Identification of *Syn*-Pimara-7,15-Diene Synthase Reveals Functional Clustering of Terpene Synthases Involved in Rice Phytoalexin/Allelochemical Biosynthesis[1]

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Rice (*Oryza sativa*) produces momilactone diterpenoids as both phytoalexins and allelochemicals. Accordingly, the committed step in biosynthesis of these natural products is catalyzed by the class I terpene synthase that converts *syn*-copalyl diphosphate to the corresponding polycyclic hydrocarbon intermediate *syn*-pimara-7,15-diene. Here, a functional genomics approach was utilized to identify a *syn*-copalyl diphosphate specific 9β-pimara-7,15-diene synthase (OsDTS2). To our knowledge, this is the first identified terpene synthase with this particular substrate stereoselectivity and, by comparison with the previously described and closely related *ent*-copalyl diphosphate specific cassa-12,15-diene synthase (OsDTC1), provides a model system for investigating the enzymatic determinants underlying the observed difference in substrate specificity. Further, *OsDTS2* mRNA in leaves is up-regulated by conditions that stimulate phytoalexin biosynthesis but is constitutively expressed in roots, where momilactones are constantly synthesized as allelochemicals. Therefore, transcription of *OsDTS2* seems to be an important regulatory point for controlling production of these defensive compounds. Finally, the gene identified here as *OsDTS2* has previously been mapped at 14.3 cM on chromosome 4. The class II terpene synthase producing *syn*-copalyl diphosphate from the universal diterpenoid precursor geranylgeranyl diphosphate was also mapped to this same region. These genes catalyze sequential cyclization steps in momilactone biosynthesis and seem to have been evolutionarily coupled by physical linkage and resulting cosegregation. Further, the observed correlation between physical proximity and common metabolic function indicates that other such class I and class II terpene synthase gene clusters may similarly catalyze consecutive reactions in shared biosynthetic pathways.

Plants produce a vast and diverse array of low-molecular weight organic compounds, the overwhelming majority of which are secondary metabolites with nonessential, yet important, functions such as defense (Croteau et al., 2000). For example, phytoalexins are produced in response to microbial infections and exhibit antimicrobial activity (VanEtten et al., 1994), while allelochemicals are secreted to the rhizosphere and suppress the growth of neighboring plants (Bais et al., 2004). Often found serving in such roles are terpenoids, which are particularly abundant in plant metabolism and form the largest class of natural products, exhibiting wide diversity in chemical structure and biological function (Croteau et al., 2000). Much of the structural variation within this class arises from the diverse carbon backbones formed by terpene synthases (cyclases). These divalent metal dependent enzymes carry out complex electrophilic cyclizations/rearrangements to create these diverse skeletal structures from relatively simple acyclic precursors (Davis and Croteau, 2000). Notably, production of a specific backbone structure either dictates, or at least severely restricts, the metabolic fate of that particular molecule. Thus, terpenoid natural products biosynthesis is often controlled by regulating terpene synthase activity (e.g. gibberellin biosynthesis; Silverstone et al., 1997).

A substantial fraction of the known terpenoids can be classified as labdane-related diterpenoids, a large group of over 5,000 known compounds that minimally contain the bicyclic hydrocarbon structure found in the labdane family of diterpenes. This structural core is formed by class II terpene synthases, which selectively produce specific stereoisomers of labdadienyl/copalyl diphosphate (CPP) from the universal diterpenoid precursor (*E*,*E*,*E*)-geranylgeranyl diphosphate (GGPP). In addition, this core structure can be further modified and/or rearranged by stereoselective CPP specific class I terpene synthases, as in the related/derived structural families (e.g. gibberellins, abietanes, and [iso]pimaradienes). Thus, class II and class I terpene synthases act sequentially in catalyzing stereochemically coupled cyclization reactions to form labdane-related skeletal backbones (e.g. Fig. 1).

While the two classes of terpene synthases are clearly phylogenetically related (Bohlmann et al., 1998a) and both catalyze electrophilic cyclization/rearrangement reactions, each utilizes completely distinct initiation mechanisms. Most commonly, terpene synthases (i.e. the class I enzymes) initiate their

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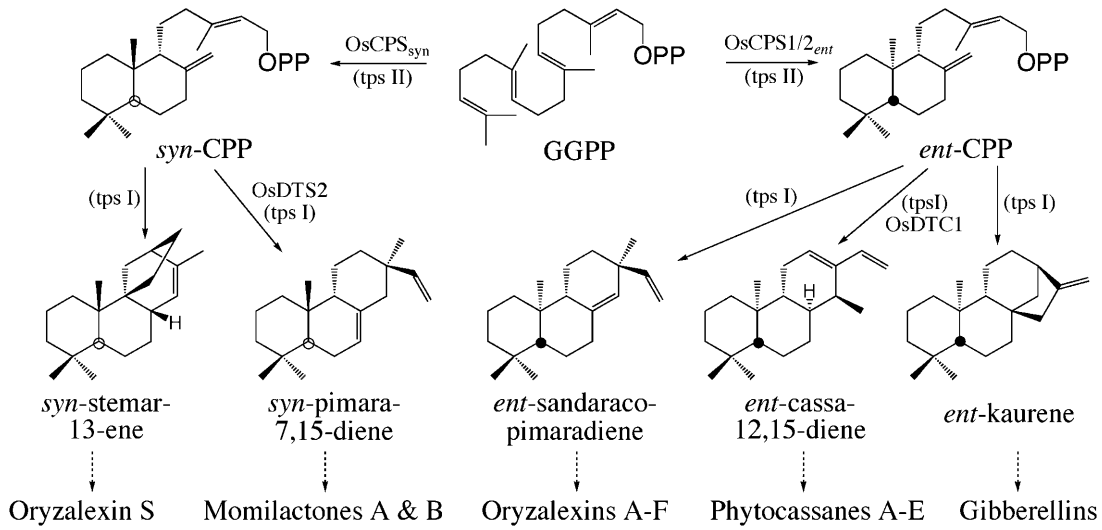

Figure 1. Known cyclization steps in labdane-related diterpenoid biosynthesis in rice. Reactions catalyzed by class II (tpsII) or class I (tpsI) terpene synthases are indicated, along with the corresponding classes of natural products derived from each of the named polycyclic hydrocarbon structures (dashed arrows indicate multiple enzymatic steps).

reactions through ionization of the allylic diphosphate moiety of their substrate. This divalent metal ion dependent reaction requires a DDXXD metal binding sequence that is the signature motif of class I terpene synthases (Davis and Croteau, 2000). In contrast, the class II enzymes initiate cyclization via protonation of the terminal carbon-carbon double bond in GGPP and contain a separately placed DXDD sequence rather than the class I associated DDXXD motif (Sun and Kamiya, 1994). Notably, prototypical class I enzymes are similar in size and contain two structurally defined domains (Starks et al., 1997; Whittington et al., 2002). However, some terpene synthases, in particular all of those involved in labdane-related diterpenoid biosynthesis (Fig. 2), contain a large amount of additional N terminal sequence termed the 'insertional' element (approximately 240 residues; Peters and Croteau, 2002).

Rice provides a model system to investigate labdane-related diterpenoid biosynthesis, as this genomically characterized plant (Goff et al., 2002; Yu et al., 2002) produces a number of such natural products beyond the ubiquitous gibberellin growth hormones (Fig. 1). These compounds include momilactones A and B (Kato et al., 1973; Cartwright et al., 1981), oryzalexins A–F (Akatsuka et al., 1985; Sekido et al., 1986; Kato et al., 1993, 1994), oryzalexin S (Kodama et al., 1992), and phytocassanes A–E (Koga et al., 1995, 1997). All of these natural products exhibit antimicrobial properties and are synthesized as part of the defensive response to leaf infection with the blast pathogenic fungus *Magneportha grisea*, thus qualifying as phytoalexins (VanEtten et al., 1994). In addition, momilactones A and B also act as allelochemicals, as these were originally identified as dormancy factors from rice seed husks (Kato et al., 1973). More recently, momilactone B has been shown to be constitutively secreted from the roots of rice seedlings (Kato-Noguchi and Ino, 2003) as an allelopathic agent (Kato-Noguchi et al., 2002). Further, secretion of antimicrobial agents to the rhizosphere may also provide a competitive advantage for root establishment through local suppression of soil microorganisms (Bais et al., 2004).

Conveniently, rice leaves produce all of these secondary metabolites after UV-irradiation as well as blast fungal infection (Cartwright et al., 1977), providing a by-now standard method for inducing biosynthesis of these natural products (Kodama et al., 1988). For example, UV-irradiation induces biosynthesis of *ent*-sandaracopimaradiene, *syn*-pimara-7,15-diene, and *syn*-stemar-13-ene, the putative precursors to oryzalexins A–F, momilactones A and B, and oryzalexin S, respectively (Wickham and West, 1992). These polycyclic diterpene hydrocarbons have been shown to be selectively produced via CPP of the corresponding stereochemistry (i.e. *ent* or *syn*; Mohan et al., 1996). Recent work has identified the class I diterpene cyclase (OsDTC1) producing *ent*-cassa-12,15-diene, precursor to phytocassanes A–E (Yajima et al., 2004), stereoselectively from *ent*-CPP (Cho et al., 2004). In addition, a very recent report detailed the identification of rice gibberellin specific enzymatic genes by mutant plant phenotype and also mapped all the potentially relevant genes on the genome, revealing clustering of certain class II and class I terpene synthases (Sakamoto et al., 2004). However, the genes involved in gibberellin biosynthesis are not clustered together. Because gene isolation and biochemical characterization were not otherwise reported, it is not known if the clustered genes operate sequentially in common metabolic path-

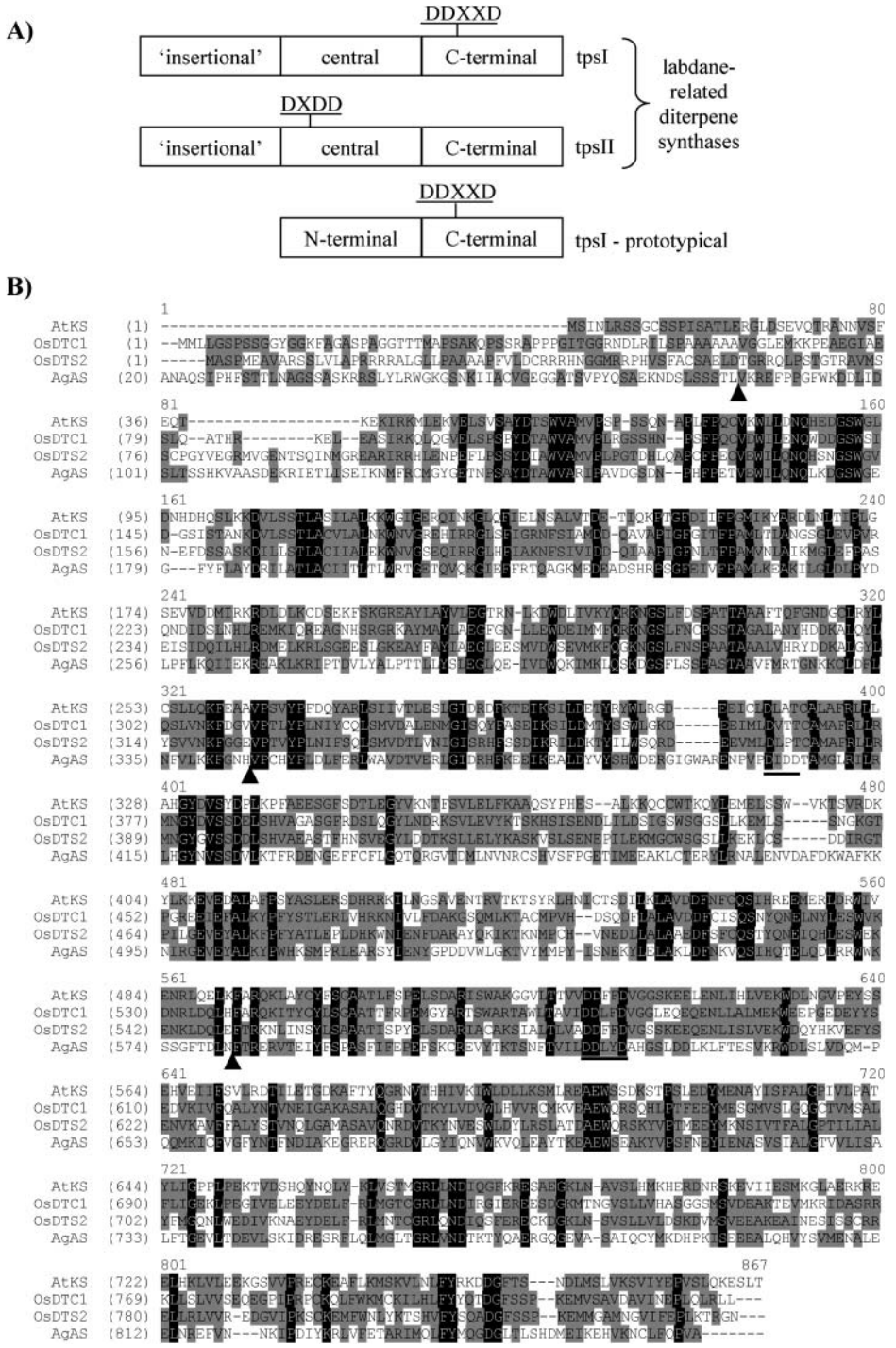

Figure 2. Terpene synthase comparisons. A, Domain schematic for prototypical class I terpene synthases (tpsI) and the class I and class II (tpsII) enzymes involved in labdane-related diterpene biosynthesis. Modeled on the structures determined for the typical class I *epi*-aristolochene and bornyl diphosphate synthases (Starks et al., 1997; Whittington et al., 2002), along with the additional insertional sequence element. Approximate locations of the Asp-rich motifs are also indicated. For clarity, the transit sequence required in mono- and diterpene synthases for their plastidal location in planta is not shown. B, Sequence comparison of OsDTS2 with other class I terpene synthases. Alignment with the previously identified rice *ent*-cassa-12,15-diene synthase (OsDTC1), Arabidopsis *ent*-kaurene synthase (AtKS), and bifunctional class II/I cyclase from *Abies grandis* abietadiene synthase (AgAS). The approximate domain boundaries, based on previous analysis of AgAS (Peters et al., 2003), are marked with arrowheads, and the class II associated DXDD and class I associated DDXXD motifs are underlined.

ways. We have functionally identified the three class II terpene synthases from rice: a *syn*-CPP synthase (OsCPS$_{syn}$) involved in phytoalexin/allelochemical biosynthesis (Xu et al., 2004), and two disparate *ent*-CPP synthases that are separately responsible for gibberellin (OsCPS1$_{ent}$) or phytoalexin (OsCPS2$_{ent}$) biosynthesis (S. Prisic, M. Xu, P.R. Wilderman, and R.J. Peters, unpublished data). Here we report functional identification of a rice class I terpene synthase gene as a *syn*-CPP specific 9β-pimara-7,15-diene synthase (OsDTS2). Both *OsDTS2* and *OsCPS$_{syn}$* map to 14.3 cM on chromosome 4 (Sakamoto et al., 2004). Thus, their shared metabolic function, catalysis of consecutive cyclization reactions to initiate biosynthesis of the phytoalexin/allelochemical

momilactones A and B, seems to have led to evolutionary coupling through physical linkage and resulting cosegregation.

RESULTS

Isolation of a Class I Terpene Synthase cDNA from UV-Irradiated Rice Leaves

The class I terpene synthases involved in labdane-related diterpenoid biosynthesis discriminate between different stereoisomers of CPP and, thus, provide a model system for investigating the underlying active site steric constraints. In addition, terpene synthases are conserved by taxonomic origin rather than biochemical function (Bohlmann et al., 1998a). Therefore, we were interested in identifying a *syn*-CPP specific class I terpene synthase from rice to complement the previously identified *ent*-CPP specific cassa-12,15-diene synthase (Cho et al., 2004). Towards this end, an initial class I terpene synthase with potential involvement in labdane-related diterpenoid biosynthesis (i.e. DDXXD motif and 'insertional' element) was found among the genes predicted from the finished sequence of rice chromosome 4 (accession no. CAD39507; Feng et al., 2002). The corresponding sequence was readily amplified from UV-irradiated rice leaves, cloned, and verified by complete sequencing of two independent isolates, demonstrating approximately 99% identity to the predicted sequence. Presumably the few observed differences are a function of intersubspecies variation between the ssp. *japonica* sequenced by Feng et al. (2002) and ssp. *indica* used here (our sequence has been deposited into the various nucleotide sequence databases as accession no. AY616862).

Sequence Comparison Suggested a Role in Labdane-Related Diterpenoid Biosynthesis

The cloned open reading frame encodes a protein of 840 amino acids that contains the 'insertional' element associated with labdane-related diterpene synthases (Fig. 2A). Also present is the catalytically requisite class I DDXXD motif, as found in the original gene prediction (accession no. CAD39507). Notably, the currently predicted gene product does not include the exon containing this metal binding motif (accession no. CAD39717), indicating that caution must be taken when analyzing predicted genes (i.e. the current prediction might be mistaken as a nonfunctional pseudo-gene). Significantly, the DXDD motif required for class II cyclization is not found. Alignment of the complete amino acid sequence with that of known 'insertional' element containing class I terpene synthases (e.g. Fig. 2B) demonstrated only weak similarity (<27% identity) to those not involved in labdane-related diterpenoid biosynthesis (Dudareva et al., 1996; Wildung and Croteau, 1996; Bohlmann et al., 1998b). Slightly higher similarity (approximately 30% identity) was found with the identified gymnosperm bifunctional class II/I diterpene synthases, which are involved in labdane-related biosynthesis (Stofer Vogel et al., 1996; Schepmann et al., 2001; Martin et al., 2004). Further, the sequence is moderately similar (39%–42% identity) to the known *ent*-kaurene synthases involved in gibberellin biosynthesis (Yamaguchi et al., 1996, 1998; Richman et al., 1999). Finally, comparison with the recently identified *ent*-CPP specific cassa-12,15-diene synthase (Cho et al., 2004) revealed significant homology (approximately 51% identity). These results suggested that the isolated sequence is also a class I labdane-related (i.e. CPP specific) diterpene synthase (OsDTS2).

Functional Characterization of OsDTS2 as *Syn*-CPP Specific 9β-Pimara-7,15-Diene Synthase

OsDTS2 was expressed both alone and as a fusion protein to glutathione-S-transferase (GST-OsDTS2). Recombinant preparations were assayed with GGPP, *ent*-CPP, or *syn*-CPP as substrate and enzymatic activity detected using gas chromatography-mass spectrometry (GC-MS) analysis of organic extracts of the assays. Only the GST-OsDTS2 fusion protein exhibited appreciable amounts of activity, indicating the transit peptide (required for plastidial import in planta) hinders folding of the full-length preprotein in the absence of the stabilizing effect provided by the fused GST structure, as found in previous studies (Williams et al., 1998; Peters et al., 2000). Further, enzymatic turnover was only observed with *syn*-CPP, as no products were detected from reactions with GGPP or *ent*-CPP. Therefore, OsDTS2 is stereoselective and represents the first identified class I terpene synthase specific for *syn*-CPP. Finally, comparison with the known synthetic standards (Mohan et al., 1996) unambiguously identified the enzymatic product as *syn*-pimara-7,15-diene (Fig. 3).

Expression Pattern of OsDTS2 mRNA

The production of 9β-pimara-7,15-diene from *syn*-CPP is the committed step in momilactone biosynthesis (Fig. 1). Therefore, regulation of the corresponding activity (i.e. OsDTS2) provides a logical point for controlling the production of these specific labdane-related diterpenoid natural products. Previous review of the relevant literature has been used to suggest that plant secondary metabolism is most often regulated at the level of transcription (Peters and Croteau, 2004). This has been demonstrated for the preceding enzyme $OsCPS_{syn}$, where the corresponding mRNA increases prior to *syn*-labdane-related diterpenoid phytochemical accumulation in UV-irradiated rice leaves (Xu et al., 2004). Nevertheless, it seemed likely that OsDTS2 would also be regulated, as OsDTC1 is similarly controlled (Cho et al., 2004), in addition to transcriptional control of the upstream enzyme $OsCPS2_{ent}$ (S.

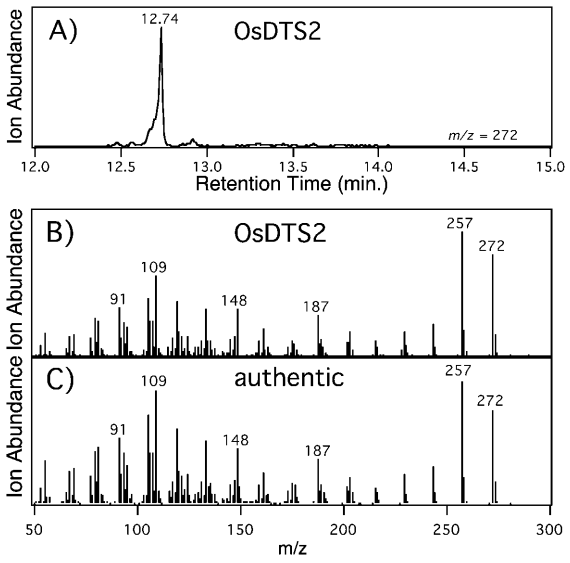

Figure 3. Production of *syn*-pimara-7,15-diene. A, GC-MS analysis (272 *m/z* extracted ion chromatograph) of the product derived from *syn*-CPP by OsDTS2. B, Mass spectrum of the GC-MS 272 *m/z* chromatograph peak for OsDTS2 (RT = 12.74 min). C, Mass spectrum from an authentic standard for 9β-pimara-7,15-diene, which also exhibits RT = 12.74 min.

Prisic, M. Xu, P.R. Wilderman, and R.J. Peters, unpublished data). Such a control mechanism was investigated through the use of UV-irradiation, which has long been appreciated to induce phytoalexin biosynthesis in rice (Cartwright et al., 1977). Further, quantitative analysis of phytochemical accumulation for the detached leaf UV-irradiation induction method used here has been previously reported (Kodama et al., 1988). Hence, the ability of UV-irradiation to induce expression of *OsDTS2* mRNA in rice leaves was characterized, demonstrating transcriptional up-regulation prior to phytoalexin accumulation (Fig. 4). To verify that such transcriptional control is part of the normal regulatory mechanism for phytoalexin biosynthesis, it was further demonstrated that *OsDTS2* mRNA levels are also increased by methyl jasmonate (MeJA), an important plant defense signaling molecule (Farmer and Ryan, 1990). MeJA has also been previously demonstrated to induce phytoalexin biosynthesis in rice cell culture (Nojiri et al., 1996), as well as transcription of the phytoalexin specific class II terpene synthases $OsCPS2_{ent}$ and $OsCPS_{syn}$ (S. Prisic, M. Xu, P.R. Wilderman, and R.J. Peters, unpublished data; Xu et al., 2004). Finally, as expected from its requisite role in constant production of an allelochemical (Kato-Noguchi and Ino, 2003), *OsDTS2* mRNA seems to be constitutively present in roots, albeit at a low level, again correlated with the expression pattern for $OsCPS_{syn}$ (Xu et al., 2004). These results strongly indicate that, in addition to the previously observed transcriptional regulation of $OsCPS_{syn}$ (Xu et al., 2004), biosynthesis of the phytoalexin/allelochemical momilactones is also more specifically controlled by transcriptional regulation of *OsDTS2*.

DISCUSSION

A class I terpene synthase containing sequence characteristics associated with labdane-related diterpene biosynthesis (*OsDTS2*) was found among the genes initially predicted from the finished sequence of rice chromosome 4 (Feng et al., 2002). The corresponding sequence was readily cloned from UV-irradiated rice leaves, which are known to produce a number of labdane-related diterpenoid phytoalexins (Kodama et al., 1988). Functional characterization demonstrated that OsDTS2 is specific for *syn*-CPP, producing 9β-pimara-7,15-diene (Fig. 3). Notably, OsDTS2 represents the first identified terpene synthase to exhibit stereoselectivity for *syn*-, rather than *ent*- or normal CPP. In addition, OsDTS2 is approximately 51% identical to the *ent*-CPP specific OsDTC1 identified by Cho et al. (2004). Hence, these provide an ideal model system for comparative investigation of the differential enzymatic determinants specifying the observed substrate selectivity for configurationally varied stereoisomers of CPP (Fig. 5). Further, radio-tracer biosynthetic studies indicate that rice produces at least seven different polycyclic diterpenes derived from *ent*- or *syn*-CPP (Mohan et al., 1996), and the essentially single product output of OsDTC1 and OsDTS2 sug-

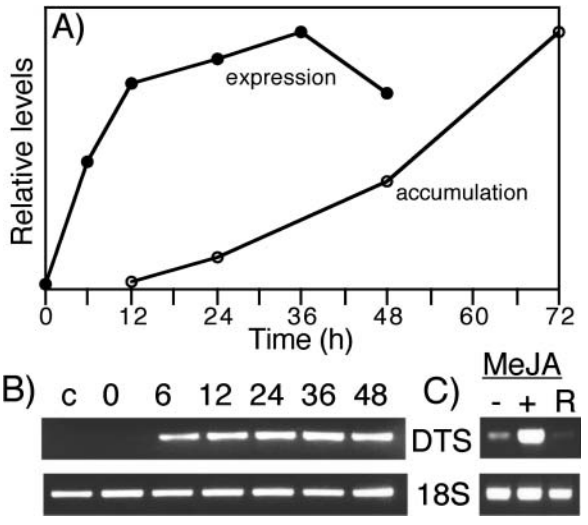

Figure 4. OsDTS2 expression analysis. A, Graphical comparison of OsDTS2 mRNA levels (black circles) and momilactone accumulation (white circles; as described by Kodama et al., 1988), in UV-irradiated detached leaves. Semi-quantitative RT-PCR analysis of OsDTS2 mRNA expression levels is shown in B and C. Specific bands corresponding to the 18S rRNA control and OsDTS2 (DTS) are indicated. B, Expression in response to UV-irradiation. Time (hours) after exposure is indicated (c = control leaves after approximately 18 h). C, Expression in untreated 4-week-old plant roots (R) or in germinated seedlings in response to application of 0.5 mM methyl jasmonate (+MeJA) or water control (−MeJA).

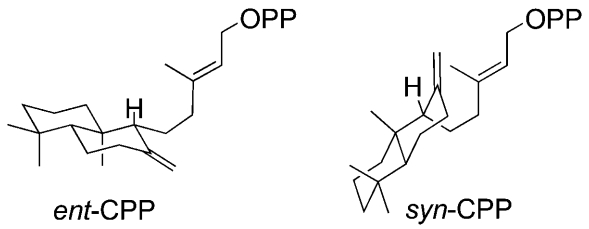

Figure 5. Configurational differences between *ent*- and *syn*-CPP. Both are depicted in the sterically less hindered boat-boat conformation with similar positions for the diphosphate group whose relative position in the active site is fixed by the conserved divalent metal ion binding sites. OsDTC1 and OsDTS2 are able to distinguish between these two stereoisomers based on these configurational differences.

gests that there will be individual stereospecific class I diterpene synthases responsible for each of these. From the extensive genomic and cDNA sequence information available for rice (Goff et al., 2002; Yu et al., 2002; Kikuchi et al., 2003) there are a total of nine putative insertional element containing class I terpene synthases, as indicated by Cho et al. (2004) and Sakamoto et al. (2004), as well as our own searches of the relevant databases. Thus, functional characterization of these genes is expected to further increase the utility of this model system by providing additional sequences and enzymatic targets for comparative analysis.

Notably, production of *syn*-pimara-7,15-diene is the committed step in momilactone biosynthesis, the end products of which exhibit both phytoalexin and allelochemical properties (Cartwright et al., 1981; Kato-Noguchi and Ino, 2003). Therefore, OsDTS2 provides a logical regulatory target for controlling production of these important phytochemicals. Expression analysis demonstrated that *OsDTS2* mRNA is only found in association with momilactone biosynthesis (Fig. 4). Accordingly, *OsDTS2* is constitutively expressed in the roots for constant allelochemical production, but is only found in leaves under conditions that induce phytoalexin biosynthesis (i.e. UV-irradiation or exposure to MeJA). These results strongly indicate that OsDTS2 activity is controlled by transcriptional regulation.

Intriguingly, in their investigation of rice gibberellin biosynthesis Sakamoto et al. (2004) mapped all terpene synthases with potential involvement in labdane-related diterpenoid biosynthesis to their chromosomal locations, demonstrating cosegregation and relatively close physical proximity for a number of these genes. In particular, the previously identified *syn*-CPP producing $OsCPS_{syn}$ (Xu et al., 2004) and the *syn*-CPP substrate specific *OsDTS2* identified here have both been mapped at 14.3 cM on chromosome 4 and are within 120 kb of each other. These enzymes catalyze sequential cyclization reactions to initiate momilactone biosynthesis (Fig. 1). Therefore, these two physically linked genes act in a common metabolic pathway, similar to the grouping of presumably consecutively acting prenyltransferases and terpene synthases noted in the genome of Arabidopsis (Aubourg et al., 2002). Cosegregation of these stereochemically coupled class II and class I terpene synthases provides an evolutionary mechanism linking their shared biosynthetic functions. Such correlation between physical proximity and sequential function in a common biosynthetic pathway is also consistent with our data supporting a specific role for the *ent*-CPP producing $OsCPS2_{ent}$ in secondary metabolism (S. Prisic, M. Xu, P.R. Wilderman, and R.J. Peters, unpublished data), since both $OsCPS2_{ent}$ and the *ent*-CPP substrate specific *OsDTC1* identified by Cho et al. (2004) have been mapped at 86 cM on chromosome 2. Two other class I terpene synthases also map to this region, with all four genes being found within a stretch of 150 kb. Hence, we speculate that these two additional enzymes will also function in *ent*-labdane-related diterpenoid secondary metabolism (i.e. specifically utilize *ent*-CPP), forming another functional grouping linked by stereochemically coupled sequential cyclization reactions. Finally, both of these terpene synthase gene clusters contain retrotransposable elements, suggesting a means by which duplication and, hence, diversification of these genes and/or clusters may have occurred. Thus, it appears the correlation between physical proximity and shared metabolic function of terpene synthases has important implications for the evolutionary mechanism underlying development of the extensive and diverse labdane-related diterpenoid secondary metabolism exhibited by rice.

CONCLUSION

In summary, we have used a functional genomics approach to identify a 9β-pimara-7,15-diene synthase (OsDTS2). Notably, the observed specificity for *syn*-CPP represents a novel substrate stereoselectivity. Thus, OsDTS2, in combination with the previously identified and relatively closely related (approximately 51% identity) *ent*-CPP specific OsDTC1 (Cho et al., 2004), offers an ideal model system for future structure/function investigations of steric constraints within the active site of class I terpene synthases. In addition, OsDTS2 catalyzes the committed step in biosynthesis of the phytoalexin/allelochemical momilactones, and its transcription seems to represent an important control point for regulation of these important metabolic processes. Finally, functional characterization of *OsDTS2*, along with the previous identification of $OsCPS_{syn}$ (Xu et al., 2004), demonstrates that the previously reported physical proximity and cosegregation of these genes reflects their consecutive action in a common metabolic pathway (i.e. momilactone biosynthesis). We further speculate that other such terpene synthase gene clusters may also share metabolic function in catalyzing sequential, stereochemically linked cyclization reactions.

MATERIALS AND METHODS

Chemicals

Synthesis of (*E*,*E*,*E*)-GGPP, *ent*- and *syn*-CPP, and the polycyclic hydrocarbon standards *ent*-kaurene, *ent*-sandaracopimaradiene, *syn*-pimara-7,15-diene, and *syn*-stemar-13-ene have been previously described (Mohan et al., 1996). Unless otherwise noted, all other chemicals were purchased from Fisher Scientific (Loughborough, Leicestershire, UK).

Plant Material

Rice plants (*Oryza sativa* L. ssp. *indica* cv IR24) and seedlings (ssp. *japonica* cv Nipponbare) were those previously described (Xu et al., 2004). Briefly, detached leaves from 4-week-old greenhouse grown plants were UV-irradiated at 254 nm from 15-cm distance for 25 min and then incubated for the indicated period of time under dark humid conditions at 30°C. Seedlings were germinated from surface sterilized seeds under sterile, humid conditions at 30°C in the dark for a week. The seedlings then underwent MeJA treatment, being sprayed with approximately 2 mL 0.1% Tween 20 ± 0.5 mM MeJA/gram of plant weight, and the seedlings then incubated for two more days under the same conditions. RNA was isolated using Concert Plant Reagent (Invitrogen, Carlsbad, CA). Semiquantitative reverse transcription (RT)-PCR expression analysis, using 0.5 μg total RNA and *OsDTS2*-specific primers or QuantumRNA 18S standard primers (Ambion, Austin, TX), was also carried out as described by Xu et al. (2004).

Cloning

A putative class I terpene synthase involved in labdane-related biosynthesis was identified by a BLAST search of the GenBank database (www.ncbi.nlm.nih.gov:80/BLAST/) with the amino acid sequence of *ent*-kaurene synthase from Arabidopsis (Yamaguchi et al., 1998). RT-PCR reactions were performed to verify expression of the predicted gene in UV-irradiated leaves by generating a fragment of the corresponding sequence. This was verified by cloning into pCR-Zero-Blunt (Invitrogen) and complete sequencing. These primers were also then used for the semiquantitative RT-PCR expression analysis. The complete open reading frame for OsDTS2 was then amplified from total RNA in an RT-PCR reaction using the GeneRacer kit (Invitrogen), cloned into pENTR/SD/D-TOPO (Invitrogen), and verified by complete sequencing. OsDTS2 was then transferred by directional recombination to the T7 based expression vectors pDEST14 and pDEST15 (Gateway system, Invitrogen).

Recombinant Expression and Functional Characterization

Expression was carried out with the BL21-derived C41 strain (Miroux and Walker, 1996), as described for $OsCPS_{syn}$ by Xu et al. (2004). Briefly, cells were grown to midlog phase at 37°C then shifted to 16°C for 1 to 2 h prior to induction (1 mM IPTG) and overnight expression. The cells were harvested by centrifugation (3,000*g*, 20 min, 5°C), resuspended in 1 mL of lysis buffer (50 mM Bis-Tris, pH 6.8, 150 mM KCl, 10 mM $MgCl_2$, 1 mM DTT), and lysed by mild sonication on ice (Branson sonifier 450: 20 s, continuous, output setting 5). The resulting lysates were cleared by centrifugation (15,000*g*, 30 min, 5°C) and filtration (0.8 μ) to yield recombinant soluble extracts. Enzymatic assays were performed with these preparations under standard conditions defined for diterpene synthase activity (e.g. Peters et al., 2000). Briefly, reactions with approximately 50 μM substrate (GGPP, *ent*-, or *syn*-CPP) were carried out in assay buffer (50 mM HEPES, pH 7.2, 100 mM KCl, 7.5 mM $MgCl_2$, 5% glycerol, and 5 mM DTT) with 25 μL of recombinant protein in a total volume of 0.2 mL. The reaction was allowed to proceed for 3 h at 30°C prior to extraction with hexanes. GC-MS analysis was performed using an HP-5 column on an Agilent (Palo Alto, CA) 6890N GC instrument with 5973N mass selective detector. Samples (5 μL) were injected at 40°C in the splitless mode. After holding 3 min at 40°C, the temperature was increased at 20°C/min to 300°C, where it was held for 3 min. MS data was collected from 50 to 500 *m/z* during the temperature ramp. The retention time and MS pattern were compared to those for *syn*-stemar-13-ene and *syn*-pimara-7,15-diene, as well as sandaracopimaradiene and *ent*-kaurene.

Sequence Analysis and Alignments

Sequence alignments and identity calculations were performed with the AlignX program in the Vector NTI software package (Invitrogen), using standard parameters. OsDTS2 was the reference sequence in all cases. The class I terpene synthases not involved in labdane-related diterpenoid biosynthesis, yet containing 'insertional' elements, are linalool synthase from *Clarkia breweri* (Dudareva et al., 1996), taxadiene synthase from *Taxus brevifolia* (Wildung and Croteau, 1996), and bisabolene synthase from *Abies grandis* (Bohlmann et al., 1998b). Bifunctional class II/I terpene synthases producing labdane-related diterpenes of normal stereochemistry are those from the gymnosperms *A. grandis* (Stofer Vogel et al., 1996), *Ginkgo biloba* (Schepmann et al., 2001), and *Picea abies* (Martin et al., 2004). The *ent*-kaurene synthases were those from *Curcurbita maxima* (Yamaguchi et al., 1996), Arabidopsis (Yamaguchi et al., 1998), and *Stevia rebaudiana* (Richman et al., 1999). Other rice class I terpene synthases with potential involvement in labdane-related diterpenoid biosynthesis were identified by BLAST queries of GenBank, and the cDNA databases at KOME (cdna01.dna.affrc.go.jp/cDNA/) and TIGR (tigrblast.tigr.org/tgi/), using Arabidopsis *ent*-kaurene synthase as the probe sequence.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AY616862.

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LITERATURE CITED

Akatsuka T, Kodama O, Sekido H, Kono Y, Takeuchi S (1985) Novel phytoalexins (oryzalexins A, B, and C) isolated from rice blast leaves infected with *Pyricularia oryzae*. Part I: isolation, characterization and biological activities of oryzalexins. Agric Biol Chem **49:** 1689–1694

Aubourg S, Lecharny A, Bohlmann J (2002) Genomic analysis of terpenoid synthase (AtTPS) gene family of *Arabidopsis thaliana*. Mol Genet Genomics **267:** 730–745

Bais HP, Park S-W, Weir TL, Callaway RM, Vivanco JM (2004) How plants communicate using the underground information superhighway. Trends Plant Sci **9:** 26–32

Bohlmann J, Crock J, Jetter R, Croteau RB (1998b) Terpenoid-based defenses in conifers: cDNA cloning, characterization, and functional expression of wound-inducible (*E*)-alpha-bisabolene synthase from grand fir (*Abies grandis*). Proc Natl Acad Sci USA **95:** 6756–6761

Bohlmann J, Meyer-Gauen G, Croteau R (1998a) Plant terpenoid synthases: molecular biology and phylogenetic analysis. Proc Natl Acad Sci USA **95:** 4126–4133

Cartwright DW, Langcake P, Pryce RJ, Leworthy DP, Ride JP (1977) Chemical activation of host defence mechanisms as a basis for crop protection. Nature **267:** 511–513

Cartwright DW, Langcake P, Pryce RJ, Leworthy DP, Ride JP (1981) Isolation and characterization of two phytoalexins from rice as momilactones A and B. Phytochemistry **20:** 535–537

Cho E-M, Okada A, Kenmoku H, Otomo K, Toyomasu T, Mitsuhashi W, Sassa T, Yajima A, Yabuta G, Mori K, et al (2004) Molecular cloning and characterization of a cDNA encoding *ent*-cassa-12,15-diene synthase, a putative diterpenoid phytoalexin biosynthetic enzyme, from suspension-cultured rice cells treated with a chitin elicitor. Plant J **37:** 1–8

Croteau R, Kutchan TM, Lewis NG (2000) Natural products (secondary metabolites). *In* B Buchanan, W Gruissem, R Jones, eds, Biochemistry and Molecular Biology of Plants. American Society of Plant Biologists, Rockville, MD, pp 1250–1318

Davis EM, Croteau R (2000) Cyclization enzymes in the biosynthesis of monoterpenes, sesquiterpenes, and diterpenes. Top Curr Chem **209:** 53–95

Dudareva N, Cseke L, Blanc VM, Pichersky E (1996) Evolution of floral

scent in *Clarkia*: novel patterns of *S*-linalool synthase gene expression in the *C. breweri* flower. Plant Cell **8:** 1137–1148

Farmer EE, Ryan CA (1990) Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc Natl Acad Sci USA **87:** 7713–7716

Feng Q, Zhang Y, Hao P, Wang S, Fu G, Huang Y, Li Y, Zhu J, Liu Y, Hu X, et al (2002) Sequence and analysis of rice chromosome 4. Nature **420:** 316–320

Goff SA, Ricke D, Lan TH, Presting G, Wang R, Dunn M, Glazebrook J, Sessions A, Oeller P, Varma H, et al (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica*). Science **296:** 92–100

Kato T, Kabuto C, Sasaki N, Tsunagawa M, Aizawa H, Fujita K, Kato Y, Kitahara Y (1973) Momilactones, growth inhibitors from rice, *Oryza sativa* L. Tetrahedron Lett **39:** 3861–3864

Kato H, Kodama O, Akatsuka T (1993) Oryzalexin E, a diterpene phytoalexin from UV-irradiated rice leaves. Phytochemistry **33:** 79–81

Kato H, Kodama O, Akatsuka T (1994) Oryzalexin F, a diterpene phytoalexin from UV-irradiated rice leaves. Phytochemistry **36:** 299–301

Kato-Noguchi H, Ino T (2003) Rice seedlings release momilactone B into the environment. Phytochemistry **63:** 551–554

Kato-Noguchi H, Ino T, Sata N, Yamamura S (2002) Isolation and identification of a potent allelopathic substance in rice root exudates. Physiol Plant **115:** 401–405

Kikuchi S, Satoh K, Nagata T, Kawagashira N, Doi K, Kishimoto N, Yazaki J, Ishikawa M, Yamada H, Ooka H, et al (2003) Collection, mapping, and annotation of over 28,000 cDNA clones from *japonica* rice. Science **301:** 376–379

Kodama O, Li WX, Tamogami S, Akatsuka T (1992) Oryzalexin-S, a novel stemarane-type diterpene rice phytoalexin. Biosci Biotechnol Biochem **56:** 1002–1003

Kodama O, Suzuki T, Miyakawa J, Akatsuka T (1988) Ultraviolet-induced accumulation of phytoalexins in rice leaves. Agric Biol Chem **52:** 2469–2473

Koga J, Ogawa N, Yamauchi T, Kikuchi N, Ogasawara N, Shimura M (1997) Functional moiety for the antifungal activity of phytocassane E, a diterpene phytoalexin from rice. Phytochemistry **44:** 249–253

Koga J, Shimura M, Oshima K, Ogawa N, Yamauchi T, Ogasawara N (1995) Phytocassanes A, B, C, and D, novel diterpene phytoalexins from rice, *Oryza sativa* L. Tetrahedron **51:** 7907–7918

Martin DM, Faldt J, Bohlmann J (2004) Functional characterization of nine norway spruce *TPS* genes and evolution of gymnosperm terpene synthases of the *TPS-d* subfamily. Plant Physiol **135:** 1908–1927

Miroux B, Walker JE (1996) Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. J Mol Biol **260:** 289–298

Mohan RS, Yee NKN, Coates RM, Ren YY, Stamenkovic P, Mendez I, West CA (1996) Biosynthesis of cyclic diterpene hydrocarbons in rice cell suspensions: conversion of 9,10-*syn*-labda-8(17),13-dienyl diphosphate to 9β-pimara-7,15-diene and stemar-13-ene. Arch Biochem Biophys **330:** 33–47

Nojiri H, Sugimora M, Yamane H, Nishimura Y, Yamada A, Shibuya N, Kodama O, Murofushi N, Omori T (1996) Involvement of jasmonic acid in elicitor-induced phytoalexin production in suspension-cultured rice cells. Plant Physiol **110:** 387–392

Peters RJ, Carter OA, Zhang Y, Matthews BW, Croteau RB (2003) Bifunctional abietadiene synthase: mutual structural dependence of the active sites for protonation-initiated and ionization-initiated cyclizations. Biochemistry **42:** 2700–2707

Peters RJ, Croteau RB (2002) Abietadiene synthase catalysis: conserved residues involved in protonation-initated cyclization of geranylgeranyl diphosphate to (+)-copalyl diphosphate. Biochemistry **41:** 1836–1842

Peters RJ, Croteau RB (2004) Metabolic engineering of plant secondary metabolism. *In* G Kishore, ed, Handbook of Plant Biotechnology: Applications of Plant Biotechnology in Agriculture, the Pharmaceutical Industry, and Other Industries, Vol 2. John Wiley & Sons, London, pp 609–628

Peters RJ, Flory JE, Jetter R, Ravn MM, Lee H-J, Coates RM, Croteau RB (2000) Abietadiene synthase from Grand Fir (*Abies grandis*): characterization and mechanism of action of the ''pseudomature'' recombinant enzyme. Biochemistry **39:** 15592–15602

Richman AS, Gijzen M, Starratt AN, Yang Z, Brandle JE (1999) Diterpene synthesis in *Stevia rebaudiana*: recruitment and up-regulation of key enzymes from the gibberellin biosynthetic pathway. Plant J **19:** 411–421

Sakamoto T, Miura K, Itoh H, Tatsumi T, Ueguchi-Tanaka M, Ishiyama K, Kobayashi M, Agrawal GK, Takeda S, Abe K, et al (2004) An overview of gibberellin metabolism enzyme genes and their related mutants in rice. Plant Physiol **134:** 1642–1653

Schepmann HG, Pang J, Matsuda SP (2001) Cloning and characterization of *Ginkgo biloba* levopimaradiene synthase, which catalyzes the first committed step in Ginkolide biosynthesis. Arch Biochem Biophys **392:** 263–269

Sekido H, Endo T, Suga R, Kodama O, Akatsuka T, Kono Y, Takeuchi S (1986) Oryzalexin D (3,7-dihydroxy-(+)-sandaracopimaradiene), a new phytoalexin isolated from blast-infected rice leaves. J Pestic Sci **11:** 369–372

Silverstone AL, Chang C-W, Krol E, Sun T-P (1997) Developmental regulation of the gibberellin biosynthetic gene GA1 in *Arabidopsis thaliana*. Plant J **12:** 9–19

Starks CM, Back K, Chappell J, Noel JP (1997) Structural basis for cyclic terpene biosynthesis by tobacco 5-epi-aristolochene synthase. Science **277:** 1815–1820

Stofer Vogel B, Wildung MR, Vogel G, Croteau R (1996) Abietadiene synthase from Grand Fir (*Abies grandis*). J Biol Chem **271:** 23262–23268

Sun T-P, Kamiya Y (1994) The Arabidopsis GA1 locus encodes the cyclase *ent*-kaurene synthetase A of gibberellin biosynthesis. Plant Cell **6:** 1509–1518

VanEtten HD, Mansfield JW, Bailey JA, Farmer EE (1994) Two classes of plant antibiotics: phytoalexins versus 'phytoanticipins'. Plant Cell **6:** 1191–1192

Whittington DA, Wise ML, Urbansky M, Coates RM, Croteau RB, Christianson DW (2002) Bornyl diphosphate synthase: structure and strategy for carbocation manipulation by a terpenoid cyclase. Proc Natl Acad Sci USA **99:** 15375–15380

Wickham KA, West CA (1992) Biosynthesis of rice phytoalexins: identification of putative diterpene hydrocarbon precursors. Arch Biochem Biophys **293:** 320–332

Wildung MR, Croteau RB (1996) A cDNA clone for taxadiene synthase, the diterpene cyclase that catalyzes the committed step of taxol biosynthesis. J Biol Chem **271:** 9201–9204

Williams DC, McGarvey DJ, Katahira EJ, Croteau R (1998) Truncation of limonene synthase preprotein provides a fully active 'pseudomature' form of this monoterpene cyclase and reveals the function of the amino-terminal arginine pair. Biochemistry **37:** 12213–12220

Xu M, Hillwig ML, Prisic S, Coates RM, Peters RJ (2004) Functional identification of rice *syn*-copalyl diphosphate synthase and its role in initiating biosynthesis of diterpenoid phytoalexin/allelopathic natural products. Plant J **39:** 309–318

Yajima A, Mori K, Yabuta G (2004) Total synthesis of *ent*-cassa-12,15-diene, a putative precursor of rice phytoalexins, phytocassanes A-E. Tetrahedron Lett **45:** 167–169

Yamaguchi S, Saito T, Abe H, Yamane H, Murofushi N, Kamiya Y (1996) Molecular cloning and characterization of a cDNA encoding the gibberellin biosynthetic enzyme *ent*-kaurene synthase B from pumpkin (*Cucurbita maxima* L.). Plant J **10:** 101–111

Yamaguchi S, Sun T, Kawaide H, Kamiya Y (1998) The GA2 locus of *Arabidopsis thaliana* encodes *ent*-kaurene synthase of gibberellin biosynthesis. Plant Physiol **116:** 1271–1278

Yu J, Hu S, Wang J, Wong GK, Li S, Liu B, Deng Y, Dai L, Zhou Y, Zhang X, et al (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. *indica*). Science **296:** 79–92