was co-expressed with OsCPR1 and all of the functional pairings of rice diterpene synthases, it was found to hydroxylate *ent*-sandaracopimaradiene, yielding the same product as CYP76M6 and -8, albeit in much smaller quantities (supplemental Fig. S2).

Given the potential phylogenetic relationship of CYP76M14 with CYP76M5–8 (Fig. 3), we applied this same synthetic gene approach to investigate the ability of CYP76M14 to react with rice diterpenes (see supplemental data for sequence). However, this CYP76M14 construct did not exhibit any such activity in the context of our metabolic engineering system. This approach was further applied to all the other rice CYP76M subfamily members with known full-length mRNA sequences (i.e. CYP76M2, -9, -10, and -13; see supplemental data for the corresponding synthetic gene sequences). However, only CYP76M2 and -10 exhibited any activity, and only with *ent*-kaurene, producing relatively small amounts of *ent*-kauren-19-ol.

We have recently reported that CYP99A3 will react with *syn*-pimaradiene and *syn*-stemodene to convert their C19 methyl group to a carboxylic acid (15). The analogous ability of CYP76M8 to hydroxylate *syn*-pimaradiene and CYP76M6 to

hydroxylate *syn*-stemodene suggests the possibility that CYP99A3 and these CYP76M subfamily members might act sequentially in rice diterpenoid metabolism. Accordingly, we tried feeding the CYP99A3 products *syn*-pimaradien-19-oic acid to CYP76M8 and *syn*-stemoden-19-oic acid to CYP76M6, as well as the CYP76M8 hydroxylated *syn*-pimaradiene and CYP76M6 hydroxylated *syn*-stemodene to CYP99A3. However, in none of these assays was any further elaborated product detected.

In our original report of CYP76M7 activity we found that this monooxygenase will infrequently produce small amounts of the further oxidized C11-keto group found in the phytocassanes (9). To determine whether CYP76M7 and -8 might act consecutively to produce C11-keto-*ent*-cassadiene, we tried feeding C11 α -hydroxy-*ent*-cassadiene to both. However, neither was found to catalyze significant further oxidation (*i.e.* to produce C11-keto-*ent*-cassadiene).

We also have recently found that OsKSL4 and OsKSL11 will react with normal (9S,10S) CPP to produce pimaradiene and sandaracopimaradiene, respectively (32). Although not found in rice, this CPP stereoisomer is produced by wheat,³ suggesting that these diterpenes might be relevant to the evolution of diterpenoid metabolism in the cereal crop plant family more broadly. Given the broad substrate range exhibited by CYP76M8, we investigated the possibility that it would react with these alternative substrates and found that it will hydroxylate both (*e.g.* that of sandaracopimaradiene is shown in supplemental Fig. S9).

Identification of Hydroxylated Products—To enable production of sufficient amounts of the various hydroxylated diterpenoid products of CYP76M5–8 for structural analysis by nuclear magnetic resonance (NMR), we increased flux into terpenoid metabolism in our *E. coli*-based metabolic engineering system. Specifically, we supplemented the endogenous methyl erythritol phosphate isoprenoid precursor supply pathway with the mevalonate-dependent pathway, which significantly increases yield, as previously described (26). This enabled production of multi-milligram amounts from reasonable quantities of these recombinant cultures (~3 liters), with the resulting diterpenoids extracted and then purified by HPLC. From the subsequent NMR analysis (supplemental Figs. S10–S13 and Tables S1–S7), it was possible to assign the position of the resulting hydroxyl group in each of the observed products. CYP76M6 catalyzes C6 β -hydroxylation of *syn*-stemodene and C7 β -hydroxylation of *ent*-sandaracopimaradiene, with the later activity also exhibited by CYP76M8 as noted above. CYP76M8 further catalyzes C7 β -hydroxylation of *ent*-pimaradiene, along with C6 β -hydroxylation of *syn*-pimaradiene, but C7 α -hydroxylation of *ent*-isokaurene, and *ent*-kaurene, as well as C9 α -hydroxylation of sandaracopimaradiene (the pimaradiene product was not structurally characterized, but presumably also occurs at the C9 α position). As noted above, CYP76M8 further catalyzes the same C11 α -hydroxylation of *ent*-cassadiene as CYP76M7, whereas CYP76M5 catalyzes the same C7 β -hydroxylation of *ent*-sandaracopimaradiene as CYP76M6 and -8 (Fig. 4).

Enzymatic Characterization of Recombinant CYP76M6 and -8—To further investigate the enzymatic properties of CYP76M5–8, *in vitro* assays were carried out, although these were only found to be possible with the robust activity exhibited by the modified CYP76M6 and -8. Specifically, when these CYP were co-expressed with OsCPR1, clarified lysates of the resulting recombinant bacterial culture were able to efficiently catalyze the hydroxylation reactions identified above. In addition, it was further possible to measure CO difference binding spectra with these preparations (supplemental Fig. S14). However, it must be noted that these spectra were inconsistent, with some preparations exhibiting activity despite the absence of the characteristic peak at 450 nm. Thus, the catalytic rates reported here from steady-state kinetic analysis (Tables 1 and 2), must be viewed with some caution, and it is not advisable to compare k_{cat} between the various CYP. Although it is possible to compare the ability of these CYP to react with their various substrates, as these assays were run in parallel, both CYP76M6 and -8 exhibited similar catalytic efficiency with all of their substrates. Thus, this does not distinguish between the various observed reactions. Notably, when compared with CYP76M7 (9), the multifunctional CYP76M8 exhibited significantly higher affinity for their common substrate *ent*-cassadiene ($K_m = 4$ versus 40 μM). On the other hand, when compared with CYP99A3 (15), CYP76M8 exhibited equivalent affinity for their common *syn*-pimaradiene substrate ($K_m = 2$ μM for both), as does CYP76M6 for the *syn*-stemodene substrate it shares in common with CYP99A3 ($K_m = \sim 8$ μM for both).

Role for CYP76M7 and -8 in Phytocassane Biosynthesis—The reactions catalyzed by CYP76M5–8 are suggestive of potential roles in at least three rice diterpenoid phytoalexin biosynthetic pathways (Fig. 5). Specifically, the CYP76M7 and -8 catalyzed C11 α -hydroxylation of *ent*-cassadiene presumably leads to the C11-keto group found in all the phytocassanes, and the C7 β -hydroxylation of *ent*-sandaracopimaradiene catalyzed by CYP76M5, -6, and -8 seems likely to be involved in the production of oryzalexins A–D, all of which have a C7 β -hydroxyl or C7-keto group. In addition, the CYP76M8-catalyzed C6 β -hydroxylation of *syn*-pimaradiene might play a role in formation of the characteristic 19,6 β -lactone ring in the momilactones, although it does not appear to do so, at least *in vitro* (*e.g.* CYP76M8 does not react with *syn*-pimaradien-19-oic acid). To further investigate the role of CYP76M7 and -8 in the inducible production of rice diterpenoid phytoalexins, RNAi-mediated knockdown of these was attempted, much as previously described for CYP99A2 and -3 (8). In particular, a vector with a trigger region of 618 bp, consisting of 123 bp from the 3'-UTR and 495 bp from the 3'-end of the open reading frame of CYP76M8 was constructed and used to transform rice cell cultures.

It has long been appreciated that rice cell cultures can be induced to produce diterpenoid phytoalexins by elicitation with chitin oligosaccharide (34), which acts at least in part by increasing mRNA levels of the relevant enzymatic genes (10, 11). Thus, such induction was applied to the generated RNAi cell culture lines, with comparison to a parental wild-type (WT) culture, to determine which exhibited reduced accumulation of CYP76M mRNA. Three lines were found that exhibited

³ Y. Wu, K. Zhou, T. Toyomasu, C. Sugawara, M. Oku, M. Usui, W. Mitsuhashi, P. M. Chandler, and R. J. Peters, submitted for publication.

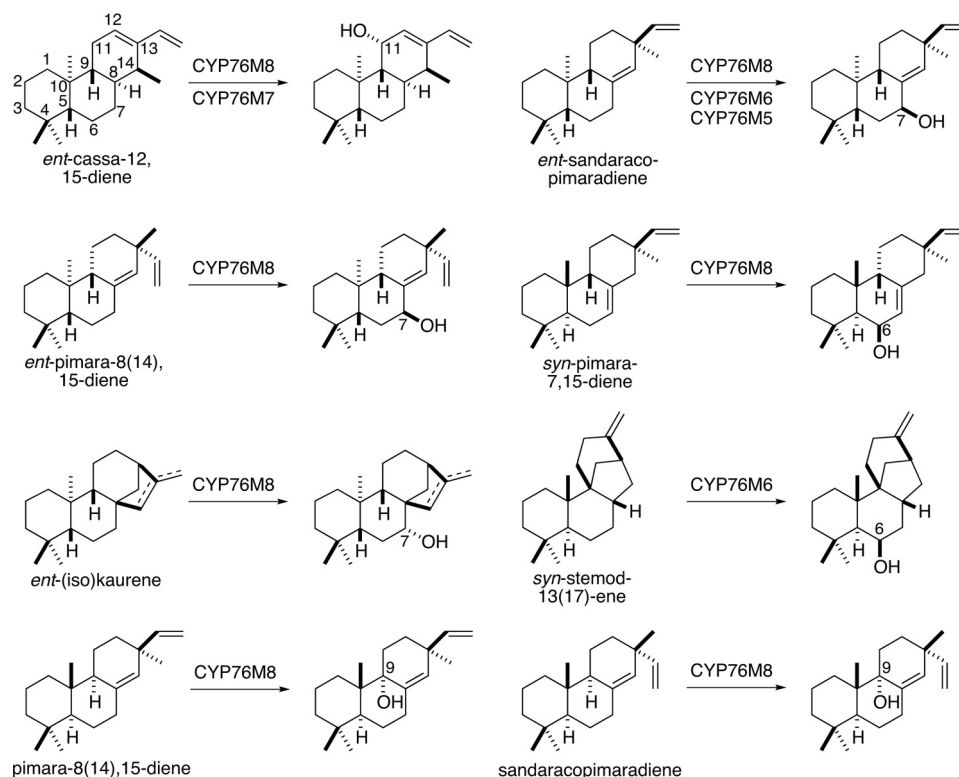


FIGURE 4. Hydroxylation reactions catalyzed by CYP76M5–8 (*ent*-kaurene and *ent*-isokaurene differ only in double bond placement, *exo*- versus *endo*-cyclic, respectively, see also Fig. 1). Numbering of ring carbons is explicitly shown for *ent*-cassadiene, and for each introduced hydroxyl group.

TABLE 1

CYP76M6 steady-state kinetic constants

Substrate	k_{cat}	K_m	k_{cat}/K_m
	s^{-1}	μM	
<i>ent</i> -Sandaracopimaradiene	0.046 ± 0.005	17 ± 8	2.7×10^3
<i>syn</i> -Stemodene	0.020 ± 0.001	7 ± 1	2.9×10^3

TABLE 2

CYP76M8 steady-state kinetic constants

Substrate	k_{cat}	K_m	k_{cat}/K_m
	s^{-1}	μM	
<i>ent</i> -Pimaradiene	0.009 ± 0.001	4 ± 2	2.3×10^3
<i>ent</i> -Isokaurene	0.015 ± 0.001	15 ± 4	1.0×10^3
<i>ent</i> -Cassadiene	0.006 ± 0.001	4 ± 2	1.5×10^3
<i>ent</i> -Sandaracopimaradiene	0.011 ± 0.001	7 ± 3	1.6×10^3
<i>ent</i> -Kaurene	0.009 ± 0.001	5 ± 2	1.8×10^3
<i>syn</i> -Pimaradiene	0.006 ± 0.001	2 ± 1	2.6×10^3

reduced levels of CYP76M7 and -8, although not the more distantly related CYP76M5, -6, or -14 (Fig. 6A). The accumulation of momilactones and phytocassanes in response to elicitation with chitin oligosaccharide was then measured by LC-MS/MS for these lines, again with comparison to WT, demonstrating reduction in induced accumulation of the phytocassanes, although a consistent change in the momilactone level was not observed (Fig. 6B). Unfortunately, oryzalexins A–E were not detected in these cultures, and even induction with the powerful cholic acid elicitor (35) did not lead to quantifiable levels of the oryzalexins.

DISCUSSION

Our results provide some insight into the assembly and continued evolution of plant biosynthetic gene clusters, with impli-

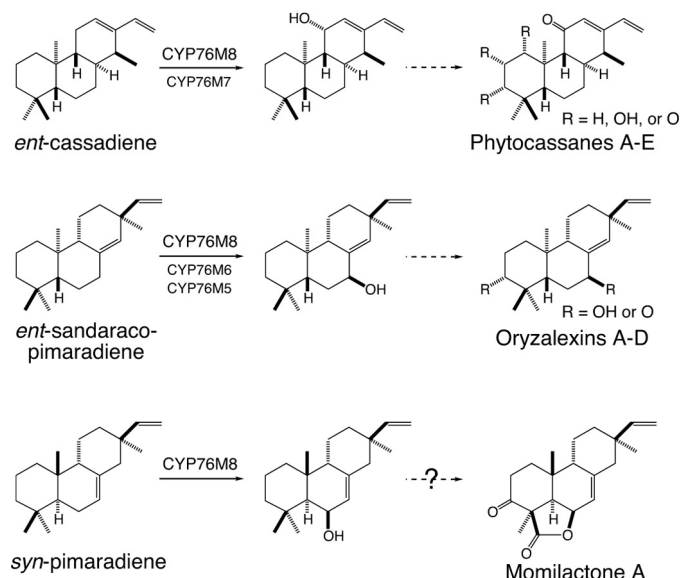


FIGURE 5. The putative relationship of the reactions characterized here to rice diterpenoid phytoalexins biosynthesis. The dashed arrow indicates the multiple biosynthetic steps leading to the indicated diterpenoid phytoalexins (?), unknown relevance of 6 β -hydroxy-*syn*-pimaradiene to momilactone biosynthesis, as discussed in the text).

cations for the evolution of plant secondary metabolism more broadly. In particular, whereas the exact role(s) of the observed reactions catalyzed by CYP76M5 and -6 to rice diterpenoid metabolism is uncertain, our results with CYP76M7 and -8 are more informative. Critically, it seems clear that at least CYP76M7 and -8 arose from a tandem gene duplication event that occurred after incorporation of an ancestral CYP76M sub-

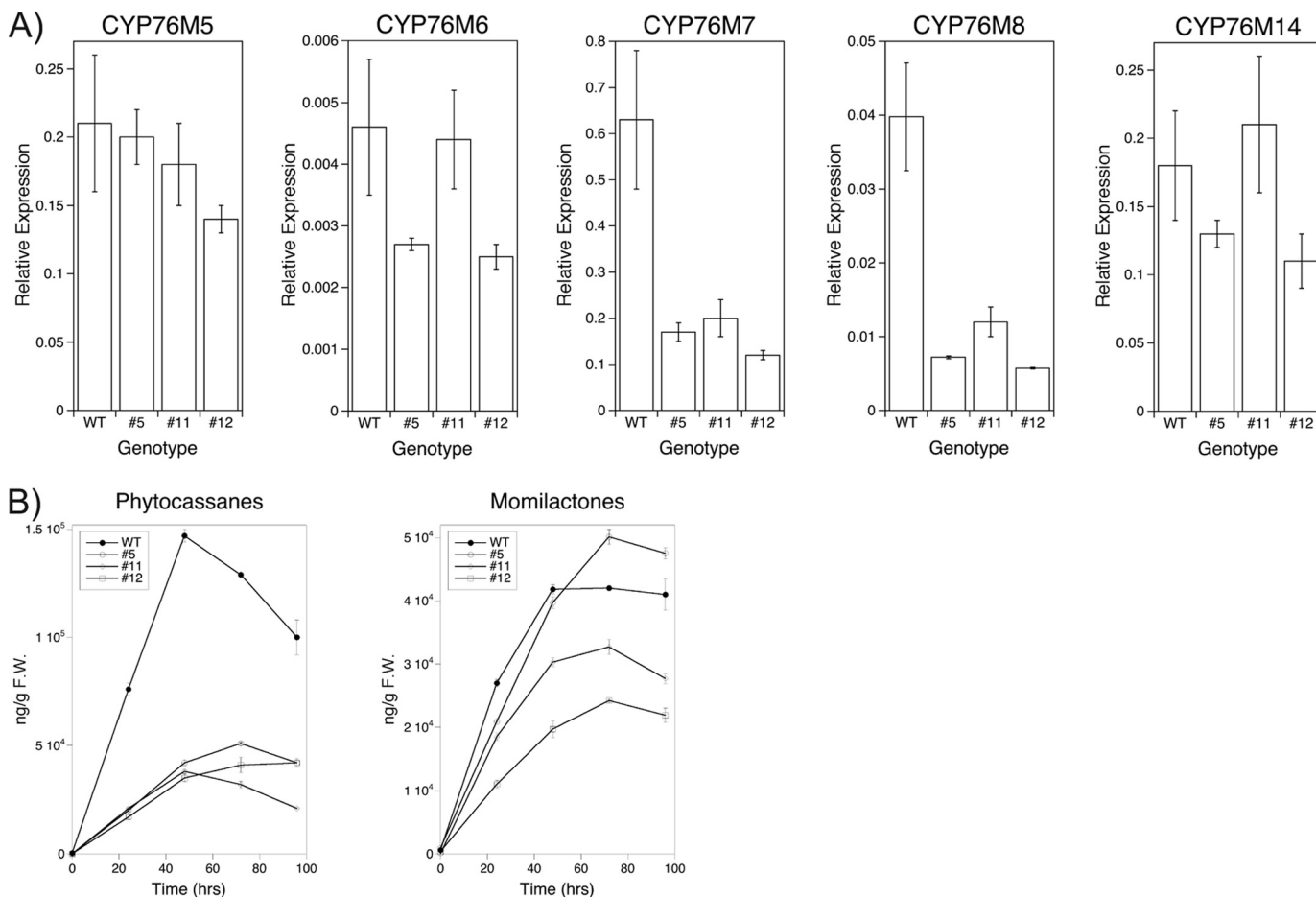


FIGURE 6. **Relative response of CYP76M7 and -8 RNAi double knockdown lines to elicitation with chitin oligosaccharide.** A, mRNA levels 8 h after elicitation (relative to ubiquitin). B, phytoalexin levels following elicitation with chitin oligosaccharide. Shown are levels in wild-type (WT) and three RNAi lines (#5, #11, and #12).

family member(s) into the chromosome 2 diterpenoid biosynthetic gene cluster (Figs. 2 and 3). Thus, our experimental results provide insight into the biochemical and resulting metabolic underpinnings of both the assembly and continued evolution of this unusual genomic organization.

Notably, only CYP76M5–8 subfamily members from the gene cluster seem to efficiently catalyze diterpene hydroxylation. This emphasizes such activity as a rationale for incorporation of these particular CYP into the biosynthetic gene cluster, which is consistent with previous reports of other plant terpenoid biosynthetic gene clusters, all of which contain early acting CYP (5, 15, 36). The selective incorporation of such CYP is substantiated here by our comprehensive investigation of the entire genomic complement of the relevant CYP76M subfamily, and is consistent with the previously observed selective induction of CYP76M5–8 transcription relative to all the other subfamily members from rice (30). However, it should be noted that such catalysis of early hydroxylation as a rationale for the initial incorporation of CYP into the biosynthetic gene cluster does not rule out later evolution of a role for these enzymes in catalyzing downstream transformations. Nevertheless, following initial assembly of the chromosome 2 biosynthetic gene cluster, the subsequent gene duplication that gave rise to CYP76M7 and -8 seems to have enabled variation on such orig-

inally catalyzed diterpene hydroxylation rather than evolution of novel downstream function(s). Hence, the spread of this gene duplication was presumably driven by an increase in the range of diterpenes that can be hydroxylated (*e.g.* the promiscuous activity of CYP76M8). This further suggests that such a role in early biosynthesis has been retained, at least during this aspect of CYP76M subfamily evolution within this gene cluster.

The rice chromosome 2 diterpenoid biosynthetic gene cluster in which CYP76M5–8 are found is unusual in several respects. Biosynthetic gene clusters are typically associated with a particular metabolic pathway leading to a specific natural product or small family of closely related compounds. By extension, it is assumed that the enzymatic genes within these clusters are dedicated to the corresponding biosynthetic pathway (1, 37). However, that is not true here. This gene cluster already has been associated with at least two metabolic pathways, *i.e.* it contains the diterpene synthases leading to both the antifungal phytocassane and the antibacterial oryzalide families of labdane-related diterpenoid phytoalexins. Intriguingly, the activity of the co-clustered CYP76M subfamily members reported here, whereas applicable to phytocassane biosynthesis (Fig. 6), does not appear to be applicable to that of the oryzalides, as these are not modified at C7 (*i.e.* as would be expected from the C7 α hydroxylation of *ent*-(iso)kaurene mediated by

CYP76M8). Instead, the results presented here suggest that some of these CYP may function in other pathways, such as that leading to the oryzalexins (Fig. 5), wherein at least one of the requisite upstream diterpene synthases is located elsewhere in the rice genome.

Furthermore, whereas most of the diterpene substrates hydroxylated by CYP76M5–8 are derived from the *ent*-CPP produced by the co-clustered OsCPS2, these also react with *syn*-CPP-derived diterpenes, for which neither of the relevant diterpene synthases are found in this chromosome 2 cluster. Accordingly, the CYP76M5–7 subfamily members found in this cluster may have roles in such pathways, which would further increase the range of rice diterpenoid phytoalexin metabolism covered by this biosynthetic gene cluster. Consistent with a role for CYP76M5–8 in such phytoalexin biosynthesis is the transcriptional induction of the encoding genes in response to elicitation with either chitin oligosaccharide (30) or methyl jasmonate (supplemental Fig. S1), much as previously reported for the upstream diterpene synthases (e.g. Ref. 30).

The implications of these deviations of the rice chromosome 2 diterpenoid biosynthetic gene cluster from the accepted norm provide some insight into the role of biosynthetic gene clusters in plant metabolic evolution. In particular, because the enzymatic genes in the known plant biosynthetic clusters are involved in more specialized/secondary metabolism (i.e. the end product is not essential for normal plant growth and development), selective pressure for their retention requires inheritance of a complete metabolic pathway (i.e. for production of a natural product whose bioactivity is advantageous). On the other hand, the production of intermediates with detrimental bioactivity is counter-selective. This then provides dual “push-pull” forces for biosynthetic gene clustering (38). Critically, as long as some function in the cluster-encoded pathways is present, this does not rule out the incorporation and/or divergent evolution, along with retention, of multifunctional enzymes with role(s) in other pathways, at least in plant biosynthetic gene clusters. By contrast, horizontal gene/operon transfer might limit the inclusion of enzymes with broad substrate range in microbial operons, as these would impose potentially deleterious effects on the metabolism of novel host organisms. But in plants, the lack of horizontal gene transfer provides an advantage for the incorporation and retention of enzymes with broad substrate range, as such multifunctionality seems to make retention of the encoding gene more likely (39).

More specifically, the presence of enzymes with broad substrate range may enable facile metabolic evolution. Accordingly, the presence of a CYP with broad diterpene substrate range provides latent metabolic plasticity that enables immediate elaboration of any such novel olefins (and potentially even downstream/elaborated diterpenoids) that might arise through evolutionary change. Strikingly, there seems to be an example of just such an event within the rice chromosome 2 gene cluster. OsKSL5 and -6 are derived from a recent tandem gene duplication event, wherein the ancestral gene presumably produced the *ent*-isokaurene precursor of the oryzalides. This function is retained by OsKSL6, whereas OsKSL5 has undergone neofunctionalization, at least in the japonica subspecies of rice, and now produces *ent*-pimaradiene (33). The presence of the mul-

tifunctional CYP76M8 then leads to ready hydroxylation of this new (at least to rice) diterpene. Furthermore, our investigation of the substrate range of the OsKSL family demonstrates a parallel capacity for facile evolution of the production of other novel diterpenes derived from a non-native normal stereoisomer of CPP (32), and these also are readily reacted upon by CYP76M8 (e.g. sandaracopimaradiene).

The incorporation of oxygen mediated by CYP imparts important functionality, as it is oxygenated diterpenoids that are almost invariably associated with biological activity. Notably, these do not have to be extensively modified (e.g. the bioactive oryzalexins D–F are diterpenoid diols only two hydroxylation reactions removed from their diterpene precursor). Thus, by tapping the latent CYP metabolic plasticity demonstrated here, rice has the capacity to readily evolve novel diterpenoid metabolism. In particular, the appearance of any novel diterpene would then immediately lead to production of a more elaborated, potentially bioactive diterpenoid(s). Accordingly, our investigation of rice diterpenoid natural products metabolism provides important insight into the interplay between biosynthetic gene clusters in plants and continuing metabolic evolution. Specifically, we hypothesize that the multifunctional/promiscuous nature of CYP76M8 provided the evolutionary advantage that drove the selective sweep of the CYP76M7 and -8 gene duplication event through the rice plant species. In addition, we speculate that the latent metabolic plasticity demonstrated here provides potential additional, albeit only intermittently applicable (i.e. at the population level), positive selection pressure.

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Plant Biology:

**Characterization of CYP76M5–8 Indicates
Metabolic Plasticity within a Plant
Biosynthetic Gene Cluster**

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