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Stemar-13-ene synthase, a diterpene cyclase involved in the biosynthesis of the phytoalexin oryzalexin S in rice

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Abstract In suspension-cultured rice cells, diterpenoid phytoalexins are produced in response to exogenously applied elicitors. We isolated a cDNA encoding a diterpene cyclase, OsDTC2, from suspension-cultured rice cells treated with a chitin elicitor. The *OsDTC2* cDNA was overexpressed in *Escherichia coli* as a fusion protein with glutathione *S*-transferase, and the recombinant OsDTC2 was indicated to function as stemar-13-ene synthase that converted *syn*-copalyl diphosphate to stemar-13-ene, a putative diterpene hydrocarbon precursor of the phytoalexin oryzalexin S. The level of *OsDTC2* mRNA in suspension-cultured rice cells began to increase 3 h after addition of the elicitor and reached the maximum after 8 h. The expression of *OsDTC2* was also induced in UV-irradiated rice leaves. In addition, we indicated that stemar-13-ene accumulated in the chitin-elicited suspension-cultured rice cells and the UV-irradiated rice leaves.

Keywords: Phytoalexin; Diterpene cyclase; Stemar-13-ene; Oryzalexin S; *Oryza sativa* L.

1. Introduction

When plants are attacked by pathogenic microorganisms, they respond by a variety of defense reactions, including production of phytoalexins that are low-molecular weight compounds serving as plant antibiotics [1,2]. Four structurally distinct types of polycyclic diterpenoid phytoalexins, oryzalexins A–F [3–5], (−)-phytocassanes A–E [6–8], momilactones A and B [9,10], and oryzalexin S [11], have been identified in extracts of the leaves of rice plants that were either infected with the rice leaf blast pathogen, *Magnaporthe grisea*, or exposed to ultra-violet (UV)-irradiation. The proposed pathways for biosynthesis of diterpenoid phytoalexins in rice are illustrated in Fig. 1. Diterpene cyclases catalyzing conversion of *ent*-copalyl diphosphate (*ent*-CDP) or *syn*-copalyl diphosphate (*syn*-CDP) to the four diterpene hydrocarbons play key roles in the biosynthesis of diterpenoid phytoalexins in rice. Previously, we reported that two species of mRNA encoding putative diterpene cyclases, OsDTC1 and OsDTC2, were expressed in chitin-elicited suspension-cultured rice cells, and that OsDTC1 functions as *ent*-cassa-12,15-diene synthase, a diterpene cyclase involved in the biosynthesis of (−)-phytocassanes [12]. Here, we show the isolation and characterization of *OsDTC2* cDNA. This gene product functioned as stemar-13-ene synthase that possibly plays a key role in the biosynthesis of oryzalexin S. We also indicated that stemar-13-ene accumulated in the chitin-elicited rice cells and UV-irradiated rice leaves.

2. Materials and methods

2.1. Cell culture

Calli of *Oryza sativa* L. cv. BL-1 and *O. sativa* L. cv. Nipponbare were cultured as described previously [12]. The rice cells, 6 days after the transfer to the fresh culture medium, were used for treatment with a chitin elicitor (*N*-acetylchitoheptaose, 10 ppm).

2.2. Isolation of a cDNA encoding the full-length ORF of OsDTC2

Previously, we reported the isolation of a 549 bp cDNA fragment encoding a putative diterpene cyclase gene, *OsDTC2* [12]. A cDNA encoding the full-length ORF of *OsDTC2* was cloned by PCR using primers designed based on the information from a rice genome database (RiceBlast.dna.affrc.go.jp), 5′-CAACGGATCCATGATGCTGCTGAGTTCCTC-3′ (forward, *Bam*HI site is underlined) and 5′-CAACGAATTCTTACTCTTGCAGGTGCAGTG-3′ (reverse, *Eco*RI site is underlined). A cDNA library from elicitor-treated rice cells (cv. Nipponbare), constructed by using HybriZAP-2.1 XR Library Construction kit (Stratagene), was used as a template. PCR was carried out using KOD-Plus-DNA polymerase (Toyobo, Tokyo, Japan) with the following program: 5 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 52 °C, and 3 min at 72 °C, followed by cooling down to 4 °C.

2.3. Expression of OsDTC2 cDNA in Escherichia coli

The *OsDTC2* ORF was subcloned into the pGEX-6P-2 vector (Amersham Bioscience, Piscataway, NJ, USA) and this vector was transformed into *E. coli* BL21. This was grown in 2× YT medium (tryptone 16 g l^{-1}, yeast extract 10 g l^{-1}, NaCl 5 g l^{-1}; pH 7.0) [13]

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Abbreviations: CDP, copalyl diphosphate; GGDP, geranylgeranyl diphosphate; GST, glutathione *S*-transferase

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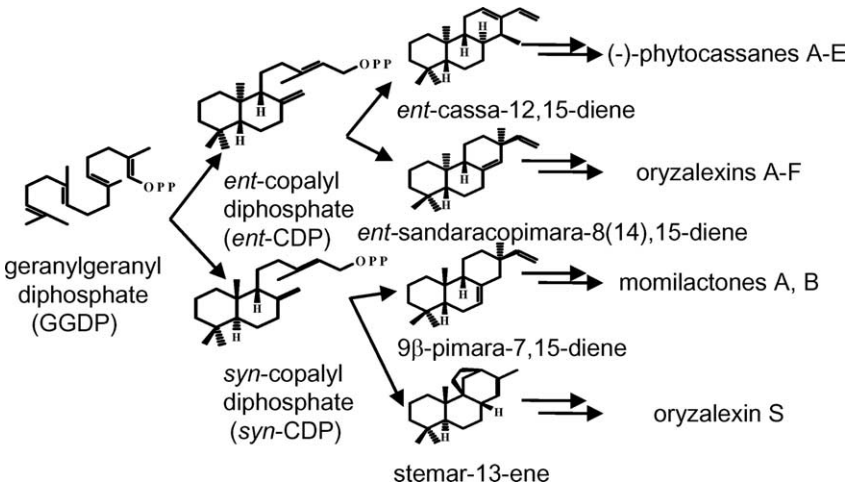

Fig. 1. Proposed pathways for biosynthesis of diterpenoid phytoalexins in rice.

containing ampicillin (50 μg ml^{-1}) at 37 °C. When the optical density at 600 nm reached 0.6, isopropyl-1-thio-β-D-galactoside was added to a final concentration of 0.1 mM and the cells were incubated at 30 °C for another 15 h. The collected cells were re-suspended in the 20 mM Tris–HCl buffer and disrupted by mild sonication on ice. After centrifugation at 15 000×*g* for 30 min, the supernatant was purified by Glutathione–Sepharose 4B (Amersham Bioscience) affinity column chromatography with the elution buffer containing 10 mM glutathione (Sigma–Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The affinity-purified GST-OsDTC2 was analyzed by 10% SDS–PAGE with Coomassie brilliant blue staining [14].

2.4. Enzyme assays of the GST-OsDTC2 fusion protein

The substrate of OsDTC2 is possibly *ent*-CDP or *syn*-CDP that is converted from geranylgeranyl diphosphate (GGDP) with *ent*-CDP synthase (*ent*-CPS) or *syn*-CPS, respectively. Since *OsCyc1* and *OsCyc2* cDNAs encoding *syn*-CPS and *ent*-CPS in rice, respectively, were cloned and characterized by our group [15], functional analysis of OsDTC2 was carried out using GGDP as a substrate in the concomitant presence of OsDTC2 and OsCyc1 or OsCyc2 as described below. The assay solution (0.5 ml) consisted of 2 μg of the substrate GGDP (Sigma–Aldrich) in a solution of dithiothreitol (2 mM), EDTA (0.5 mM), proteinase inhibitor cocktail (1/100 tablet; Complete™, Roche, Basel, Switzerland), $MgCl_2 \cdot 6H_2O$ (5 mM), and Tris–HCl buffer (100 mM, pH 7.5). After the affinity-purified protein (GST-OsDTC2 with or without GST-OsCyc1; GST-OsDTC2 with GST-OsCyc2; and GST with GST-OsCyc1) was added to the assay solution, the assay mixture was incubated at 30 °C for 1 h. The solution was extracted with *n*-hexane and subjected to gas chromatography-mass spectrometry (GC-MS) analysis. When GGDP was incubated with GST-OsDTC2 and GST-OsCyc2, production of *ent*-CDP was indicated as follows. The assay mixture, after incubation at 30 °C for 1 h, was dephosphorylated by incubation with bacterial alkaline phosphatase (6 unit, Toyobo) at 37 °C for 1 h. The resultant solution was extracted with *n*-hexane and subjected to GC-MS to identify *ent*-copalol that was the dephosphorylated form of *ent*-CDP.

2.5. Expression analysis of OsDTC2 mRNA in elicited suspension-cultured rice cells

Reverse transcription was performed with SuperScript II RT (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions using poly(A)$^+$ RNA (1 μg) prepared from suspension-cultured rice cells (cv. BL-1) 0, 3, 6, 8, 10, and 12 h after adding the chitin elicitor (10 ppm). PCR was carried out using *Ex Taq* DNA polymerase (TaKaRa, Ohtsu, Japan) with the following program: 5 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 56 °C, and 1 min at 72 °C, followed by cooling down to 4 °C. A pair of the gene-specific primers, 5′-ATGATGCTGCTGAGTTCCTCCT-3′ (forward) and 5′-CGCTGCTGTTGTGGAAGGCGA-3′ (reverse), was used to amplify a 786-bp *OsDTC 2* cDNA fragment. As an internal standard, the rice actin gene *ACT1* was amplified by RT-PCR using the gene-specific primers *ACT1-P1* (5′-CATGCTATCCCTC-GTCTCGACCT-3′) and *ACT1-P2* (5′-CGCACTTCATGATGGAG-TTGTAT-3′) [16].

2.6. Expression analysis of OsDTC2 mRNA in the UV-irradiated rice leaves (UV+)

Rice plants (cv. Nipponbare) were cultured in a greenhouse. At the sixth-leaf stage, the fourth and fifth leaves were detached and UV-irradiated as described previously [12]. Control rice leaves (UV−) were handled similarly except that they were not exposed to UV irradiation. Reverse transcription was performed with SuperScript III RT (Invitrogen) according to the manufacturer's instructions using total RNA prepared from these leaves. PCR was carried out using KOD-Plus-DNA polymerase (Toyobo) with the following program: 2 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C, and 1 min at 68 °C, followed by cooling down to 4 °C. A pair of the gene-specific primers, 5′-TCGCCCTCCCTCTACTTAAG-3′ (forward) and 5′-TTGCCCATCAATGGCAACCG-3′ (reverse), was used to amplify a 619-bp *OsDTC2* cDNA fragment. The rice actin gene *ACT1* was amplified as an internal standard as described in Section 2.5.

2.7. GC-MS

To analyze diterpene hydrocarbons produced in the enzyme assays of the GST-OsDTC2 fusion protein and diterpene hydrocarbons in the elicited rice cells (Figs. 2 and 4A), a JMS-Auto Mass 150 GC-MS system (ionization voltage 70 eV; JEOL, Tokyo, Japan) was used, fitted with a fused silica chemically bonded capillary column (DB-5; 0.25 mm in diameter, 15 m long, 0.25 μm film thickness; J&W Scientific Inc., Folsom, CA, USA). Each sample was injected onto the column at 60 °C in the splitless mode. After a 2-min isothermal hold at 60 °C, the column temperature was programmed at 20 °C min^{-1} to 180 °C, at 2 °C min^{-1} to 210 °C, and at 20 °C min^{-1} to 270 °C with a 3-min isothermal hold at 270 °C. The head pressure of the helium carrier gas was 65 kPa. To analyze reaction products in the incubation of GGDP with GST-OsDTC2 and GST-OsCyc2, GC-MS was conducted as reported previously [15]. To analyze diterpene hydrocarbons in the UV-irradiated rice leaves (Fig. 4B), GC-MS was carried out using an Agilent 6890 N GC-5973 N MSD system fitted with a fused silica chemically bonded capillary column (DB-WAX; 0.25 mm in diameter, 60 m long, 0.25 μm film thickness; J&W Scientific Inc.). The column temperature was programmed as follows: 80 °C for 2 min, from 80 to 250 °C at 2 °C min^{-1}, and then 250 °C for 10 min. The flow rate of the helium carrier gas was 1 ml min^{-1}.

For GC-MS analysis of the diterpene hydrocarbons, 9.9 g fresh weight of the suspension-cultured rice cells treated with the chitin elicitor (10 ppm) for 48 h (Fig. 2C), approximately 2 g fresh weight each of the rice cells treated with the chitin elicitor (10 ppm) for 0, 8, 12, and 24 h (Fig. 4A), or 5.0 g fresh weight each of UV-irradiated and control rice leaves (Fig. 4B) were extracted with methanol, and the methanol extracts were purified as described previously [17].

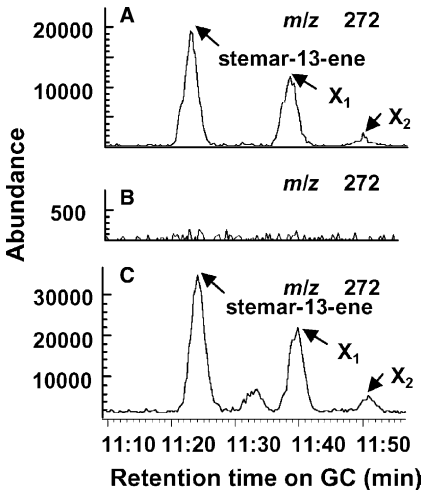

Fig. 2. GC-MS analysis of diterpene hydrocarbons obtained by incubation of GGDP with the recombinant OsDTC2 and OsCyc1 (A) or the recombinant OsDTC2 and OsCyc2 (B), and diterpene hydrocarbons in the methanol extract from suspension-cultured rice cells 24 h after addition of the elicitor *N*-acetylchitoheptaose (C). Diterpene hydrocarbons were monitored at *m*/*z* 272. Full-scan mass spectra of stemar-13-ene, X1, and X2 were as follows [*m*/*z* (relative abundance)]: stemar-13-ene, 272 (M^+, 32), 257 (100), 229 (39), 201 (21), 187 (32), 161 (60), 105 (96); X1, 272 (M^+, 74), 257(58), 229(5), 201 (31), 177 (57), 148 (100), 105 (97); X2, 272 (M^+, 48), 257 (29), 229 (28), 187 (61), 159 (53), 105 (100).

3. Results and discussion

3.1. Isolation of a cDNA encoding a diterpene cyclase, OsDTC2, from suspension-cultured rice cells treated with a chitin elicitor

As already reported [12], we exhibited that at least two diterpene cyclase genes tentatively named *OsDTC1* and *OsDTC2* were expressed in the elicited rice (*O. sativa* L. cv. BL-1) cells. A cDNA encoding the full-length ORF of *OsDTC1* was cloned and indicated to encode *ent*-cassa-12,15-diene synthase. With regard to *OsDTC2*, a 549-bp cDNA fragment was amplified by RT-PCR, but isolation of the cDNA encoding the full-length ORF was not successful.

In the course of this study, the rice (cv. Nipponbare) genome database was opened to the public (http://RiceBLAST.dna.affrc.go.jp/). In our search of the database, we found that the nucleotide sequence of the 549-bp *OsDTC2* cDNA fragment from cv. BL-1 was completely identical to that of cv. Nipponbare. We therefore changed the plant material from cv. BL-1 to cv. Nipponbare, and succeeded in the isolation of a cDNA containing the full-length ORF of *OsDTC2* by PCR using primers designed based on the information from rice genome database and a cDNA library from elicitor-treated rice (cv. Nipponbare) cells as a template. The nucleotide sequence data of *OsDTC2* cDNA reported in this paper will appear in the Genome Sequence Data Base (GSDB), DNA Data Bank of Japan (DDBJ), European Molecular Biology Laboratory (EMBL), and National Center for Biotechnology Information (NCBI) nucleotide sequence databases with the Accession No. AB118056.

The *OsDTC2* ORF encodes 820 amino acid residues. There is a transit peptide-like sequence at the N-terminus of OsDTC2, indicating that OsDTC2 protein would be targeted to plastids [18]. It is thus suggested that OsDTC2 is localized in plastids, as are OsDTC1 (*ent*-cassa-12,15-diene synthase) [12] and other plant diterpene cyclases such as *ent*-CPS and *ent*-kaurene synthase (*ent*-KS) [19,20]. Quite recently, it was suggested that several diterpene cyclase genes found in the rice genome are involved in the phytoalexin biosynthesis in rice [21], one of them tentatively named *OsKS8* being identical to *OsDTC2*. However, functional analysis of *OsKS8* has not been carried out.

3.2. Sequence comparison with other plant diterpene cyclases

The amino acid sequence of OsDTC2 was compared with those of other plant diterpene cyclases. Similarity was found with OsDTC1 (*ent*-cassa-12,15-diene synthase) (48% identity), *ent*-KS (39–41% identity) [22–24], abietadiene synthase (AS) [25] (32% identity), and *ent*-CPS [26,27] (29% identity). AS has two active sites responsible for KS-type and CPS-type activities. The DDXXD motif responsible for KS-type activity is conserved in OsDTC2, but the DXDDTA motif responsible for CPS-type activity is not present in OsDTC2 as in the case of OsDTC1 [12]. Thus, the function of OsDTC2 is probably to catalyze the cyclization of *ent*-CDP or *syn*-CDP into a diterpene hydrocarbon that is a key intermediate of the diterpenoid phytoalexins in rice.

3.3. Functional analysis of OsDTC2 protein

The OsDTC2 protein was overexpressed in *E. coli* as a glutathione *S*-transferase (GST) fusion protein (GST-OsDTC2). The fusion protein was affinity-purified as described in Section 2.3 and a 116-kDa protein (GST-OsDTC2) was detected by SDS–PAGE. Also, the *E. coli* BL21 harboring the control plasmid pGEX-c yielded a 26-kDa protein (GST). We used the affinity-purified GST-OsDTC2 fusion protein for enzyme assays.

Enzyme assays of GST-OsDTC2 for diterpene cyclase activity were performed with GGDP as a substrate. The identification of the reaction products was carried out by GC-MS analysis. The incubation of GGDP with GST-OsDTC2 and GST-OsCyc2, which catalyzes the conversion of GGDP into *ent*-CDP, did not give any diterpene hydrocarbons (Fig. 2B). However, the same reaction followed by dephosphorylation using alkaline phosphatase gave *ent*-copalol (data not shown). These results indicate that the incubation of GGDP with GST-OsDTC2 and GST-OsCyc2 yielded *ent*-CDP but not any diterpene hydrocarbons. On the other hand, the incubation of GGDP with GST-OsDTC2 and GST-OsCyc1, which catalyzes the conversion of GGDP into *syn*-CDP, gave the diterpene hydrocarbon stemar-13-ene, a putative precursor of oryzalexin S, as a major product. Unknown diterpene hydrocarbon-like compounds X1 and X2 were yielded as minor products (Fig. 2A), which were also detected as minor components in the elicited rice cells (Fig. 2C). The incubation of GGDP with GST-OsCyc1 and the control GST gave no diterpene hydrocarbon (data not shown), suggesting that the GST domain of GST-OsDTC2 did not contribute to its diterpene cyclase activity. In addition, we confirmed that GST-OsDTC2 without GST-OsCyc1 did not convert GGDP into stemar-13-ene (data not shown). It is thus concluded that OsDTC2 functions as stemar-13-ene synthase in rice.

3.4. Expression levels of OsDTC2 mRNA

To analyze the expression levels of *OsDTC2* mRNA in chitin-elicited suspension-cultured rice cells (cv. BL-1), we performed RT-PCR using a pair of gene-specific primers.

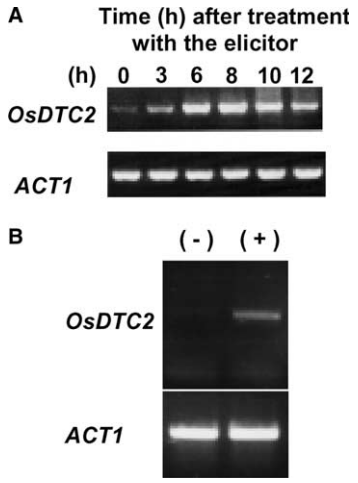

Fig. 3. Expression levels of *OsDTC2* (stemar-13-ene synthase) mRNA in suspension-cultured rice cells treated with the elicitor *N*-acetylchitoheptaose (A) and UV-irradiated rice leaves (B). (A) RT-PCR was performed using poly(A)$^+$ RNA (1 μg) prepared from the rice cells treated with the chitin elicitor (10 ppm) for the indicated period of time and a pair of the gene-specific primers. As an internal standard, the rice actin gene *ACT1* was amplified by using the gene-specific primers. (B) RT-PCR was performed using total RNA (1 μg) isolated from the UV-irradiated (UV+) and control (UV−) rice leaves and a pair of the gene-specific primers. As an internal standard, the rice actin gene *ACT1* was amplified by using the gene-specific primers.

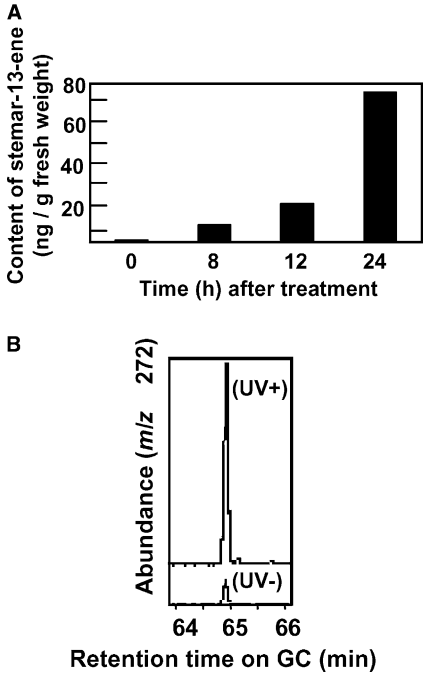

Fig. 4. Accumulation of stemar-13-ene in the elicited suspension-cultured rice cells (A) and the UV-irradiated rice leaves (B). (A) Suspension-cultured rice cells treated with the chitin elicitor (10 ppm) for the indicated time. The purified methanol extracts from the respective materials were subjected to GC-MS. An aliquot of each sample, equivalent to 20 mg fresh weight, was used for each scanning. The content of stemar-13-ene in each material was estimated based on total ion current (TIC). (B) The methanol extracts from the UV-irradiated (UV+) and control (UV−) rice leaves were purified and subjected to GC-MS. An aliquot of each sample, equivalent to 0.8 mg fresh weight, was used for each scanning. Stemar-13-ene was identified by comparison of the retention time on GC and the full-scan mass spectrum with those of the authentic sample.

Levels of *OsDTC2* mRNA began to increase 3 h after addition of the elicitor, and the level reached the maximum after 8 h, and gradually decreased (Fig. 3A). On the other hand, stemar-13-ene began to accumulate in suspension-cultured rice cells 8 h after addition of the elicitor and the levels continued to increase for at least 16 h (Fig. 4A).

Rice leaves produce diterpenoid phytoalexins after UV irradiation as well as infection with the blast fungus *M. grisea* [4,5]. UV irradiation is a convenient method for obtaining tissues for investigating rice phytoalexin biosynthesis. By RT-PCR analysis, the induction of *OsDTC2* mRNA after UV irradiation in rice leaves was clearly indicated (Fig. 3B). The induction of *OsKS8* (*OsDTC2*) mRNA by UV irradiation was also reported by Sakamoto et al. [21] quite recently. GC-MS analysis indicated that the production of stemar-13-ene was induced in the rice leaves after UV irradiation (Fig. 4B). It was thus indicated that OsDTC2 functions as stemar-13-ene synthase not only in the elicited suspension-cultured rice cells but also in the UV-irradiated rice leaves.

In this study, we isolated a cDNA encoding a diterpene cyclase from the chitin-elicited suspension-cultured rice cells, and indicated that the gene product functioned as stemar-13-ene synthase in the elicited suspension-cultured rice cells and the UV-irradiated rice leaves. The stemar-13-ene synthase cDNA isolated here will be a useful tool to investigate the regulatory mechanisms of the biosynthesis of the diterpenoid phytoalexin oryzalexin S in rice. Based on the information from a rice genome database, at least 10 diterpene cyclase genes are expressed in rice including genes encoding *ent*-cassa-12,15-diene synthase [12], stemar-13-ene-synthase [this study], OsCyc1 (*syn*-CPS), OsCyc2 (*ent*-CPS involved in phytoalexin biosynthesis), and *ent*-CPS involved in gibberellin biosynthesis [15,21]. Cloning and functional analysis of other diterpene cyclase cDNAs are now under way.

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