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

Yisheng Wu, Matthew L. Hillwig, Qiang Wang, Reuben J. Peters *

Department of Biochemistry, Biophysics, & Molecular Biology, Iowa State University, Ames, IA 50011, USA

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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.

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).

The presence of biosynthetic gene clusters is an emerging theme in plants [4], and rice is known 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 (Fig. 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 Cllα-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

* Corresponding author. Address: Mol. Biol. Bldg., Rm. 4216, Ames, IA 50011, USA. Tel.: +1 515 294 8580; fax: +1 515 294 0453.
E-mail address: rjpeters@iastate.edu (R.J. Peters).

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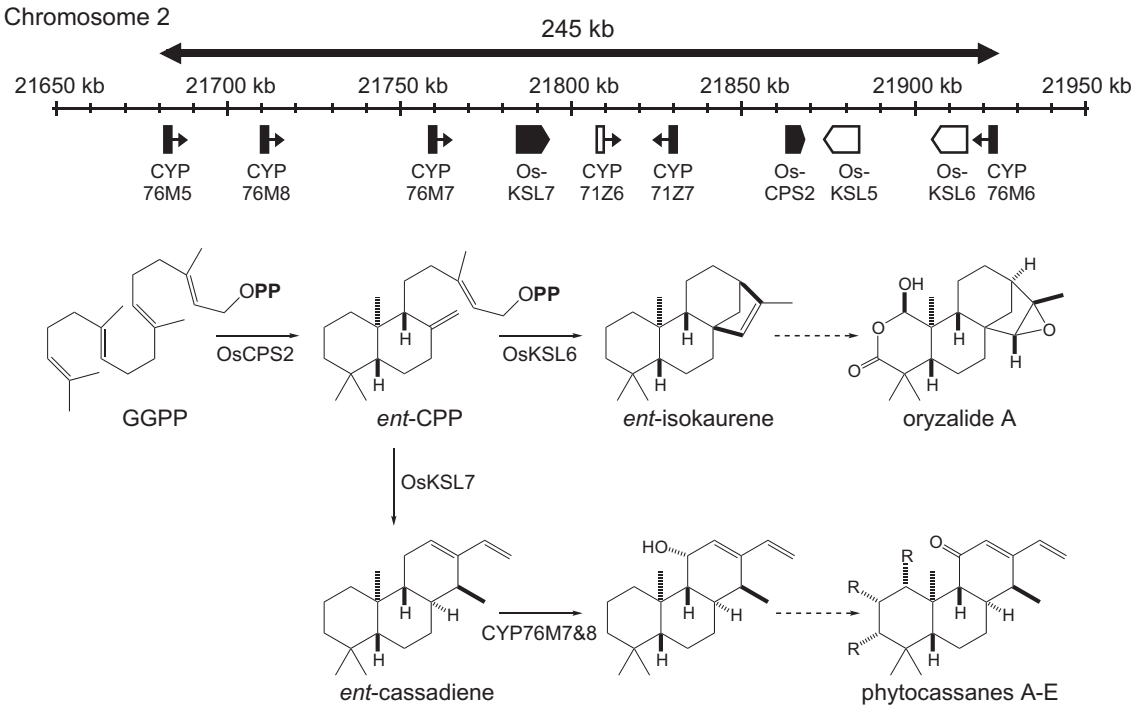

Fig. 1. Rice chromosome 2 labdane-related diterpenoid gene cluster and associated biosynthesis pathways. The gene map is adapted from [7], with the filled boxes representing genes whose mRNA accumulates in response to the fungal cell wall component chitin oligosaccharide, while the open boxes those whose mRNA levels are unchanged [16].

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 *Escherichia 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 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