Cyclic diterpenoids are commonly biosynthesized from geranylgeranyl diphosphate (GGDP) through the formation of carbon skeletons by specific cyclases and subsequent chemical modifications, such as oxidation, reduction, methylation, and glucosidation. A variety of diterpenoids are produced in higher plants and fungi. Rice produces four classes of diterpene phytoalexins, phytocassanes A to E, oryzalexins A to F, oryzalexin S, and momilactones A and B. The six diterpene cyclase genes involved in the biosynthesis of these phytoalexins were identified and characterized. Fusicoicin A was produced by the phytopathogenic Phomopsis amygdali and served as a plant H\textsuperscript{+}-ATPase activator. A PaFS, encoding a fungal diterpene synthase responsible for fusicoicin biosynthesis, was isolated. The PaFS is an unusual chimeric diterpene synthase that possesses not only terpene cyclase activity (the formation of fusicoicadiene, a biosynthetic precursor of fusicoicin A), but also prenyltransferase activity (the formation of GGDP). Thus, we identified a unique multifunctional diterpene synthase family in fungi.

**Key words:** diterpene cyclase; fungus; gene cluster; higher plant; phytoalexin

The terpenoids are a very large family of natural compounds. Terpenoid structures are diverse and range from relatively simple linear hydrocarbon chains to highly complex ring structures. Terpenoids are biosynthesized from isoprene units, derived through the mevalonate pathway and/or methylerythritol pathway.\textsuperscript{1)} Cyclic terpenoids are constructed through formation of the prenyl chain by prenyltransferases, formation of the cyclic carbon skeleton by terpene cyclases, and then chemical modification by enzymes, including oxidases, reductases, methyltransferases, and glucosyltransferases. Prenyltransferases catalyze the sequential condensation of isopentenyl diphosphate (IPP, 5 carbons) with allylic diphosphates, including dimethylallyl diphosphate (DMAPP, 5 carbons), geranyl diphosphate (GDP, 10 carbons), and farnesyl diphosphate (FDP, 15 carbons), into the respective linear prenyl diphosphates, GDP, FDP, and geranylgeranyl diphosphate (GGDP, 20 carbons). Generally, monoterpenes, sesquiterpenes, and diterpenes are derived from GDP, FDP, and GGDP respectively. Terpene cyclases are key branch-point enzymes, catalyzing complex intramolecular cyclization of linear prenyl diphosphates into cyclic hydrocarbons.

A variety of diterpenes with unique biological activities have been isolated from higher plants and fungi. For example, gibberellins (GAs, phytohormone),\textsuperscript{2)} momilactones (phytoalexin; allelopathic substance, Fig. 1),\textsuperscript{3–5} oryzalexins (phytoalexin, Fig. 1),\textsuperscript{6–9} and phytocassanes (phytoalexin, Fig. 1)\textsuperscript{10–12} have been isolated from plants. Similarly, fungal GAs,\textsuperscript{13} aphidicolin (a specific inhibitor of DNA polymerase \(\alpha\)),\textsuperscript{14,15} and fusicoicin A (a diterpene glucoside that acts as a plant plasma membrane H\textsuperscript{+}-ATPase activator)\textsuperscript{16,17} have been isolated from fungi. The cyclic carbon skeletons of these diterpenoids are elaborated from a common precursor, GGDP, and the formation of the cyclic hydrocarbons is catalyzed by specific diterpene cyclases. This review highlights the labdane-related diterpene cyclase gene family in rice, and an unusual chimeric diterpene synthase involved in fusicoicin biosynthesis in a fungus.

### I. Labdane-Related Diterpene Cyclase Gene Family in Rice

Many diterpenoids have been identified in rice (*Oryza sativa* L.), including phytohormone GAs and phytoalexins. Phytoalexins are low molecular weight compounds produced after attack by microorganisms and exposure to elicitors and ultraviolet (UV) irradiation. It has been suggested that they serve as plant antibiotics. The
Diterpenoid phytoalexins identified in rice include oryzalexins, momilactones, and phytocassanes (Fig. 1). Oryzalexins A to F, phytocassanes A to E, momilactones A and B, and oryzalexin S are biosynthesized from GGDP via their respective hydrocarbon precursors, ent-sandaracopimaradiene, ent-cassa-12,15-diene, 9βH-pimar-7,15-diene, and stemar-13-ene (Fig. 2A). GA is a diterpene phytohormone that regulates many aspects of development in higher plants. It is biosynthesized from GGDP via ent-kaur-16-ene (Fig. 2B). These five hydrocarbon precursors are synthesized from GGDP through the formation of the bicyclic intermediates ent-CDP or syn-CDP (Fig. 2A, B). syn-CDP is a diastereomer of ent-CDP. The first cyclization step in this pathway, of GGDP to ent- or syn-CDP, is initiated by the addition of a proton to the terminal olefin bond of GGDP (type B); the second cyclization, of ent- or syn-CDP to each hydrocarbon precursor, is initiated by elimination of the diphosphate group (type A). The cDNAs encoding the ent-CDP synthase and ent-kaurene synthase enzymes that act in GA biosynthesis were first isolated from Arabidopsis and pumpkin, and were designated CPS and KS, respectively. The amino acid motifs DxDD, near the N-terminal region, and DDxxD, near the C-terminal region, are known as aspartate-rich motifs and are important in catalysis by CPS (type-B) and KS (type-A), respectively. Further, they are targeted to the plastid in plant cells by N-terminal transit peptides. Thus the diterpene hydrocarbon precursors of rice phytoalexins was regarded to be biosynthesized by two types of diterpene cyclase (CPS type and KS type) from GGDP, similarly to the GAs. Searches of The Institute for Genomic Research (TIGR) database (http://www.tigr.org/tab/e2k1/osa1/) showed that the japonica rice genome contains four CPS-type genes and eleven KS-type genes. In contrast to rice, the Arabidopsis thaliana genome contains only one set of such labdane-related diterpene cyclase genes, AtCPS and AtKS, which are required for gibberellin biosynthesis. We isolated from japonica rice and characterized the cDNAs OsCPS4/OsCyc1 (Os04g09900), OsCPS2/OsCyc2 (Os02g36210), OsKSL4 (Os04g10060), OsKSL7/OsDTC1 (Os02g36140), OsKSL8/OsDTC2 (Os1g28530), and OsKSL10 (Os12g30824), encoding syn-CDP synthase, ent-CDP synthase, 9βH-pimara-7,15-diene synthase, ent-cassa-12,15-diene synthase, stemar-13-ene synthase, and ent-sandaracopimaradiene synthase, respectively (Fig. 2A). The transcript levels of all of these genes increased markedly after UV treatment in rice leaves and after elicitor treatment in cultured rice cells, both of which induce phytoalexin biosynthesis. Thus these genes are likely to be involved in rice diterpene phytoalexin biosynthesis. Another group isolated these cDNAs independently from indica rice. OsCPS1 (Os04g52680) and OsKS1 (Os04g52230) were first identified as GA biosynthetic genes; their knockout mutants showed severe GA-deficient dwarf phenotypes. After UV and after elicitor treatment, the levels of the OsCPS1 and OsKS1 transcripts did not increase. In addition, recombinant OsCPS1 and OsKS1 have been found to convert GGDP into ent-CDP and ent-CDP into ent-kaur-16-ene, respectively (Fig. 2B). All of these rice diterpene cyclases include transit peptide-like sequences at the N-terminus. Transient assays using the
green fluorescent protein system showed that all were transported into plastids (unpublished data).

*OsKSL5* (Os02g36220) and *OsKSL6* (Os02g36264), the expression of which was not increased by UV treatment, encode ent-pimara-8(14),15-diene synthase and ent-kaur-15-ene (ent-isokaurene) synthase, respectively (Fig. 2C). 30) *OsKSL5* and *OsKSL6* indica types encode ent-isokaurene synthases. 28) Xu et al. reported that the difference between the function of japonica *OsKSL5* and that of the indica type was due to a single amino acid residue change, Thr in the japonica type for ent-pimaradiene synthesis and Ile in the indica type for ent-isokaurene synthesis.33) It has been suggested that *OsCPSL3* (Os09g15050), *OsKSL2* (Os04g52240), *OsKSL3* (Os04g52210), *OsKSL9* (Os11g28500), and *OsKSL11* (Os12g30800) are pseudogenes.

Biosynthesis of GA, oryzalexins A to F, and phytocassanes A to E share the conversion step from GGDP to ent-CDP. It has long been unclear whether a single ent-CDP synthase is involved in the biosynthesis of both GA and phytoalexin. The above results suggest the presence of two genes encoding ent-CDP synthases in rice, one (*OsCPS1*) involved in the biosynthesis of GAs, and the other (*OsCPS2/OsCyc2*) in the biosynthesis of phyto-
alexins. Further, we characterized the enzymatic properties of recombinant OsCPS2/OsCyc2 and compared them with those of OsCPS1.34) Several enzymatic properties of OsCPS2/OsCyc2, including the optimal pH (neutral), optimal temperature (30°C), and divalent cation requirement (Mg²⁺), were very similar to those of OsCPS1. The $K_m$ and $k_{cat}$ values of OsCPS1 and OsCPS2/OsCyc2 were calculated to be $14.2 \pm 1.5 \mu M$ and $4.5 \times 10^{-2}/s$, and $21.1 \pm 5.6 \mu M$ and $7.8 \times 10^{-2}/s$, respectively. However, the enzymatic properties of OsCPS2/OsCyc2 were different from those of OsCPS1 with regard to inhibition by the substrate GGDP and Amo-1618 (a GA biosynthesis inhibitor). OsCPS2/OsCyc2 activity was not inhibited by 50–60 μM GGDP, which did inhibit OsCPS1 activity.34) The ent-CDP synthases are localized in the plastid, as mentioned above. In the plastid, large amounts of carotenoid and phytol, which provide accessory pigments and the chlorophyll side chains for photosynthesis respectively, are synthesized from GGDP. The substrate inhibition of ent-CDP synthase responsible for GA biosynthesis is perhaps a mechanism regulating GA levels through a restricted flow of GGDP into GA biosynthesis, but the physiological role of the substrate inhibition of ent-CDP synthase remains unclear. Our results suggest that the flow of GGDP into phytoalexin biosynthesis is less restricted than that into GA biosynthesis in rice. On the other hand, OsCPS1 activity exhibited approximately 70% inhibition with 100 μM Amo-1618, while OsCPS2/OsCyc2 activity was inhibited by approximately 10%.34) Amo-1618 is a quaternary ammonium compound that inhibits CPS activity, a step in GA biosynthesis.35) It is noteworthy that Amo-1618, which can suppress fungal ent-CDP synthase activity,35) inhibits OsCPS2/OsCyc2 activity much less effectively than it does OsCPS1 activity. Hence, OsCPS2/OsCyc2 is a useful tool in investigating the mode of inhibition of Amo-1618.

The OsKSL7/OsDTC1, encoding ent-cassa-12,15-diene synthase, is located on chromosome 2 of the rice genome near OsCPS2/OsCyc2, which encodes ent-CDP synthase. Similarly, OsKSL4 encoding 9βH-pimara-7,15-diene synthase is located on chromosome 4 near OsCPS4/OsCyc1, which encodes syn-CDP synthase. Six (CYP71Z7, 8, CYP76M5, 6, 7, and 8) and two (CYP99A2, and 3) P450 monooxygenase-like genes are found near the cyclase genes on chromosomes 2 and 4, respectively. Further, a dehydrogenase-like gene is located near these genes on chromosome 4. These findings suggest that phytocassane and momilactone biosynthesis genes are clustered on chromosomes 2 (86 cM) and 4 (14.3 cM), respectively. We found that the dehydrogenase-like gene encodes momilactone A synthase (OsMAS), which converts 3β-hydroxy-9βH-pimara-7,15-dien-19,6β-olide into momilactone A, and that knock-down of CYP99A2 and CYP99A3 by RNAi resulted in lower levels of production of momilactones A and B in cultured rice cells. In addition, the levels of CYP99A2, CYP99A3, and OsMAS transcripts increased markedly after UV and elicitor treatment. These results suggest that the momilactone biosynthetic genes are clustered on chromosome 4.36) It is likely that six P450-like genes on chromosome 2, transcript levels of which also increased after UV and elicitor treatment (unpublished data), are involved in phytocassane biosynthesis. In contrast to momilactone and phytocassane biosynthesis genes, GA biosynthesis genes are not clustered in the rice genome39 as in other plant species, such as Arabidopsis.19)

It has been found that momilactone B is excluded from rice roots and exhibits allelopathic activity against the growth of dicot plant seedlings.3) We found that phytocassanes A to E and momilactones A and B are released from roots.37) In contrast to momilactone B, phytocassanes do not show inhibitory activity against dicot seedling growth.37) The rice blast fungus Magnaporthe grisea, which is believed to attack the aerial parts of rice, causes a devastating disease in infected rice plants, but a previous study found that M. grisea also invades rice roots by a typical root-specific pathway.38) Since all of the phytocassanes A to E and momilactones A and B possess antimicrobial activity against the rice blast M. grisea, phytocassanes might be released from roots to defend against soil pathogens, such as M. grisea, and momilactones might act not only as allelopathic, but also as antimicrobial substances. Expression analyses of diterpene cyclase genes (Fig. 2A) suggest that phytocassanes and momilactones found in roots are primarily biosynthesized in those roots.37)

II. An Unusual Chimeric Diterpene Synthase Family in Fungi

Fusicoccins A and J (Fig. 3) are representative metabolites produced by the phytopathogenic fungus Phomopsis (Fusicoccom amygdali (Del.).16,39) Fusicoccin A not only has potent higher plant plasma membrane

Fig. 3. Proposed Biosynthetic Pathway of Fusicoccins.
encoded KS P. betae are clustered in the genome of
We have suggested that aphidicolin biosynthetic genes
on amphibian embryogenesis. 40) The aglycon of fusicoccins is a tricyclic diterpene derived from GGDP. It has been found that fusicocca-2,10(14)-diene (Fig. 3) is a biosynthetic intermediate of fusicoccins. 41–43) We isolated the gene encoding the diterpene cyclase from the new fungus that causes Bakanae disease, 13) and the G. fujikuroi synthase from P. amygdali. 46) Recombinant PaDC3:GGS converts GGDP into phomopsene, a novel tetracyclic diterpene hydrocarbon isolated from the P. amygdali mycelia (unpublished results).

Cyclization of GGDP by both PaDC1 and PaDC2 is initiated by protonation of GGDP (Fig. 4), while that by PaDC3:GGS can be initiated by ionization of the allylic diphosphate group (unpublished results). The conversion of fusicocca-2,10(14)-diene from GGDP has been shown to be initiated by ionization of the allylic diphosphate group. 48) We isolated a homolog of PaDC3:GGS, by RT-PCR with degenerate primers, which we named PaFS. It has been demonstrated that recombinant PaFS converts GGDP into fusicocca-2,10(14)-diene. 49) Furthermore, we found an unusual diterpene synthase PaFS, a multi-functional enzyme with both prenyltransferase and diterpene cyclase activities. 49) This recombinant enzyme converted isoprene units sequentially into GGDP and then into fusicocca-2,10(14)-diene. Functional analysis of truncated mutants and site-directed mutagenesis indicated that PaFS consists of two domains: a terpene cyclase domain at the N-terminus and a prenyltransferase domain at the C-terminus (Fig. 5). These findings suggest that fusicoccadiene can be produced efficiently in the fungus using C5 precursors regardless of GGDP availability. In fact, heterologous expression of PaFS alone resulted in the accumulation of fusicocca-2,10(14)-diene in Escherichia coli cells. Previously, an artificially constructed chimeric enzyme containing an FDP synthase domain and a tobacco epi-aristolochene synthase domain was found to produce the sesquiterpene epi-aristolochene from GDP and IPP. 50) This previous study also suggested that the chimeric enzyme was more efficient than a mixture of the two separate enzymes in converting GDP and IPP into epi-aristolochene in vitro. We suggest that multifunctional fungal enzymes exhibiting both prenyltransferase and terpene cyclase activity contribute to the efficient production of bioactive terpenes, regardless of the availability of GGDP in the cell. Our database survey suggested that such multifunctional terpene biosynthesis enzymes also occur in other fungal species, implying that this hypothetical mechanism for the efficient production of terpenes is not restricted to P. amygdali. In addition, we found a PaGGS3 at the 3‘-end, which we named PaDC3:GGS.

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Fig. 5. Schematic of the Two Domains in the Unusual Chimeric Diterpene Synthase PaFS.

PaGGS3 at the 3′-end, which we named PaDC3:GGS. Recombinant PaDC3:GGS converted GGDP into phomopsene, a novel tetracyclic diterpene hydrocarbon isolated from the P. amygdali mycelia (unpublished results).

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like gene, a fungal 2-oxoglutarate-dependent dioxygenase-like gene, a short chain dehydrogenase/reductase-like gene, and an α-mannosidase-like gene in the regions flanking the \textit{PaFS} gene, suggesting that fusicoccin biosynthetic genes are clustered in the genome of \textit{P. amygdali}.

III. Conclusions and Future Prospects

We isolated several diterpene cyclase genes from higher plants and fungi, and characterized the proteins encoded by these genes. This study included the first identification of rice phytoalexin biosynthetic genes, and a proposal for an unusual chimeric diterpene synthase family in fungi. The diterpene cyclase genes responsible for phytoalexin biosynthesis can serve as tools for a reverse genetic approach to examine the roles of these phytoalexins in the pathogen defense system in rice. We found that heterologous expression of \textit{PaFS} alone was sufficient to produce a large amount of fusicoccin-2,10(14)-diene in \textit{E. coli}. Since the Cs isoprene units are thought to be synthesized in all organisms as universal precursors to isoprenoids, \textit{PaFS} should be a powerful and flexible tool in producing fusicocins in heterologous systems. Furthermore, the crystal structures of these diterpene cyclases should provide useful information on the mechanism of the formation of these complex carbon skeletons, and ideas on manipulating enzymes to create novel carbocyclic diterpenes.

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