論文の内容の要旨

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論文題目

Functional analysis of diterpenoid biosynthetic enzymes and the regulatory mechanisms of diterpenoid production in rice
(イネにおけるジテルペン生合成酵素の機能解析とジテルペン生産の制御機構)

Terpenoids are one of the large family of natural compounds which are produced in living organisms. The structures of terpenoids are varying from relatively simple linear hydrocarbon chains to complicated ring structures. Among the terpenoids, a variety of cyclic diterpenoids, the plant hormones gibberellins (GAs), phytoalexins, and the constitutively produced antimicrobial compounds oryzalides have been isolated and identified from rice plants. These compounds with unique biological activities have been suggested to be biosynthesized from a common precursor, geranylgeranyl diphosphate (GGDP) via two-step sequential cyclization followed by several oxidation steps. However, some of important biosynthetic enzymes have not been functionally identified, and the details of regulatory mechanisms of their biosynthesis remain to be clarified. In this study, functional analysis of P450s which are involved in biosynthesis of the biologically important rice diterpenoids GAs and phytoalexins was performed, and involvement of cytokinin in induction of production of the diterpenoid phytoalexins in rice was investigated.

I. Functional analysis of OsKO2

Gibberellins are biosynthesized from GGDP *via* the diterpene hydrocarbon precursor *ent*-kaurene, which is converted into bioactive GAs through several oxidation steps catalyzed by P450s and dioxygenases. Recently, it was shown that some of P450 genes in CYP701A family are involved in the steps from *ent*-kaurene to *ent*-kaurenoic acid *via* *ent*-kaurenol and *ent*-kaurenal. Itoh et al. found five *CYP701A* genes including

ent-kaurene oxidase (KO) gene form a cluster on chromosome 6 of rice (*Oryza sativa* L. cv. Nipponbare), and indicated that both *OsKO1* and *OsKO2* complement the dwarf phenotype in the *KO*-mutant *d35*$^{Tan\text{-}ginbozu}$. Expression analysis of the genes has shown that *OsKO2* is expressed in whole organs, and that *OsKO1* expression is restricted to the panicles and roots, suggesting that *OsKO2* encodes the main KO that contributes to the GA biosynthesis for vegetative growth in rice. However, the functional identification of OsKO2 has not been carried out yet. Therefore, identification of OsKO2 *in vitro* enzyme activity was attempted using the methylotrophic yeast *Pichia pastoris* expression system.

The cDNA of *OsKO2* and a fungal P450 reductase gene (*PhCPR*) were introduced into the expression vectors pPICZ and pPIC6 for yeast expression, respectively, both of which were expressed under an alcohol oxidase promoter and as a c-myc epitope tagged fusion protein. After the induction using MeOH, the yeast cells were harvested to prepare the microsomal fraction. Expression of OsKO2-myc and PhCPR-myc was confirmed by western blot analysis. Enzyme assays using *ent*-kaurene or [$^2H_2$]*ent*-kaurenol as a substrate were carried out. Incubation of the microsomal fraction with [$^2H_2$]*ent*-kaurenol gave [$^2H_2$]*ent*-kaurenoic acid. When the microsomal fraction was incubated with *ent*-kaurene, *ent*-kaurenoic acid was detected as a reaction product. These results indicate that OsKO2 is involved in the 3-step oxidation from *ent*-kaurene to *ent*-kaurenoic acid *via ent*-kaurenol and *ent*-kaurenal in the GA biosynthetic pathway in rice (Fig. 1).

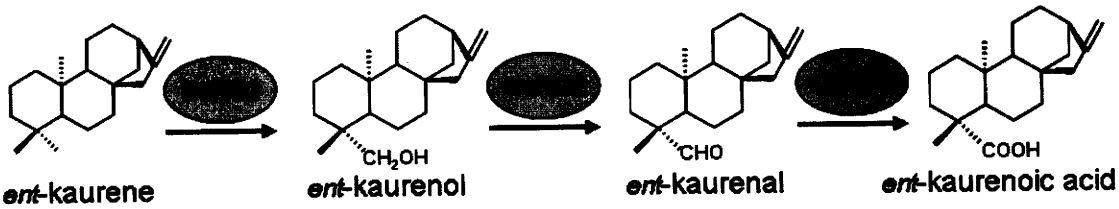

Figure 1. Involvement of OsKO2 in the 3-step oxidation from *ent*-kaurene to *ent*-kaurenoic acid in the GA biosynthetic pathway in rice.

II. Functional analysis of CYP99A2 and CYP99A3

When plants are attacked by pathogenic microorganisms, they protect themselves by a variety of defense responses including production of the antimicrobial secondary metabolites phytoalexins. In rice, 14 diterpenoid phytoalexins have been identified in suspension-cultured cells treated with biotic elicitors such as a chitin oligosaccharide *N*-acetylchitooctaose elicitor and/or from leaves that were either infected with the rice blast fungus *Magnaporthe oryzae* or exposed to UV irradiation. These diterpenoid phytoalexins are classified into four groups based on their basic carbon frameworks: phytocassanes A–E,

oryzalexins A–F, momilactones A and B, and oryzalexin S, the major diterpenoid phytoalexins being phytocassanes and momilactones. Concerning enzymes involved in biosynthesis of the diterpenoid phytoalexins, all the six diterpene cyclases catalyzing the conversion of the common precursor GGDP to the four diterpene hydrocarbon precursors *via ent-* or *syn*-CDP have been identified. On the other hand, concerning the enzymes catalyzing the downstream oxidation of the diterpene hydrocarbons, involvement of microsomal P450s and/or dehydrogenases has been suggested. However, none of the enzymes involved in the downstream oxidation have been identified. It was previously reported that two diterpene cyclase genes involved in phytocassane biosynthesis, *OsKSL7* and *OsCPS2,* are located in a narrow region on chromosome 2, and that two diterpene cyclase genes involved in momilactone biosynthesis, *OsKSL4* and *OsCPS4,* are located in a narrow region on chromosome 4. In addition, six and two elicitor-inducible P450 genes have been found near the diterpene cyclase genes on chromosomes 2 and 4, suggesting that the phytocassane and momilactone biosynthesis genes are clustered on chromosomes 2 and 4, respectively. To investigate this hypothesis, I focused on the gene cluster on rice chromosome 4 and attempted to perform functional analysis of the two P450 genes *CYP99A2* and *CYP99A3.*

Utilizing the Rice Genome Automated Annotation System (RiceGAAS), it was found that the genes *OsCPS4, CYP99A3,* the putative dehydrogenase gene AK103462, *OsKSL4,* and *CYP99A2* are linearly arranged within a 168-kb region on chromosome 4. The transcriptional expression of the clustered five genes was up-regulated in suspension-cultured rice cells after treatment with a chitin oligosaccharide elicitor and in rice leaves after UV irradiation. To examine the involvement of CYP99A2 and CYP99A3 in the chitin oligosaccharide elicitor-inducible production of diterpenoid phytoalexins in rice cells, RNAi-mediated knockdown of *CYP99A2* and *CYP99A3* was attempted. Since the ORFs of the two genes share 87% identity at the nucleotide sequence level, RNAi-mediated knockdown of *CYP99A2* resulted in production of the double knockdown of the two genes. The accumulation of the major diterpenoid phytoalexins momilactones and phytocassanes in culture media of the double knockdown cell lines after chitin oligosaccharide elicitor treatment was determined by HPLC-MS/MS to indicate that momilactone biosynthesis is specifically suppressed in the double knockdown lines. Functional analysis of the AK103462 protein was performed by one of my colleagues, in parallel with this study, to demonstrate that the AK103462 gene encodes momilactone A synthase (OsMAS), which catalyzes conversion from 3β-hydroxy-9βH- pimara-7,15-dien-19,6β-olide to momilactone A. These results strongly suggests that CYP99A2 and/or CYP99A3 are involved in biosynthetic steps between 9βH-pimara-7,15-diene and 3β-hydroxy-9βH-pimara-7,15-dien-19,6β-olide in the momilactone biosynthetic pathway. It was thus indicated that *CYP99A2* and *CYP99A3* form

a momilactone biosynthetic gene cluster together with the momilactone synthase gene *OsMAS* and the diterpene cyclase genes *OsCPS4* and *OsKSL4* on chromosome 4 (Figure 2). In addition, I succeeded in heterologous expression of the recombinant CYP99A2 and CYP99A3 using a baculovirus expression system. Functions of CYP99A2 and CYP99A3 will be able to be determined by enzyme assays when their substrates are available.

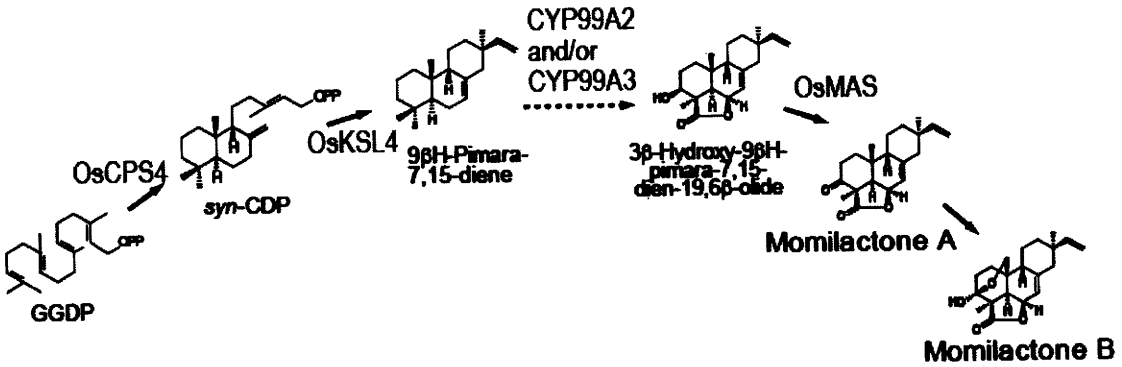

Figure 2. Hypothetical momilactone biosynthetic pathway and the possible function of CYP99A2 and/or CYP99A3.

III. Involvement of cytokinin in production of diterpenoid phytoalexins in rice

Plant hormones are often involved in the stress responses, which are caused by biotic and abiotic stresses. Defense-associated signaling molecules such as salicylic acid, jasmonic acid, and ethylene have been investigated extensively and suggested to play crucial roles in plant defense mechanisms. In addition, cytokinin is also believed to play important roles in plant defense responses. In fact, cytokinin was suggested to act downstream of a small GTP-binding protein in defense signal transduction pathways in tobacco. Therefore, I investigated the possibility that cytokinin is involved in defense responses, such as phytoalexins production, in rice as well.

Both of the synthetic cytokinin benzyladenine (BA) and the natural cytokinin *trans*-zeatin (*tZ*) induced accumulation of the major diterpenoid phytoalexins phytocassanes and momilactones in culture media of suspension-cultured rice cells and rice leaves in a dose-dependent manner up to 100 μM. In time course study using suspension-cultured rice cells, the levels of diterpenoid phytoalexins increased from 24 h through 96 h in response to BA treatment. Whereas in the chitin oligosaccharide elicitor *N*-acetylchitooctaose treatment, the levels of diterpenoid phytoalexins began to increase from 8 h and peaked at 72 h. The four diterpene cyclase genes that are involved in phytocassane and momilactone biosynthesis showed strong expression at 8-12 h after chitin oligosaccharide elicitor treatment, whereas in the BA treatment, the expression levels of the genes increased from 8

or 12 h through 96 h after the treatment. It was also confirmed that the expression of four diterpene cyclase genes were induced in rice leaves at 48 and 72 h after the BA treatment. In rice leaf disks, BA also led to increases in the accumulation levels of the diterpenoid phytoalexins and in the expression levels of the four diterpene cyclase genes at 48 h and 72 h after treatment. These effects of BA on accumulation of the diterpenoid phytoalexins in rice leaves were similar to those of exogenously applied JA. It was thus indicated that BA-induced accumulation of the diterpenoid phytoalexins is caused by *de novo* biosynthesis not only in suspension-culture rice cells but in rice leaves. In rice plants, accumulation of diterpenoid phytoalexins was induced after infection with the pathogen *M. oryzae*. It was also reported that endogenous cytokinin levels increased in response to infection with *M. oryzae* in rice plants. These results suggest that cytokinin might play a role as a signal molecule in the pathogen-induced production of diterpenoid phytoalexins in rice.

IV. Conclusions

In this study, functional analysis of P450s which are involved in biosynthesis of the biologically important rice diterpenoids GAs and phytoalexins was performed, leading to functional identification of *OsKO2*, a causal gene for the GA-deficient mutant *d35*$^{Tan\text{-}ginbozu}$, and to identification of the momilactone biosynthetic gene cluster. The clustered genes for momilactone biosynthesis exhibit a temporally coordinated expression in suspension-cultured rice cells after elicitor treatment. Such coordinated, stress-inducible clustered gene expression, responsible for the biosynthesis of one particular compound, has not previously been reported in plants. In addition, involvement of cytokinin in pathogen-induced production of the diterpenoid phytoalexins in rice was suggested. This might provide a new insight into plant hormone-dependent defense mechanisms against pathogens in rice.