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Molecules of Interest

Uncovering the complex metabolic network underlying diterpenoid phytoalexin biosynthesis in rice and other cereal crop plants

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Abstract

Rice (*Oryza sativa*) is a staple food crop and serves as a model cereal crop plant for scientific study. Phytochemical investigations of the agronomically devastating rice blast disease have identified a number of rice phytoalexins exhibiting significant direct anti-fungal activity against the causative agent, *Magneporthe grisea*. Current evidence strongly indicates that these phytoalexins, largely a family of labdane-related diterpenoids, are important as general antibiotics, and that similar phytoalexins are produced more broadly throughout the cereal crop family. From the extensive sequence information available for rice it has been possible to functionally identify the genes for the enzymes catalyzing the two consecutive cyclization reactions that initiate biosynthesis of these labdane-related diterpenoid phytoalexins. This has led to several insights into the underlying evolution of diterpene biosynthesis throughout the cereal crop family. The hydrocarbon olefins resulting from cyclization must be further elaborated to form bioactive natural products and, because not much is currently known, necessarily speculative biosynthetic pathways for these processes are presented. Given the significant antibiotic activity of the labdane-related diterpenoid phytoalexins from rice, and the presence of similar secondary metabolism throughout the cereal crop plant family, study of this type of biosynthesis will continue to be an area of active investigation.

Keywords: *Oryza sativa*; Poaceae; Diterpenoids; Labdane-related diterpenoids; Phytoalexins; Biosynthesis; Terpene synthases

1. Introduction

Rice is an important source of food, providing 20% of the total direct human caloric intake world-wide, as well as being the predominant staple food for many developing countries (FAO, 2004). Furthermore, rice also has become a model plant for the widespread grass plant family (Poaceae), which covers ~20% of the earth's land mass surface area. In particular, the relatively small size of the rice genome (~430 Mb), ease of transformation, and extensive genetic resources, along with agronomic importance, led to its selection for both public and private genomic sequencing (Goff et al., 2002; Yu et al., 2002). This has stimulated the additional development of a host of related resources, such as the availability of large numbers of defined full-length cDNAs (Kikuchi et al., 2003) and various types of mutant rice lines (Hirochika et al., 2004). Thus, rice is not only important for its own sake, but also as a model system for study of all the cereal crop plants, which altogether provide approximately half of the global total direct human caloric intake (FAO, 2005), and further provides critical sources of forage and processed feed for many agronomically important domesticated animals.

Among the many factors contributing to plant fitness and, specifically, disease resistance, seems to be the deployment of antimicrobial small molecules. These are termed phytoalexins if their biosynthesis is induced by microbial

Abbreviations: CPP, copalyl diphosphate; CPS, copalyl diphosphate synthase; CPSL, copalyl diphosphate synthase-like; P450, cytochrome P450; GGPP, geranylgeranyl diphosphate; KO, kaurene oxidase; KOL, kaurene oxidase-like; KS, kaurene synthase; KSL, kaurene synthase-like; MeJA, methyl jasmonate; MYA, million years ago.

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infection, and phytoanticipins if preformed (VanEtten et al., 1994). Not surprisingly, there are a number of reports in the literature detailing the isolation and identification of rice phytoalexins. These studies have generally focused on finding compounds that exhibit antibiotic activity against *Magneporthe grisea* (originally known as *Pyricularia oryzae*), a filamentous ascomycete fungus that is the causal agent of rice blast disease. This devastating disease has been estimated to cause the loss of 10–30% of the total rice harvest, and *M. grisea* also has been observed to infect wheat, barley, and millet crops, producing a similar blast disease and loss of grain production (Talbot, 2003). Accordingly, there is considerable interest in elucidating any means by which *M. grisea* infections might be contained, which includes early work demonstrating the generalized production of phytoalexins by rice plants in response to *M. grisea* (Uehara, 1958). Of particular relevance here is identification of the corresponding natural products, along with elucidation of the associated biosynthetic pathways.

2. Identified rice phytoalexins

The first identified rice phytoalexins were the 9,10-*syn*-pimarane diterpenoids momilactones A and B (Fig. 1). These compounds were originally isolated and identified as plant growth inhibitors from rice seed husks (Kato et al., 1973). Later work has demonstrated that at least momilactone B acts as an allelochemical, inhibiting seed germination of other plant species (Kato-Noguchi et al., 2002), as well as being constitutively secreted from rice roots (Kato-Noguchi and Ino, 2003). Nevertheless, work by Cartwright and co-workers, as first communicated to Nature (Cartwright et al., 1977) and later detailed in Phytochemistry (Cartwright et al., 1981), demonstrated that momilactones A and B are also phytoalexins. In particular, the momilactones exhibit antifungal activity against *M. grisea* and only appear in rice leaves after infection. In their initial report Cartwright et al. (1977) also reported that biosynthesis of these two phytoalexins was 'primed' by treatment with the systemic antifungal WL 28325 (2,2-dichloro-3,3-dimethyl cyclopropane carboxylic acid), which does not induce production of momilactones directly but rather increases the amounts produced following infection. Indeed, the authors suggest that this 'priming' of phytoalexin biosynthesis is the mode of action accounting for dichlorocyclopropane-induced blast resistance. In both reports Cartwright et al. also demonstrated that momilactone biosynthesis could be induced by UV-irradiation, an observation that was later reported to extend to a wider range of rice phytoalexins (Kodama et al., 1988), and has proven useful in a number of the following studies.

Many of the known rice phytoalexins were identified by Tadami Akatsuka, Osamu Kodama, and co-workers. Their efforts were initiated by the isolation of a group of four compounds from *M. grisea* infected rice leaves, wherein the first, oryzalexin A, was suggested to be a pimarane diterpenoid in their initial report (Akatsuka et al., 1983). Further spectroscopic analysis demonstrated that oryzalexin A was configured with *enantio*-stereochemistry (Fig. 2) and, thus, was an *ent*-pimarane diterpenoid (Kono et al., 1984). Oryzalexins B and C were also identified as *ent*-pimarane diterpenoids, and found in *M. grisea* infected, but not healthy rice leaves (Akatsuka et al., 1985; Kono et al., 1985); likewise for oryzalexin D (Sekido et al., 1986). Accordingly, all four oryzalexins have been classi-

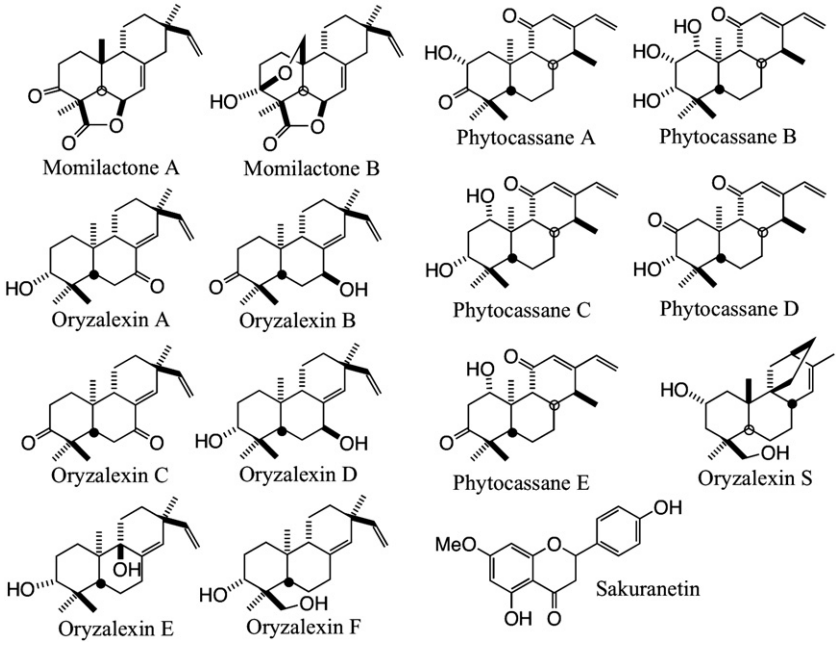

Fig. 1. Known rice phytoalexins.

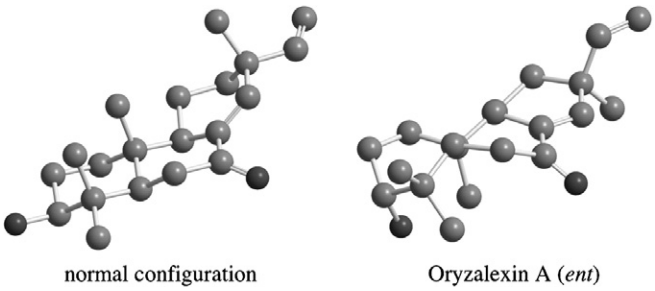

Fig. 2. Comparison of the originally assumed normal configuration and actual enantiomeric form of oryzalexin A. Carbon atoms are lighter grey, while oxygen atoms are darker and hydrogens atoms are not shown.

fied as rice phytoalexins. Critically, in the course of these studies production of rice phytoalexins was found to be localized to the blast disease lesions (e.g. Akatsuka et al., 1985), rather than being systemic, which is consistent with the observed direct antifungal activity of these compounds, and further supports a physiologically relevant role for these natural products in plant disease resistance.

The Akatsuka and Kodama group also was responsible for demonstrating the aforementioned wider induction of rice diterpenoid phytoalexin biosynthesis by UV-irradiation (Kodama et al., 1988). In this report, the presence of a novel UV-irradiation inducible natural product was noted. This compound was found to be the *syn*-stemarane diterpenoid orzyalexin S, which was classified as a phytoalexin since it exhibited potent antifungal activity against *M. grisea* and its biosynthesis was also induced by blast infection (Kodama et al., 1992a). Later, detailed fractionation of UV-irradiated leaves by this same group also led to the identification of two more *ent*-pimarane diterpenoid phytoalexins, oryzalexins E and F (Kato et al., 1993, 1994). In addition, the flavanone sakuranetin also was found in UV-irradiated rice leaves, and demonstrated to be a rice phytoalexin whose biosynthesis is induced by blast infection and which exhibits potent antibiotic activity against *M. grisea* (Kodama et al., 1992b). Notably, sakuranetin was observed to accumulate to high levels, leading Kodama et al. (1992a) to suggest that this compound is particularly important in rice defense against *M. grisea*. However, relatively little follow up work has been done on this natural product, which is derived in a single step from the core flavonoid intermediate (2*S*)-naringenin by an inducible 7-*O*-methyltransferase that has not yet been identified (Rakwal et al., 1996, 2000), and sakuranetin will not be further discussed here.

A final group of rice phytoalexins was identified by an independent group led by Jinichiro Koga. Their efforts were similarly initiated by the isolation of four novel antifungal natural products from *M. grisea* infected rice leaves or *Rhizoctonia solani* infected rice stems. These four compounds met the criteria for phytoalexin classification and were initially identified as cassane diterpenoids: phytocassanes A–D. Interestingly, production of these compounds was not only induced by *M. grisea* infection, but seemed to correlate with the effectiveness of the defensive response, as these phytoalexins accumulated to higher levels in *M. grisea* resistant rice strains than in susceptible ones (Koga et al., 1995). In addition, these compounds also exhibited antibiotic activity against *R. solani*, suggesting that these, and possibly the other identified rice phytoalexins, will prove to be broadly effective antifungal agents. This research group also later identified an additional cassane diterpenoid rice phytoalexin, phytocassane E (Koga et al., 1997). Subsequently, through spectroscopic comparison to synthetic (+)-deoxyphytocassane A, it was found that the rice phytocassanes were configured with *ent*-, rather than the originally suggested normal stereochemistry and, thus, are enantiomeric to the originally suggested chemical structures (Yajima and Mori, 2000).

3. Dual cyclization reactions in rice diterpenoid phytoalexin biosynthesis

Like the structurally related gibberellic acid (GA) phytohormones, the rice diterpenoid phytoalexins fall into the labdane-related super-family (Fig. 3). In particular, biogenesis

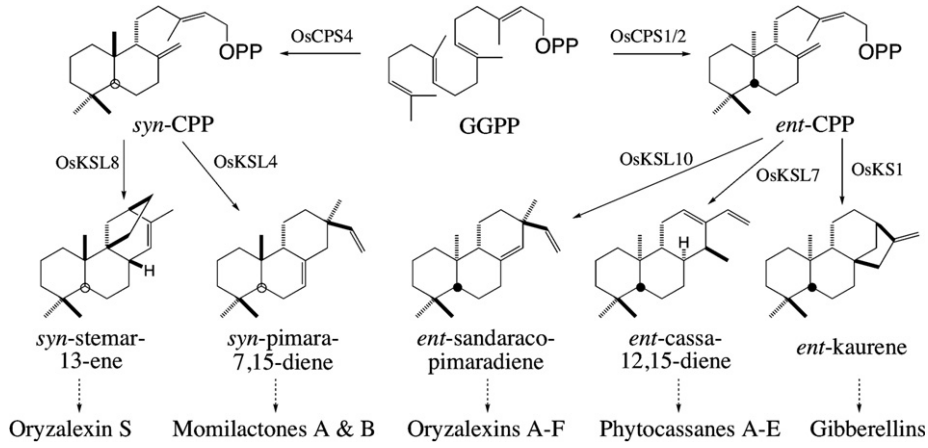

Fig. 3. Dual cyclization reactions in rice labdane-related diterpenoid phytoalexin biosynthesis. The corresponding enzymes are indicated, along with the derived products (dashed arrows indicate multiple enzymatic steps).

of these natural products was predicted to proceed through a consecutive pair of cyclization reactions (Tamogami et al., 1993; Wickham and West, 1992). Starting from the universal diterpenoid precursor (*E*,*E*,*E*)-geranylgeranyl diphosphate (GGPP) a mechanistically unusual protonation-initiated cyclization reaction catalyzed by class II diterpene cyclases first produces either *ent*- or *syn*-labdadienyl/copalyl diphosphate (CPP). This is followed by a diphosphate ester ionization initiated cyclization reaction catalyzed by more typical (i.e. class I), yet CPP stereospecific, terpene synthases to produce polycyclic diterpene olefins corresponding to the various base structures underlying the identified groups of rice diterpenoid phytoalexins.

Evidence for such a dual cyclization reaction biogenetic mechanism was provided by Wickham and West (1992), who demonstrated that activity of the corresponding diterpene synthases/cyclases in cell-free extracts from rice leaves was induced by UV-irradiation. In particular, the predicted base diterpenes were produced from GGPP, as well as the expected subset from *ent*-CPP. Accordingly, both class II and class I labdane-related diterpene synthase activity is inducible. Wickham and West (1992) were able to identifiy, by comparison of the enzymatic products to authentic samples provided by Robert Coates and co-workers, *syn*-pimara-7,15-diene and *ent*-sandaracopimara-8(14),15-diene, the presumed precursors to momilactones A and B, and oryzalexins A–F, respectively, along with the *ent*-kaurene intermediate of GA biosynthesis. While *ent*-kaurene production was found to be constitutive and not inducible, that of *syn*-pimaradiene and *ent*-sandaracopimaradiene was clearly induced by the UV-irradiation treatment. Wickham and West (1992) also reported that the major product of inducible cyclase activity was an unidentified diterpene derived from *ent*-CPP, which they suggested to be a tricyclic pimaradiene or derived structure on the basis of their spectral evidence. This product almost certainly corresponds to *ent*-cassa-12,15-diene, the presumed precursor for the later identified phytocassanes, based on the reported GC-MS data (Wickham and West, 1992; Yajima et al., 2004). Notably, the significant production of this putative phytocassane precursor reported by Wickham and West (1992) is consistent with the importance of this particular class of rice diterpenoid phytoalexins suggested by Koga et al. (1995).

Subsequent work led by Coates and West then demonstrated the intermediacy of 9,10-*syn*-CPP in production of rice labdane-related diterpenes with the corresponding stereochemical conformation (Mohan et al., 1996). Along with those diterpenes already identified by Wickham and West, Mohan et al. (1996) also identified *syn*-stemar-13-ene, the presumed precursor to oryzalexin S, as another diterpene whose production was induced by fungal elicitors in rice suspension-cell culture. Intriguingly, in addition to *syn*-pimaradiene and *syn*-stemarene, which provided presumed precursors for all the known rice diterpenoid phytoalexins with *syn* stereochemical configuration, Mohan et al. (1996) also found significant induced production of another, unidentified *syn*-CPP derived diterpene, suggesting the existence of an additional group of rice phytoalexins.

Building on this early work with cell-free extracts, all of the genes encoding the diterpene synthases/cyclases responsible for the known rice diterpenoid phytoalexins have now been identified. Many of these genes were first reported, albeit without biochemical characterization, in a comprehensive study of rice GA metabolism enzymatic genes (Sakamoto et al., 2004). Based on the unique domain structure of labdane-related diterpene synthases (i.e. relative to other terpene synthases these all contain additional sequence, ~250 amino acids, at their amino termini), Sakamoto et al. (2004) reported finding four CPP synthase (CPS) and nine kaurene synthase (KS) genes in the rice genome, and these were simply designated OsCPS1-4 and OsKS1-9. However, because inactivation of either OsCPS1 or OsKS1 led to severely dwarfed, GA-responsive plants, only these individual class II and class I enzymes, out of each gene family, is involved in GA biosynthesis (Sakamoto et al., 2004). Similar results with OsKS1 also were reported by another group (Margis-Pinheiro et al., 2005). Transcription of some of the other diterpene synthase genes were shown to be induced by either UV-irradiation or fungal elicitor, suggesting a role in phytoalexin production (Sakamoto et al., 2004). All the functional class II (i.e. OsCPS) genes have been shown to produce either *ent*- or *syn*-CPP (Otomo et al., 2004b; Prisic et al., 2004; Xu et al., 2004). However, the results from in planta mutagenesis indicate that, of the class I labdane-related diterpene synthase (i.e. OsKS) genes, only OsKS1 actually produces kaurene and can be properly termed KS (Margis-Pinheiro et al., 2005; Sakamoto et al., 2004). In addition, due to the essentially simultaneous publication of functional identification of several rice diterpene synthases/cyclases, other nomenclature has been suggested (i.e. OsDTC1 and 2, OsDTS2, and OsCyc1 and 2). Margis-Pinheiro et al. (2005) also used the OsKS designation, but with a somewhat different numbering scheme for the class I labdane-related diterpene synthases they identified. To avoid confusion, it has been suggested that the non-kaurene producing class I labdane-related diterpene synthase family members be termed kaurene synthase-like (KSL), with the corresponding number from Sakamoto et al. (2004) where appropriate (Morrone et al., 2006). This convention, along with use of OsCPS1-4 for the class II genes (although OsCPS3 appears to be a pseudo-gene), will be followed here (Table 1).

A consortium of several different groups led by Hisakazu Yamane cloned two class I labdane-related diterpene synthase genes from fungal elictor induced rice suspension-cell culture. This was done using degenerate primers based on highly conserved sequence motifs contained in the additional N-terminal 'insertional' sequence element found in labdane-related diterpene synthases. Consistent with a role in phytoalexin biosynthesis, transcription of both genes was induced by either fungal elicitor or UV-irradiation. The first of these genes to be characterized

Table 1
Rice kaurene synthase-like gene family

Name[a]	Product	Accession number	Rice gene locus	Alternative name(s)	Reference
OsKS1	*ent*-Kaurene	NR[b]	Os04g52230	–	Sakamoto et al. (2004)
		AY347876			Margis-Pinheiro et al. (2005)
OsKSL4	*syn*-Pimaradiene	AY616862	Os04g10060	OsDTS2	Wilderman et al. (2004)
		AB126934		OsKS4	Otomo et al. (2004)
OsKSL7	*ent*-Cassadiene	DQ823354	Os02g36140	OsDTC1	Cho et al. (2004)
OsKSL8	*syn*-Stemarene	AB118056	Os11g28530	OsDTC2	Nemoto et al. (2004)
OsKSL10	*ent*-Pimaradiene	DQ823355	Os12g30824	OsKS10	Otomo et al. (2004)

[a] Gene numbering based on Sakamoto et al. (2004), with kaurene synthase-like (KSL) nomenclature for the non-kaurene producing family members, as previously suggested (Morrone et al., 2006).
[b] NR = not reported, Sakamoto et al. (2004) did not report any sequence information.

was demonstrated to encode an *ent*-CPP specific cassa-12,15-diene synthase, originally termed OsDTC1 but referred to here as OsKSL7 (Cho et al., 2004). Identification of this enzymatic product was enabled by comparison to an authentic standard synthesized for this purpose (Yajima et al., 2004). Later the second identified gene was shown to be a *syn*-CPP specific diterpene synthase, originally named OsDTC2 but referred to here as OsKSL8, that produces stemarene as its major product, along with significant production of two other, unidentified diterpenes (Nemoto et al., 2004). However, given the differences in GC-MS mass spectral patterns, neither of these is the unidentified *syn*-CPP derived diterpene noted by Mohan et al. (1996).

Largely the same consortium of groups, in efforts led by Tomonobu Toyomasu, cloned two CPP synthases from UV-irradiated rice leaves (Otomo et al., 2004b). This was carried out using degenerate primers previously used to isolate a fungal bifunctional class II/I diterpene cyclase (Kawaide et al., 1997). Again consistent with a role in phytoalexin biosynthesis, transcription of these two rice class II diterpene synthase genes was induced by UV-irradiation. These were originally termed OsCyc1 and OsCyc2, corresponding to OsCPS4 and OsCPS2, which were shown to produce *syn*- and *ent*-CPP, respectively. Also cloned was a truncated version of OsCPS1, which was utilized to demonstrate the production of *ent*-CPP and lack of transcriptional induction by UV-irradiation expected from its role in GA biosynthesis (Otomo et al., 2004b). A later communication from this consortium also reports cloning a number of additional class I labdane-related diterpene synthase genes first identified by bioinformatic approaches. The two genes whose transcription was induced by UV-irradiation, OsKS(L)4 and the newly identified OsKS(L)10, were then further functionally characterized as encoding *syn*-pimaradiene and *ent*-sandaracopimaradiene synthases, respectively (Otomo et al., 2004a). Notably, this last communication completed functional identification of labdane-related diterpene synthases for biosynthesis of all the known rice diterpenoid phytoalexins (Fig. 3).

My own research group, in collaboration with that of Robert Coates, was concurrently taking a functional genomics approach towards identifying rice labdane-related diterpene synthases. Using the CPS and KS genes from *Arabidopsis* as probe sequences for BLAST searches, three full-length putative class II (i.e. OsCPS) genes and seven full-length putative class I genes (i.e. OsKSL) were identified in silico. Sequence comparisons within and between these general plant gene families revealed that the functionally cryptic N-terminal 'insertional' sequence element ubiquitously found in class II labdane-related diterpene synthases exhibits a significantly higher degree of conservation in the class II enzymes than is found across this region with class I terpene synthases that also contain such an 'insertional' sequence element. This degree of conservation suggests a role for the 'insertional' sequence element in the enzymatically distinct mechanism of class II cyclases.

Fortuitously, the first gene we cloned was a class II cyclase that produces the biosynthetically novel *syn* stereoisomer of CPP, originally called $OsCPS_{syn}$ but referred to here as OsCPS4 (Xu et al., 2004). Thus, along with the novel biosynthetic activity, we were able to report the specific conservation of the 'insertional' sequence element in class II labdane-related diterpene synthases. The second gene we cloned was a class I labdane-related diterpene synthase, originally termed OsDTS2 but referred to here as OsKSL4, that was demonstrated to specifically accept *syn*-CPP and produce pimaradiene (Wilderman et al., 2004). Both OsCPS4 and OsKSL4 are involved in production of the dual function phytoalexin/allelochemical momilactones. Not surprisingly then, we found that mRNA for both OsCPS4 and OsKSL4 is constitutively present in roots, and is upregulated by either UV-irradiation or treatment with methyl jasmonate (MeJA) in leaves. OsCPS4 and OsKSL4 are also clustered together in the rice genome, as first reported by Sakamoto et al. (2004), and our biochemical analysis demonstrated that these act consecutively (see Fig. 3), thus, forming a functional gene pairing (Wilderman et al., 2004).

Upon cloning the two remaining class II cyclases, both were found to produce *ent*-CPP, yet each exhibited distinct transcriptional responses to either UV-irradiation or MeJA treatment (Prisic et al., 2004). Specifically, transcription of OsCPS1, consistent with its role in GA biosynthesis, was not affected, while that of OsCPS2 was induced by these treatments. Combined with the finding by Sakamoto et al. (2004) that loss of OsCPS1 abolishes GA production, our results strongly indicate that OsCPS1 operates solely in GA metabolism and OsCPS2 operates solely in phytoalexin

biosynthesis. Intriguingly, OsCPS2 is clustered with the three class I labdane-related diterpene synthases OsKSL5, OsKSL6, and OsKSL7; and OsKSL7 previously had been demonstrated to encode the *ent*-CPP specific cassadiene synthase (Cho et al., 2004). Therefore, we further speculated that OsKSL5 and OsKSL6 will similarly encode *ent*-CPP specific diterpene synthases (Wilderman et al., 2004), which has recently been verified (Kanno et al., 2006; Xu et al., submitted for publication). Hence, the two gene clusters noted by Sakamoto et al. (2004) as containing both class II and class I labdane-related diterpene synthases each represent functional biosynthetic modules containing enzymes that act to produce and then use specific stereoisomers of CPP. In addition, from sequence comparison of the OsCPS gene family with the previously identified CPS gene An1/ZmCPS1 from maize (*Zea mays*) that is involved in GA biosynthesis (Bensen et al., 1995), we suggested that there had been an early duplication of an ancestral CPS gene in the cereal crop plant family, and further speculated that this was associated with the development of separate primary (GA phytohormone) and secondary (phytoalexin) biosynthetic pathways (Prisic et al., 2004).

Although not yet identified, given the similar transcriptional regulation of the phytoalexin associated labdane-related diterpene synthase genes and their presumably common evolutionary origin (see below), it seems likely that their promoters may share regulatory elements. Identification of these sequence motifs would enable investigation of the corresponding transcription factors and upstream signaling pathways, allowing further insight into the molecular control mechanisms underlying regulation of phytoalexin biosynthesis. This might also assist efforts to identify the unknown enzymes catalyzing the remaining steps of the relevant biosynthetic pathways.

4. Elaboration of labdane-related diterpenes to bioactive phytoalexins

Production of the final, bioactive diterpenoid phytoalexins clearly requires further elaboration of the cyclized hydrocarbon olefins that result from action of the labdane-related diterpene synthases described above (cf. Figs. 1 and 3). In particular, the introduction of oxygen, which is most likely incorporated by microsomal cytochromes P450 (P450s), and subsequent formation of keto groups and heterocycles, at least one of which requires a soluble dehydrogenase (Atawong et al., 2002). Relatively little information about the enzymes catalyzing these downstream reactions has been reported, although speculative biosynthetic pathways can be drawn (Fig. 4).

For oryzalexins A–F, it has been demonstrated that *ent*-sandaracopimaradien-3α-ol can be isolated from the UV-irradiated rice leaves and further hydroxylated by inducible P450s at either the C7ß or C9ß position to yield oryzalexins D and E, respectively (Kato et al., 1995). Different kinetics for the separate hydroxylation activities and accumulation of corresponding product indicate that there are separate P450s for each hydroxylation reaction. The C7-hydroxylated oryzalexin D is presumably an intermediate in biosynthesis of oryzalexins A, B, and C, which all similarly contain a C7-oxy group (Fig. 4a). The presence of a 3-oxy group in all the lower (i.e. A–F) oryzalexins indicates that *ent*-sandaracopimaradien-3α-ol is a common precursor to all of these phytoalexins; although it does not exhibit any antifungal activity against *M. grisea* itself (Kato et al., 1995). Notably, all the rice diterpenoid phytoalexins, with the sole exception of oryzalexin S, have 3-oxy moieties, suggesting a common early C3α hydroxylation step (Fig. 4), although given the differences in cyclized structures, perhaps not all catalyzed by the same cytochrome P450. In momilactone biosynthesis, oxidation of the C3 hydroxyl group of endogenously found 3-hydroxy-*syn*-pimara-7,15-dien-19,6ß-olide by an inducible soluble dehydrogenase to produce momilactone A, with its characteristic 3-keto group, has been reported (Atawong et al., 2002). Momilactone A itself is potentially an intermediate en route to momilactone B, as C20 hydroxylation would enable intramolecular cyclization via spontaneous ring closure between the C20-hydroxyl and C3-keto to the hemiketal heterocycle characteristic of momilactone B (Fig. 4b). For the phytocassanes, the ubiquitous C11-keto group and common C2-oxy moiety suggests early incorporation of oxygen at these positions as well (Fig. 4c).

While none of the enzymes involved in elaboration of the rice diterpenoid phytoalexins has yet been identified, the rice genome encodes five genes homologous to kaurene oxidase (KO), the initial cytochrome P450 in GA biosynthesis that transforms the C4α methyl (i.e. C18) of kaurene into a carboxylic acid (Fig. 4e). As reported by Sakamoto et al. (2004), only one of the rice KO homologs (OsKO2) is involved in GA metabolism. In a separate study, transcription of the two most divergent paralogs, termed KO-like (i.e. OsKOL4 and 5), were found to be induced by UV-irradiation and/or fungal elicitor. In addition, expression of OsKOL4 in an OsKO2 mutant plant did not reverse the dwarf phenotype (i.e. OsKOL4 can not convert kaurene to kaurenoic acid), all of which indicates a role for OsKOL4 and 5 in phytoalexin, rather than GA biosynthesis (Itoh et al., 2004). The proximity of the C3α hydrogen to the C4α methyl targeted by bona fide kaurene oxidases (e.g. OsKO2) lends itself to speculation that the nearly ubiquitous, presumably similarly early C3α hydroxylation of rice diterpenoid phytoalexins is catalyzed by the OsKOL P450s (Fig. 4). If any of the rice KO P450s have a role in diterpenoid phytoalexin biosynthesis, it is interesting to note that these enzymes appear to be specifically targeted by the rice dwarf virus protein P2, which might then suggest a role for these phytoalexins in viral, as well as fungal plant defense (Zhu et al., 2005).

Finally, the distinct sub-cellular locations for the varied enzymatic activities involved in labdane-related diterpenoid production (i.e. plastid localized synthases/cyclases

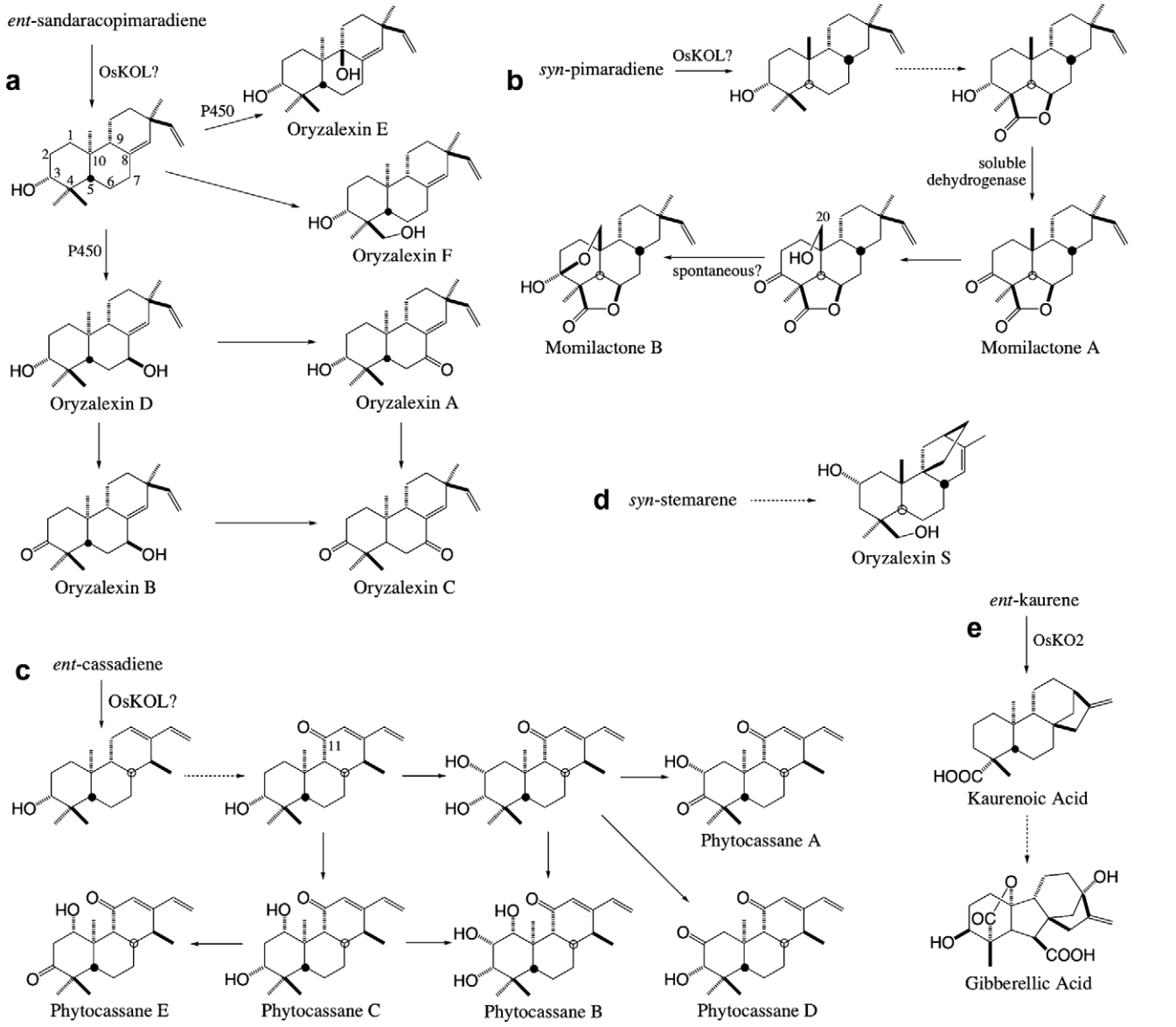

Fig. 4. Elaboration of rice labdane-related phytoalexins. Shown are hypothetical biosynthetic pathways, with indicated enzymatic type or speculative enzymatic assignments, for (a) oryzalexins A–F, (b) momilactones A and B, (c) phytocassanes A–E, (d) oryzalexin S, and (e) related biosynthesis of the phytohormone gibberellic acid (dashed arrows indicate multiple enzymatic steps).

and, presumably, endoplasmic reticulum localized P450s) indicates the need for intracellular transport during biosynthesis. The direct anti-fungal activity of the rice phytoalexins also suggests that the bioactive compounds may be secreted, and certainly momilactone B is secreted from root cells (Kato-Noguchi and Ino, 2003), which further highlights a role for transporters. However, nothing is known about the corresponding transport mechanisms, let alone the identity of the responsible genes.

5. Evolution of labdane-related diterpenoid phytoalexins in Poaceae

Extensive phylogenetic analysis of the grass plant family indicates that rice and maize are individually representative of the two major lineages (the BEP and PACCAD clades, respectively) within Poaceae (Fig. 5). Together these two clades encompass over 95% of the constituent genera from Poaceae, and include all the cereal crop plants (GPWG, 2001). Therefore, comparisons between rice and maize allows some insight into the evolution of labdane-related diterpenoid phytoalexin biosynthesis within Poaceae more generally. In particular, how this is related to GA metabolism, which presumably provided the initial genetic material (i.e. biosynthetic genes) whose duplication led to the derived production of such alternative labdane-related diterpenoid natural products.

It has been established that maize produces labdane-related diterpenes in response to fungal infection (Mellon and West, 1979). Although these were demonstrated not to act as antifungal agents in their own right, the precedent

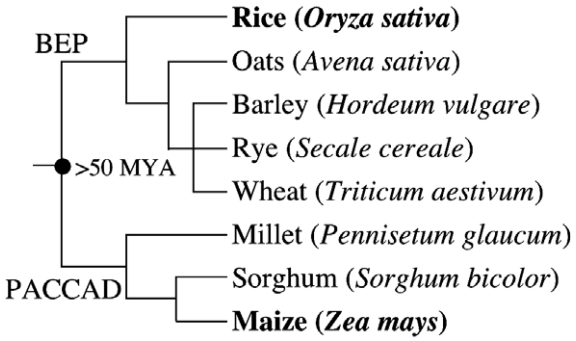

Fig. 5. Phylogenetic relationships among cereal crop plants. Illustrated by an approximate phylogenetic tree adapted from GPWG (2001) with the major BEP and PACCAD clades as indicated (MYA, million years ago).

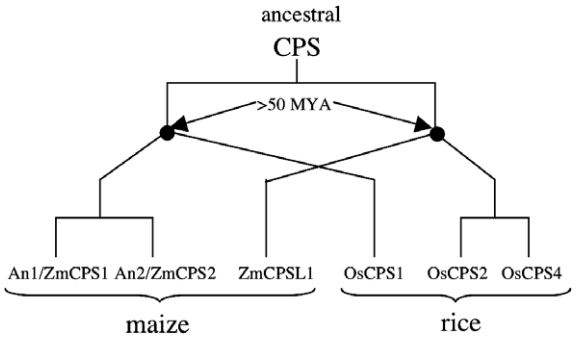

Fig. 6. Putative phylogenic relationship of class II labdane-related diterpene cyclases in Poaceae. Approximate cladogram with gene duplication events indicated by horizontal lines and speciation events by diagonal lines (MYA, million years ago).

set by rice indicates that these diterpene olefins may similarly serve as precursors to more elaborated, bioactive maize phytoalexins. Accordingly, rice and maize each produce labdane-related diterpenoids as both primary (GA phytohormone) and secondary (phytoalexin) metabolites. Identification of functionally distinct GA and phytoalexin CPS genes (i.e. OsCPS1 and OsCPS2, respectively) in rice enabled analysis of the evolution of class II labdane-related diterpene cyclases in Poaceae (Prisic et al., 2004). Sequence comparisons revealed that the rice GA specific OsCPS1 was significantly more closely related to the maize An1/ZmCPS1 similarly involved in GA metabolism than to the OsCPS2 and OsCPS4 involved in phytoalexin biosynthesis. OsCPS2 and OsCPS4 are significantly more closely related to each other than either OsCPS1 or An1/ZmCPS1. This conservation pattern suggests early duplication of an ancestral CPS gene, i.e. prior to the divergence of the separate lineages leading to rice and maize, which has been estimated, from evidence in the fossil record, to have occurred over 50 million years ago (Stebbins, 1981). The case for such an early gene duplication event has been bolstered by a recent report that maize contains a CPS-like gene (ZmCPSL1) more closely related to OsCPS2 and OsCPS4 than to An1/ZmCPS1 and OsCPS1 (Fig. 6). However, fungal infection of maize induces transcription of an An2/ZmCPS2 gene, which encodes an *ent*-CPP synthase and is most closely related to An1/ZmCPS1 and OsCPS1, rather than ZmCPSL1 (Harris et al., 2005). Thus, the early CPS gene duplication event does not seem to have led to evolution of labdane-related diterpenoid phytoalexin biosynthesis in the same manner in rice and maize.

Barley (*Hordeum*) and wheat (*Triticum*) also fall within the BEP clade of Poaceae and are clearly more closely related to rice than maize. While neither barley nor wheat have been shown to produce labdane-related diterpenoids other than GA, chromosome mapping in barley, wheat, and rice with barley gene probes has demonstrated that barley and wheat similarly contain multiple copies of CPS-, KS-, and KO-like genes (Spielmeyer et al., 2004). Furthermore, some of these barley genes seem to be most homologous (i.e. map most strongly) to rice genes now known to be involved in phytoalexin biosynthesis. Hence, although no labdane-related diterpenoid phytoalexins have yet been reported in barley or wheat, it seems possible that defense related production of this type of natural product also occurs in these plants, which would further suggest that production of labdane-related diterpenoid phytoalexins is widespread in the cereal crop family, and would be consistent with early evolution and broad long-term retention of this type of biosynthesis in Poaceae. Regardless, either broad long-term retention or convergent evolution of such defensive phytochemical production indicates that labdane-related diterpenoid natural products are particularly good scaffolds for assembling effective antibiotics.

In discussing the small labdane-related diterpene synthase gene clusters found in the rice genome, Sakamoto et al. (2004) noted the presence of multiple retrotransposon-like sequences within these clusters. Retrotransposition then was suggested to provide a mechanism for the observed extensive duplication of CPS and KS genes in rice. Consistent with such a mechanism, Sakamoto et al. (2004) further noted the frequent occurrence of small pieces of CPS-like and KS-like sequences in the rice genome, as could be expected from retrotransposition mediated gene family expansion. Such mobility also has been suggested to underlie the observed functional clustering of consecutively acting class II and class I labdane-related diterpene synthases (Wilderman et al., 2004). In addition, Sakamoto et al. (2004) pointed out that these gene clusters contain cytochromes P450 that are similar to geraniol-10-hydroxylase (i.e. terpenoid hydroxylases), which they and others (Otomo et al., 2004a) have suggested also may act in labdane-related diterpenoid phytoalexin biosynthesis. Finally, while similar gene clustering has not yet been demonstrated in any other cereal crop plant, the barley, wheat, and rice chromosomal gene mapping of CPS- and KS-like genes placed some of the mapped homologs in regions known to be syngenic with the rice labdane-related diterpenoid gene clusters (Spielmeyer et al., 2004). This suggests that these clusters may have arisen at least prior to the split between the rice containing Ehrhartoideae and wheat/barley containing Pooideae subfamilies in the BEP clade of Poaceae (Fig. 5). As sequence information becomes available in these syngenic regions

for barley and wheat, as well as in maize, the timing of such clustering, and presumably associated evolution of labdane-related diterpenoid secondary metabolism, will become clearer.

6. Conclusions

With the recent identification of genes responsible for early biosynthetic steps in labdane-related diterpenoid phytoalexin biosynthesis, there is now an opportunity to test the role of these natural products, both generally and for individual groups, in rice blast disease resistance, as well as other processes. In particular, the available evidence suggests a broader role for these compounds as more general antibiotics, potentially including anti-viral activity, and as allelochemicals. This last role is intriguing, as not only the *syn*-CPP producing OsCPS4, but also the *ent*-CPP producing OsCPS2 (M. Xu and R.J.P., unpublished results), along with some *ent*-CPP specific KSL family members (Margis-Pinheiro et al., 2005), are constitutively expressed in roots. Accordingly, the associated compounds may be constitutively secreted from roots, which would suggest a role for these labdane-related diterpenoids in the rhizosphere. For example, through antibiotic activity mediated local suppression of soil microorganisms, enabling root establishment and access to nutrients, and/or as signaling molecules facilitating the establishment of beneficial plant-microbe interactions. Some insight into the roles played by these natural products may be gained by study of the molecular regulation mechanisms (promoters, transcription factors, signaling pathways, etc.) that control the corresponding metabolic pathways. Elucidation of these physiological roles will be important not only for rice, but, given the wide-spread occurrence of labdane-related diterpenoid metabolism, also should apply to the rest of the cereal crop and greater grass plant family as well. Thus, while much progress has been made, much more remains to be done!

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