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Parsing a multifunctional biosynthetic gene cluster from rice: Biochemical characterization of CYP71Z6 & 7

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Abstract

Rice (*Oryza sativa*) contains a biosynthetic gene cluster associated with production of at least two groups of diterpenoid phytoalexins, the antifungal phytocassanes and antibacterial oryzalides. While cytochromes P450 (CYP) from this cluster are known to be involved in phytocassane production, such mono-oxygenase activity relevant to oryzalide biosynthesis was unknown. Here we report biochemical characterization demonstrating that CYP71Z6 from this cluster acts as an *ent*-isokaurene C2-hydroxylase that is presumably involved in the biosynthesis of oryzalides. Our results further suggest that the closely related and co-clustered CYP71Z7 likely acts as a C2-hydroxylase involved in a latter step of phytocassane biosynthesis. Thus, CYP71Z6 & 7 appear to have evolved distinct roles in rice diterpenoid metabolism, offering insight into plant biosynthetic gene cluster evolution.

Keywords

Cytochromes P450; terpenoid metabolism; genome organization; phytoalexin biosynthesis

1. Introduction

When attacked by microbial pathogens, plants produce antibiotic natural products in response – i.e., phytoalexins, which in rice largely consist of labdane-related diterpenoids [1,2]. Biosynthesis of these compounds is distinguished by the use of a pair of sequential cyclization reactions [3]. Most characteristic is the initial bicyclization of the general diterpenoid precursor (*E*,*E*,*E*)-geranylgeranyl diphosphate (GGPP) by a class II diterpene cyclase. This most often results in production of the eponymous labadienyl/copalyl diphosphate (CPP), as catalyzed by CPP synthases (CPS). This is followed by further transformations catalyzed by a more typical class I diterpene synthase, often termed kaurene synthase like (KSL) for their resemblance to the presumably ancestral *ent*-kaurene synthases required for gibberellin phytohormone biosynthesis. In addition, the production of bioactive natural products almost invariably requires further elaboration; typically the incorporation of oxygen catalyzed by cytochromes P450 (CYP), with the introduced hydroxyl group(s) often further oxidized by short chain dehydrogenases (SDR).

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The presence of biosynthetic gene clusters is an emerging theme in plants [4], and rice is know to contain two such clusters involved in labdane-related diterpenoid production [5–7]. That located on chromosome 4 appears to be dedicated to momilactone biosynthesis, containing the relevant, sequentially acting *syn*-CPP synthase OsCPS4 and *syn*-pimaradiene synthase OsKSL4 [5]. In addition, this region contains two CYP (CYP99A2 & 3), one or both of which are required for momilactone biosynthesis, and an SDR that catalyzes the final step in production of momilactone A [6]. We also have recently demonstrated that CYP99A3 catalyzes conversion of *syn*-pimaradiene to *syn*-pimaradien-19-oic acid, presumably en route to the 19,6-olide moiety – i.e., the eponymous lactone ring [8].

The other biosynthetic gene cluster, located on chromosome 2, is quite different. In particular, unlike other such clusters, this region contains enzymatic genes associated with multiple biosynthetic pathways (Figure 1). This includes that leading to the oryzalides whose production is increased in response to infection with the bacterial leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae* [9], as well as the phytocassanes produced in response to the fungal blast pathogen *Magnaporthe oryzae* [1,2]. Specifically, this cluster contains OsCPS2, which produces the common *ent*-CPP precursor [10,11], as well as the subsequently acting OsKSL5-7, which produce *ent*-pimaradiene, *ent*-isokaurene, and *ent*-cassadiene, respectively [12–14]. OsKSL6 & 7 then catalyze the committed step in oryzalide and phytocassane biosynthesis, respectively. In addition, this region further contains six CYP – CYP76M5-8 and CYP71Z6 & 7.

We have previously shown that CYP76M7 acts as an *ent*-cassadiene C11α-hydroxylase [7], and further analysis of the rice CYP76M sub-family has not only verified a role for CYP76M7 & 8 in phytocassane biosynthesis, but suggested one for CYP76M5-8 in other diterpenoid pathways as well [15]. However, this does not include a role for any rice CYP76M sub-family member in oryzalide biosynthesis. This is consistent with previously reported investigation of transcriptional induction by the fungal cell wall component chitin oligosaccharide, which increases mRNA levels of *OsKSL7* and *CYP76M5-8*, as well as *CYP71Z7*, although not that of *CYP71Z6* or *OsKSL6* [16]. Here we report biochemical characterization of CYP71Z6 & 7, revealing that CYP71Z6 is an efficient *ent*-isokaurene C2-hydroxylase presumably involved in an early step in oryzalide biosynthesis. On the other hand, while CYP71Z7 will act as a C2-hydroxylase of *ent*-cassadiene, it does so quite inefficiently, and we hypothesize that it actually functions to catalyze a later step in phytocassane biosynthesis.

2. Materials and Methods

2.1 General

Unless otherwise noted, chemicals were purchased from Fisher Scientific (Loughborough, Leicestershire, UK), and molecular biology reagents from Invitrogen (Carlsbad, CA, USA). The gene mapping and nomenclature used here has been previously described [7,15]. Gas chromatography (GC) with a Varian (Palo Alto, CA) 3900 GC with Saturn 2100 ion trap mass spectrometer (MS) in electron ionization (70 eV) mode, was carried out as previously described [7,8,15].

2.2 Recombinant constructs

CYP71Z6 & 7 were obtained from the KOME rice cDNA databank (GenBank accessions AK107418 and AK070167, respectively), and completely recoded versions to optimize codon usage for *E. coli* expression synthesized (GenScript; see Supporting Data for sequence). These were cloned into pENTR/SD/D-TOPO, and derived N-terminally modified constructs made for each, with removal of the first 32 codons and replacement with ten new

codons (encoding the amino acid sequence "MAKKTSSKGK"), based on the modifications used for bacterial expression of the mammalian CYP2B sub-family [17], as previously described [7,8,15,18]. All full-length and N-terminally modified genes were then transferred into a previously described pCDF-Duet vector containing a DEST cassette and rice cytochrome P450 reductase (OsCPR1) in the first and second multiple cloning sites, respectively [7].

2.3 Recombinant expression and screening

All native and recoded CYP71Z6 & 7 and N-terminally modified variants were recombinantly expressed in the OverExpress C41 strain of *E. coli* (Lucigen, Middleton, WI). Taking advantage of our previously described modular diterpene metabolic engineering system [19], these were co-expressed not only with OsCPR1, but also a GGPP synthase and CPS carried on co-compatible pGGxC vectors, along with rice KSL expressed from the additionally co-compatible pDEST14 or pDEST15 (i.e., for expression as either alone or as a fusion to GST, respectively). This enabled facile assessment of their ability to react with the resulting diterpene olefins, using 50 mL expression cultures, as previously described [7,8,15].

2.4 Diterpenoid production

To obtain enough oxygenated product for NMR analysis, the functional combinations of gene expression vectors were co-transformed with the additionally compatible pMBI, which contains the "bottom half" of the mevalonate dependent isoprenoid precursor supply pathway from *Sacchromyces cerevisiae* [20]. This enabled increased flux into isoprenoid metabolism by feeding these cultures mevalonate, much as previously described [21]. The resulting cultures were grown and extracted as previously described [7,8,15]. The organic phase was separated, combined, and then dried by rotary evaporation. The resulting residue was resuspended in hexanes and fractionated over a 4 g-silica column by flash chromatography, using a Reveleris system (Grace, Deerfield, IL), with a hexane:acetone gradient. The hydroxylated diterpenoids eluted in 10% acetone, and these fractions were combined and dried under N_2. This material was resuspended in acetonitrile, and the hydroxylated diterpenoids purified by high performance liquid chromatography with an Agilent 1100 series instrument equipped with autosampler, fraction collector, diode array UV detection, and a ZORBAX Eclipse XDB-C8 column (4.6 × 150 mm, 5 μm), also as previously described [7,8,15].

2.5 NMR analysis

The purified diterpenoids were dried under N_2, then brought up in 0.5 mL deuterated chloroform ($CDCl_3$) and placed into NMR tubes (Wilmad LabGlass; Vineland, NJ) or microtubes (Shigemi; Allison Park, PA) for analysis. NMR spectra for the diterpenoids were recorded at 25 °C on either a Bruker Avance 500 or 700 (CYP71Z7 product) spectrometer equipped with a 5-mm HCN cryogenic probe for ^{1}H and ^{13}C. Chemical shifts were referenced using known chloroform (^{13}C 77.23, ^{1}H 7.24 ppm) signals offset from TMS (Tables S1&2). Structural analysis was performed using 1D ^{1}H, 2D DQF-COSY, HSQC, HMQC, HMBC, and NOESY experiment spectra acquired at 500 or 700.13 MHz, and 1D ^{13}C and DEPT135 spectra (125.5 MHz or 174 MHz) using standard experiments from the Bruker TopSpin v1.3 or 1.4 software, respectively. Correlations from the HMBC spectra were used to propose a partial structure, while resonance signals between protonated carbons were obtained from DQF-COSY data to complete the partial structure and assign proton chemical shifts. The structure was further verified using HSQC, and DEPT135 spectra to confirm assignments.

2.6 Kinetic analysis

Kinetic analysis was carried out with the functional synthetic and N-terminally modified CYP71Z6 & 7 constructs, co-expressed with OsCPR1, much as previously described [7,8,15,18]. The amount of active CYP in the resulting clarified lysates was measured by reduced CO difference spectrum, using the standard extinction coefficient of (ΔA_{450}–ΔA_{490})/0.091 (nmol of CYP per ml) [22]. Enzymatic assays were carried out with 150 nM CYP, much as previously described [7,8,15,18], but using a substrate range of 1–50 μM *ent*-isokaurene and 1–200 μM for *ent*-cassadiene. The reactions also were supplemented with an NADPH regeneration system consisting of 1 mM glucose-6-phosphate and 2 μg/mL yeast glucose-t-phosphate dehydrogenase (Roche), along with 0.4 mM NADPH, 5 μM FAD, 5 μM FMN, and 1 mM DTT.

3. Results

3.1. Tandem gene duplication origin for CYP71Z6 & 7

Our success in functional characterization of the role of CYP76M5-8 in rice diterpenoid phytoalexin production promoted us to investigate the remaining two CYP within the rice chromosome 2 biosynthetic gene cluster, CYP71Z6 & 7. These are most closely related to each other, sharing 94% identity at the nucleotide sequence level. In addition, *CYP71Z6 & 7* are located adjacent to each other, indicating that they were derived from a tandem gene duplication event. Nevertheless, their differential transcriptional response to elicitation with chitin oligosaccharide suggests that CYP71Z6 & 7 might have distinct roles, and we were particularly interested in the potential role of these in production of the orzyalides, for which no relevant CYP activity had yet been identified.

3.2. Functional recombinant expression

Previous attempts at recombinant expression of CYP71Z7 in both yeast (*Saccharomyces cerevisiae*) and insect cells (*Spodoptera frugiperda*) were unsuccessful [7]. On the other hand, we have had success with recombinant expression of plant CYP in *E. coli* [7,8,15,18]. Accordingly, our initial attempts at characterization of CYP71Z6 & 7 were directly carried out in *E. coli*, using our previously developed metabolic engineering system [19]. This enabled co-expression of these CYP with all functional pairings of upstream diterpene cyclases/synthases from rice to provide potential substrates (Figure S1), as well as the requisite CYP reductase (specifically OsCPSR1 from rice). However, even with N-terminal modification for bacterial expression, such recombinant expression of the native *CYP71Z6 & 7* genes did not lead to any detectable activity (i.e., hydroxylation of diterpene olefins).

We have previously 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 [8,15]. Thus, we had such gene constructs synthesized for CYP71Z6 & 7, which were also N-terminally modified for bacterial expression. These were again incorporated into our metabolic engineering system as described above. Notably, with the N-terminally modified synthetic constructs CYP activity was observed. In particular, CYP71Z6 was found to react with *ent*-isokaurene and CYP71Z7 with *ent*-cassadiene, with reasonable conversion of these diterpene olefins to an apparently hydroxylated diterpenoid (MW = 288 Da) in each case (Figure 2). Notably, neither CYP71Z6 or 7 exhibited activity with the any other of the diterpenes found in rice (e.g., CYP71Z6 does not react with *ent*-cassadiene and nor does CYP71Z7 react with *ent*-isokaurene).

3.3. Identification of hydroxylated products

To produce enough of these diterpenoids for structural characterization by NMR, we increased flux towards isoprenoid metabolism in our metabolic engineering system by

incorporating the "bottom half" of the yeast mevalonate dependent (MEV) pathway and feeding the cultures mevalonate, as previously described [23]. It was then possible to produce and purify approximately a milligram of each product by extraction from reasonable quantities of these recombinant cultures (3-L each). From the subsequent NMR analysis (Figures S2&3 and Tables S1&2), it was possible to assign the position of the resulting hydroxyl group in each of the observed products. Hence, we were able to determine that CYP71Z6 catalyzes C2-hydroxylation of *ent*-isokaurene and CYP71Z7 similarly catalyzes C2-hydroxylation of *ent*-cassadiene (Figure 3).

3.4. Enzymatic characterization of CYP71Z6 & 7

To further characterize the hydroxylase activity observed with CYP71Z6 & 7, we carried out *in vitro* enzymatic analysis. These assays relied on co-expression with OsCPR1 and determination of the level of functional CYP present by measurement of the CO difference binding spectra from the resulting clarified lysates (Figure S4). These preparations were then used for steady-state kinetic characterization of enzymatic activity. This revealed that, whereas CYP71Z6 efficiently reacts with its *ent*-isokaurene substrate (K_M = 12 ± 7 μM, k_{cat} = 0.01 ± 0.005 s^{-1}), CYP71Z7 is much less efficient, and only poorly recognizes *ent*-cassadiene as a substrate (K_M = 200 ± 100 μM, k_{cat} = 0.11 ± 0.03 s^{-1}).

4. Discussion

In the oryzalides, C2 is actually replaced by the ester linkage of the eponymous –olide (i.e., lactone ring) moiety. However, this almost certainly proceeds via initial hydroxylation of C2 in *ent*-isokaurene, as rice contains a number of related diterpenoids that contain such a C2-hydroxyl group, as well as the C2,3-ring opened oryzalic acids (Figure 4). Thus, consistent with the previously reported co-regulation of the *ent*-isokaurene producing OsKSL6 and CYP71Z6 [16], the ability of CYP71Z6 to efficiently catalyze C2-hydroxylation of *ent*-isokaurene presumably reflects a role in mediating such an early step in oryzalide biosynthesis (Figure 4). This then provides the first evidence for a CYP involved in oryzalide production, and further expands the enzymatic genes from the rice chromosome 2 cluster associated with this pathway. In addition, an initial hydroxylation role for CYP71Z6 in oryzalide biosynthesis is consistent with similarly early roles for the CYP found in other plant terpenoid biosynthetic gene clusters [8,15,24,25].

On the other hand, CYP71Z7 only poorly recognizes *ent*-cassadiene (K_M = 200 μM), which then seems unlikely to be its true substrate *in planta*. Moreover, of the five known phytocassanes, only three contain a C2-oxy group (i.e., either a C2-hydroxyl or C2-keto), while all five have C11-keto and C3-oxy groups in common. This suggests that oxygen is first inserted at C3 and C11, and only then at C2. Consistent with early hydroxylation at C11, the CYP76M7 & 8 also found in the rice chromosome 2 gene cluster efficiently catalyze C11α-hydroxylation of *ent*-cassadiene (e.g., CYP76M8 exhibits K_M = 4 μM), and have been shown to play a role in phytocassane biosynthesis [15]. We have previously suggested that C2-hydroxylation occurs with a C3α-hydroxy-C11-keto-*ent*-cassadiene intermediate [1], and hypothesize here that this is the true substrate for CYP71Z7 (Figure 4B). Such a role in phytocassane biosynthesis would be consistent with the observed chitin induced transcriptional accumulation of *CYP71Z7* along with the *ent*-cassadiene producing *OsKSL7*, as well as *CYP76M7 & 8*.

Our results strongly suggest that, despite their close phylogenetic relationship, CYP71Z6 & 7 play distinct roles in rice diterpenoid phytoalexin biosynthesis. This is consistent with the previously reported difference in their transcript accumulation in response to the fungal cell wall elicitor chitin oligosaccharide [16], as well as the differing substrate specificities observed here (Figure 3). Given their striking difference in affinity for the diterpene olefin

substrates characterized here, we hypothesize that CYP71Z6 & 7 also differ in playing early versus late roles in the relevant biosynthetic pathways, respectively (Figure 4). Interestingly, consistent with the presence of an early acting CYP in all other characterized biosynthetic gene clusters in plants [8,15,24,25], an early role for CYP71Z6 in oryzalide biosynthesis suggests that this may represent the initial function for which a CYP71Z sub-family member was recruited into the rice chromosome 2 biosynthesis gene cluster. By contrast, the hypothesized later role for CYP71Z7 in phytocassane biosynthesis presumably reflects subsequent evolutionary duplication and functional diversification within the rice chromosome 2 biosynthetic gene cluster, indicating flexibility in its evolutionary trajectory following initial assembly, which is consistent with the previously observed roles for the co-clustered CYP76M5-8 sub-family members in not only phytocassane, but also oryzalexin biosynthesis [15].

Highlights

- Characterization of closely related CYP71Z6 & 7 demonstrates different activity
- Biochemical activity suggests distinct roles in rice diterpenoid metabolism
- Results provide insight into plant biosynthetic gene cluster evolution

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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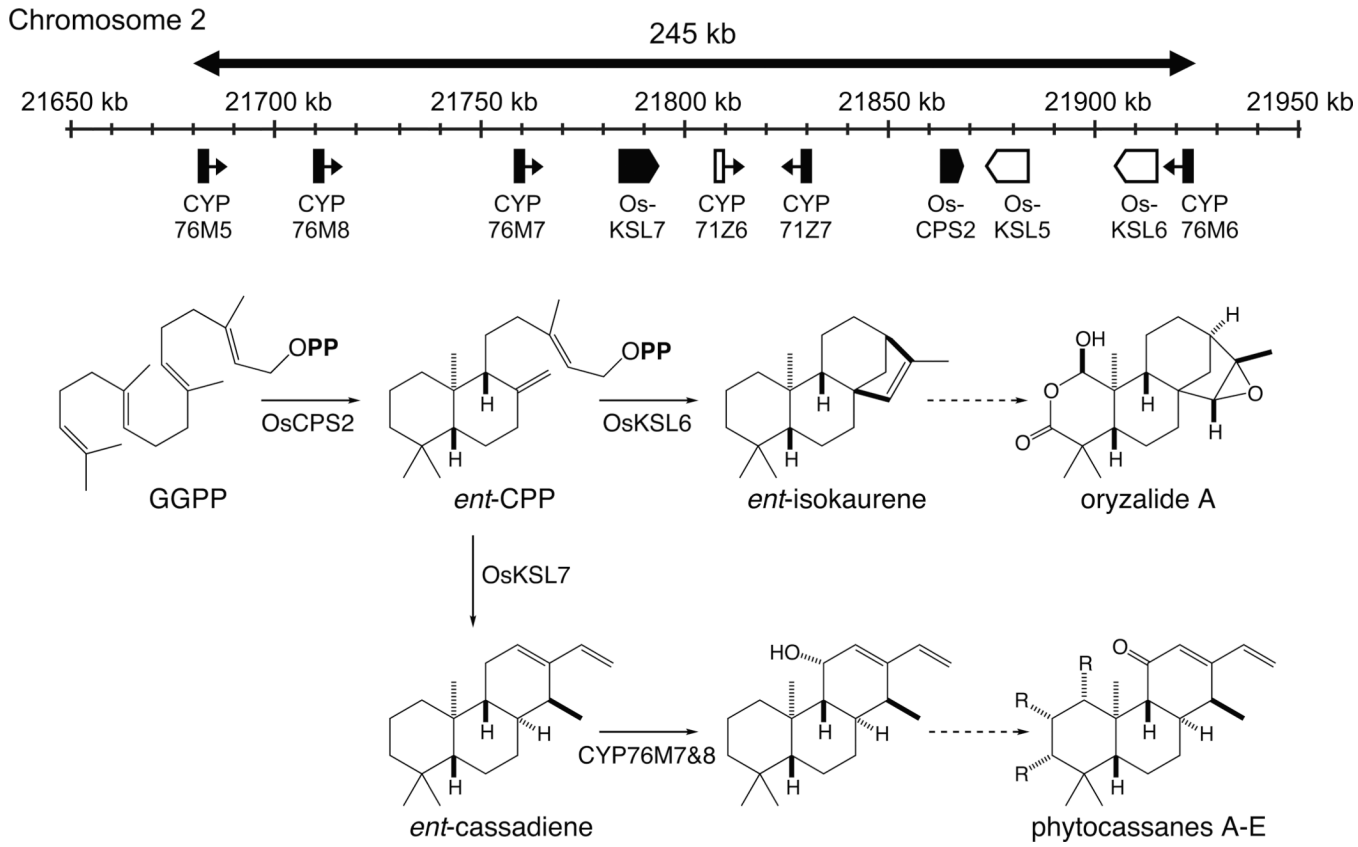

Figure 1.

Rice chromosome 2 labdane-related diterpenoid gene cluster and associated biosynthesis pathways. The gene map is adapted from [7], with the filled boxes representing genes whose mRNA accumulates in response to the fungal cell wall component chitin oligosaccharide, while the open boxes those whose mRNA levels are unchanged [16].

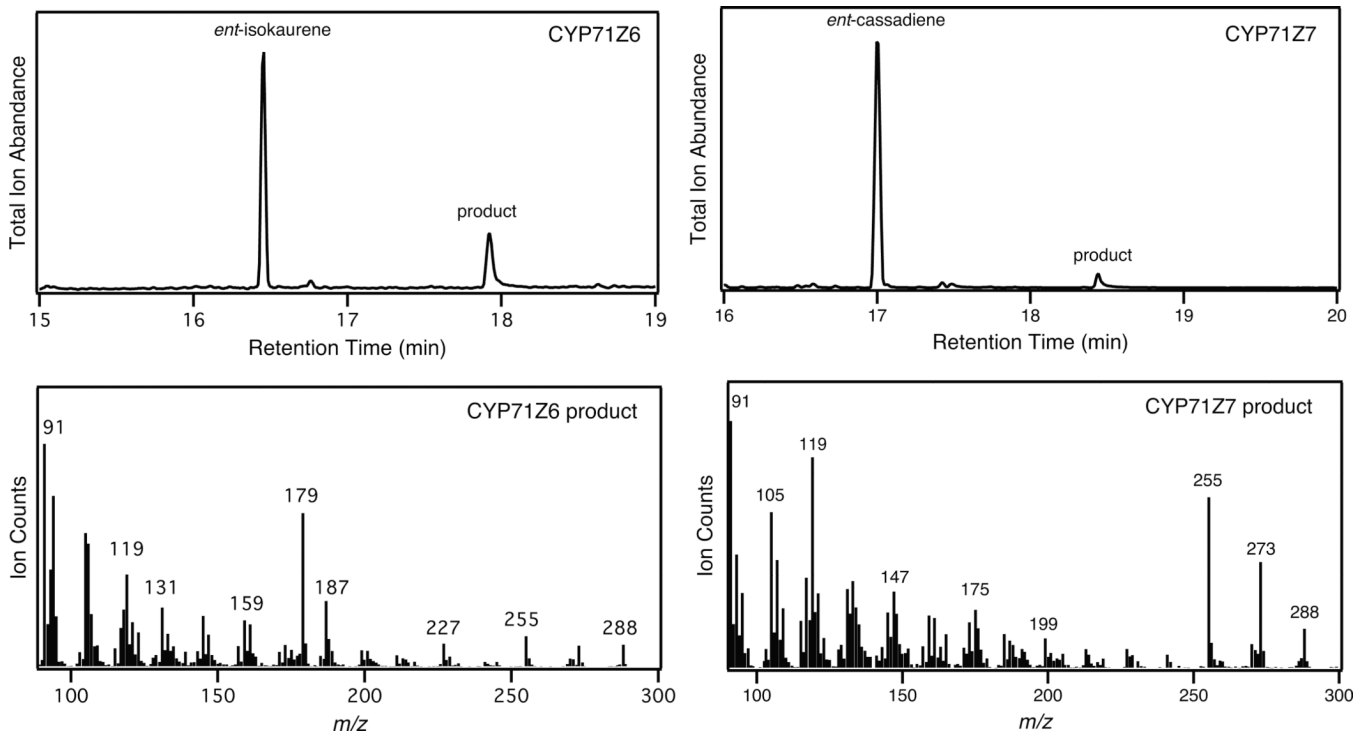

Figure 2.

Observed CYP71Z6 & 7 activity. Shown is GC-MS analysis of extracts from cultures engineered to produce the indicated diterpene olefin and co-express OsCPR1 with the indicated CYP. Chromatograms indicate relative turnover, and mass spectra the *m/z* peak for the molecular ion at 288 Da.

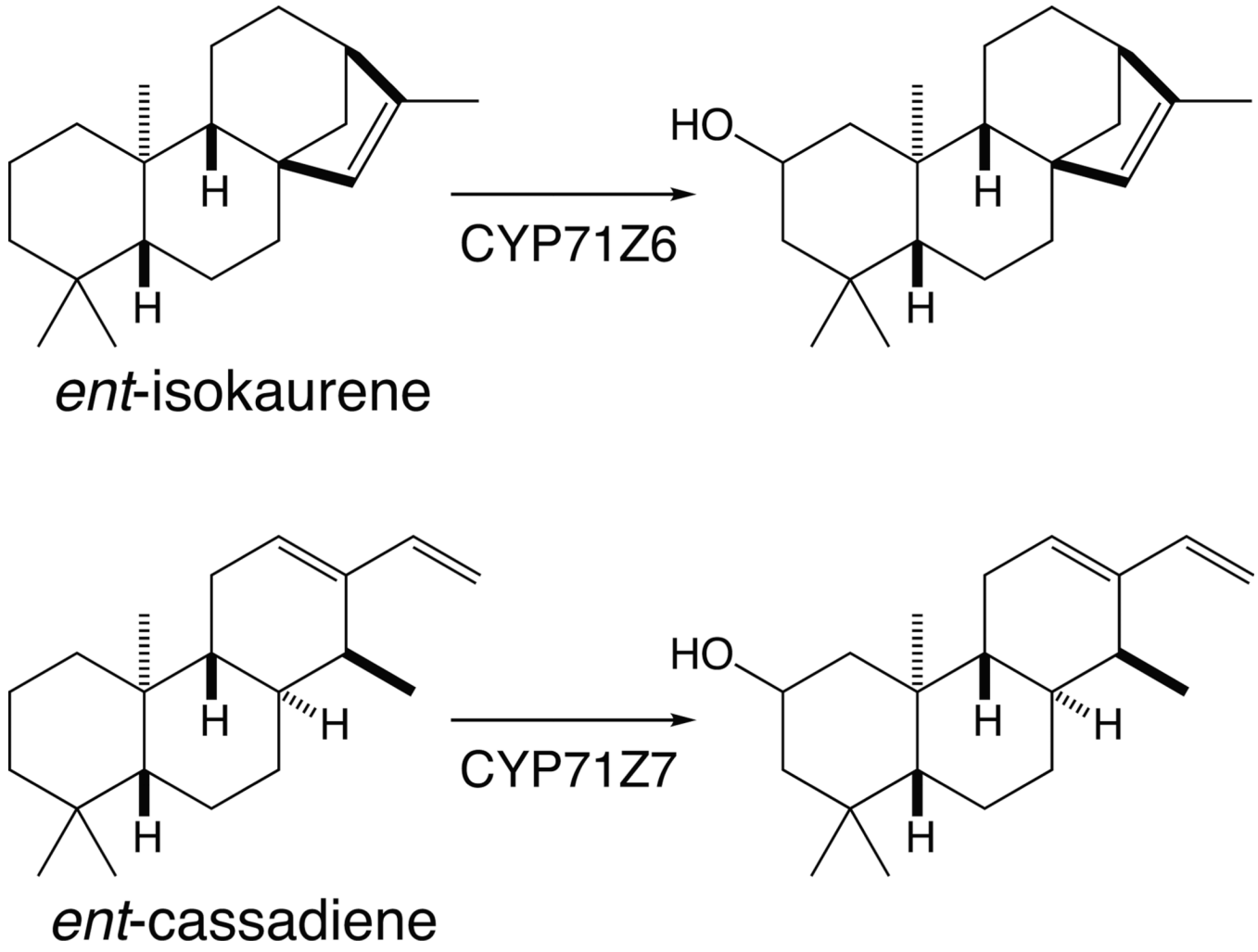

Figure 3.
Reactions catalyzed by CYP71Z6 and CYP71Z7.

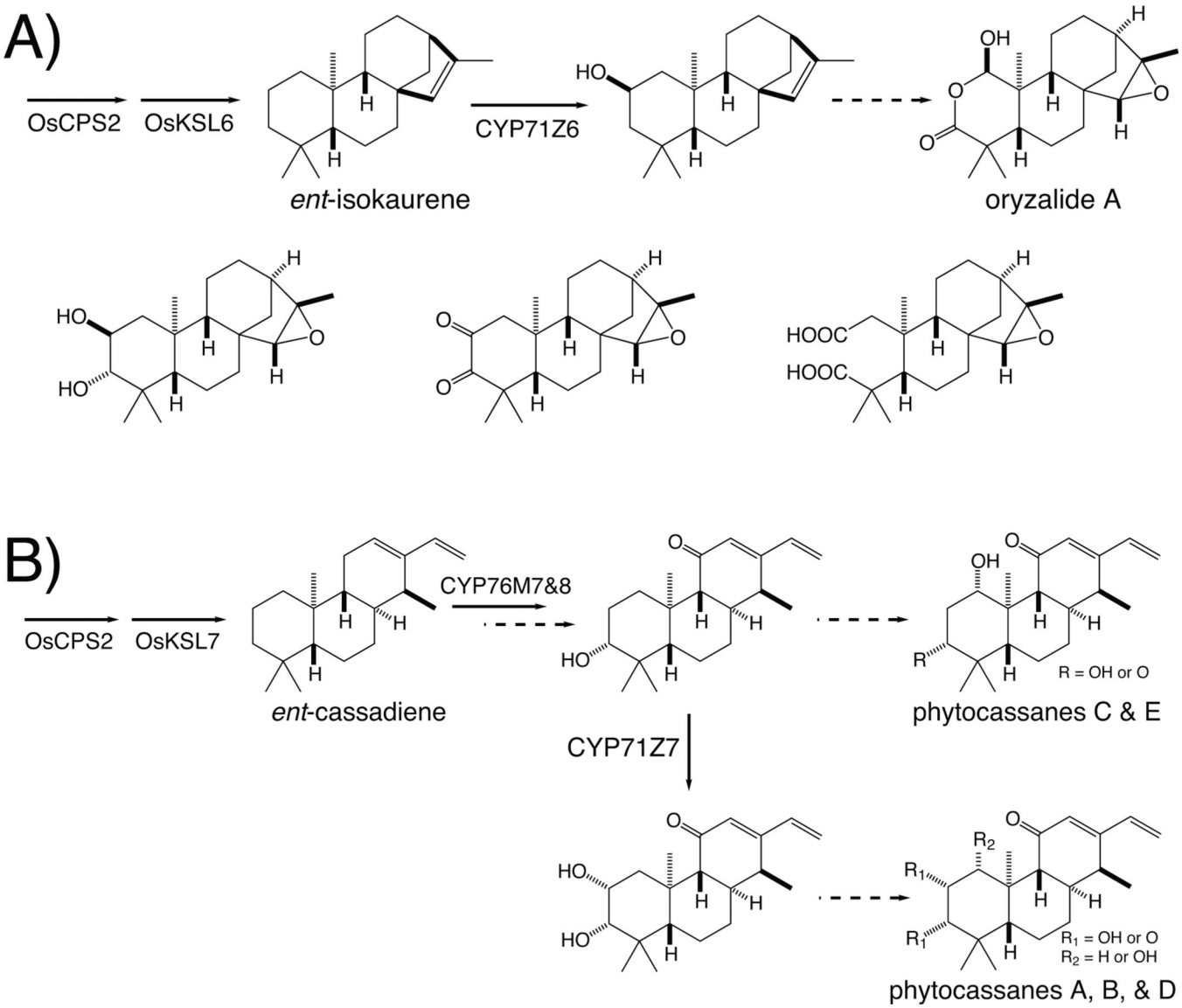

Figure 4.
Putative biosynthetic roles. (a) For CYP71Z6 in oryzalide production (also shown are other known *ent*-isokaurene derived metabolites from rice). (b) For CYP71Z7 in phytocassane biosynthesis.