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Award Review

Biosynthesis of Phytoalexins and Regulatory Mechanisms of It in Rice

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We performed extensive functional characterization of diterpenoid phytoalexin biosynthetic genes in rice, and found that the genes for the biosynthesis of the major diterpenoid phytoalexins, phytocassanes and momilactones, are clustered on chromosomes 2 and 4, and that their expression is coordinately induced in rice cells after elicitation. Isopentenyl diphosphate, an early precursor of diterpenoid phytoalexins, was found to be synthesized through the plastidic methylerythritol phosphate pathway. We also found that chitin elicitor receptor kinase OsCERK1 and a mitogen-activated protein kinase cascade, the OsMKK4-OsMPK6 cascade, play essential roles in the elicitor-induced production of diterpenoid phytoalexins. In addition, a basic leucine zipper transcription factor, OsTGAP1, was identified as a key regulator of the coordinated expression of the clustered genes and the methylerythritol phosphate pathway genes. Naringenin 7-*O*-methyltransferase (OsNOMT) was also identified as a key enzyme in the biosynthesis of another major rice phytoalexin, sakuranetin.

Key words: jasmonates; methylerythritol phosphate pathway; naringenin 7-*O*-methyltransferase; phytoalexin biosynthetic gene cluster; rice

Plants respond to pathogen infection through a variety of defense responses that include the production of phytoalexins, antifungal secondary metabolites. Fifteen phytoalexins have been identified in rice (*Oryza sativa* L.) plants infected by the blast fungus *Magnaporthe oryzae* and/or rice leaves irradiated with ultraviolet (UV) light.[1–11] Although one of these compounds, sakuranetin, is a flavonoid, the remaining 14 are diterpenoids that can be classified into four groups based on basic carbon skeleton: phytocassanes A to E, oryzalexins A to F, momilactones A and B, and oryzalexin S (Fig. 1).[12] Sakuranetin, the phytocassanes, and the momilactones are the dominant groups. In rice plants, the production of phytoalexins, including sakuranetin, is induced by pathogen infection, UV irradiation, jasmonates, and $CuCl_2$.[13] In suspension-cultured rice cells, the production of diterpenoid phytoalexins, but not of sakuranetin, is induced by a chitin oligosaccharide.[14]

In the biosynthetic pathways of diterpenoid phytoalexins (Fig. 2), common precursor geranylgeranyl diphosphate (GGDP) is postulated to be sequentially cyclized *via ent*-copalyl diphosphate (*ent*-CDP) to *ent*-cassa-12,15-diene and *ent*-sandaracopimaradiene, producing phytocassanes A to E and oryzalexins A to F respectively. GGDP is also probably cyclized *via syn*-CDP, to 9βH-pimara-7,15-diene and stemar-13-ene, producing momilactones A and B and to oryzalexin S respectively.[12] In fact, Mohan *et al.* (1996) demonstrated that enzyme preparations from chitin-elicited suspension-cultured rice cells converted *ent*-CDP to *ent*-sandaracopimaradiene, a putative diterpene hydrocarbon precursor of oryzalexins A to F.[15] Similarly *syn*-CDP was converted to 9βH-pimara-7,15-diene and stemar-13-ene, putative diterpene hydrocarbon precursors of momilactones A and B and oryzalexin S respectively, but their study did not examine the conversion of *ent*-CDP to *ent*-cassa-12,15-diene by the enzyme preparation,[15] because the phytocassanes were not identified in rice leaves infected by the blast fungus until later.[5–7] It has also been suggested that microsomal cytochrome P450 monooxygenases and/or a dehydrogenase are involved in the downstream oxidation of diterpene hydrocarbons, producing the bioactive phytoalexins.[16,17]

Sakuranetin is biosynthesized from naringenin by *S*-adenosyl-L-methionine–dependent naringenin 7-*O*-methyltransferase (NOMT). Because naringenin is a biosynthetic intermediate to a variety of flavonoids, NOMT plays a key role in sakuranetin biosynthesis at a branch point from the common flavonoid biosynthetic pathway (Fig. 3). Purification of NOMT from crude extracts of UV-irradiated wild-type rice leaves has been attempted, but was unsuccessful.[18,19]

In the early 2000s, information about the structure of rice phytoalexins and their biosynthetic pathways was accumulating, but no rice phytoalexin biosynthetic enzyme genes had been identified. Hence we performed extensive functional identification of rice phytoalexin biosynthetic enzymes in collaboration with Professors Toyomasu and Sassa of Yamagata University. In addition, we obtained key information about signal

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Abbreviations: bZIP, basic leucine zipper; CDP, copalyl diphosphate synthase; COMT, coffeic acid 3-*O*-methyltransferase; CPS, CDP synthase; DMAPP, dimethylallyl diphosphate; EIE, early-induced expression; LIE, late-induced expression; GAs, gibberellins; GGDP, geranylgeranyl diphosphate; IPP, isopentenyl diphosphate; KS, kaurene synthase; KSL, kaurene synthase-like; MAPK, mitogen activated protein kinase; MEP, methylerythritol phosphate; MKK, MAPK kinase; MVA, mevalonic acid; NOMT, naringenin 7-*O*-methyltransferase; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; UV, ultraviolet

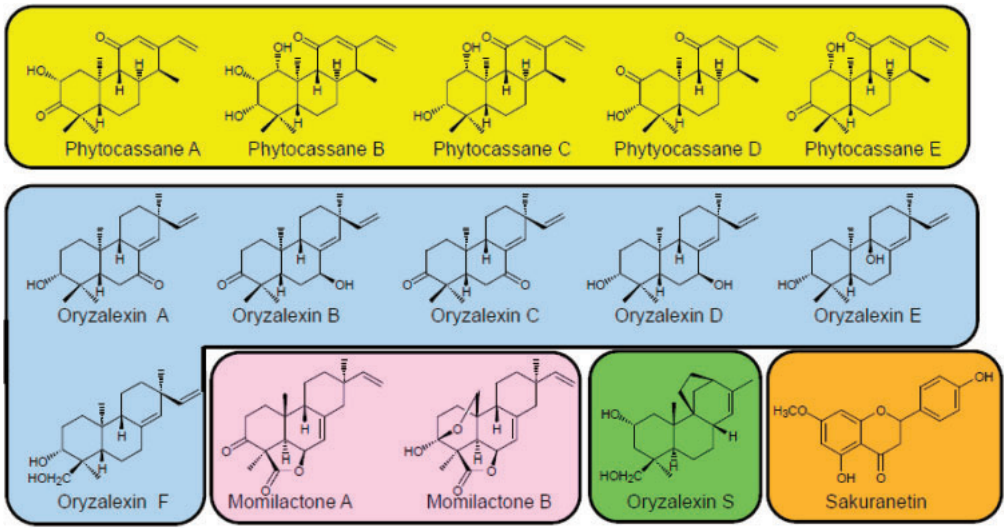

Fig. 1. Chemical Structures of Rice Phytoalexins.

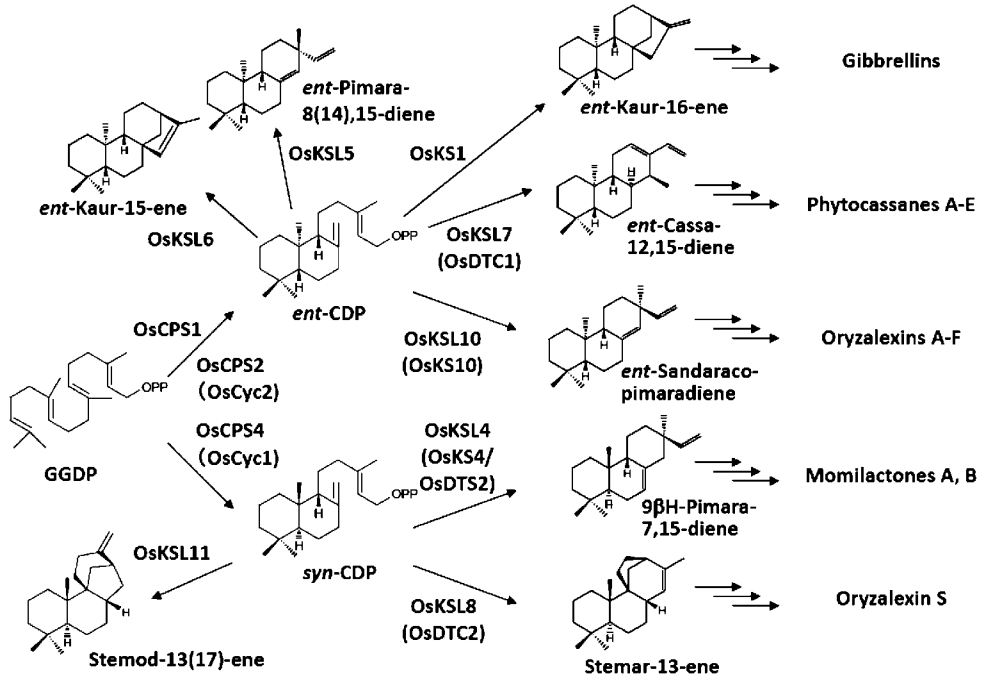

Fig. 2. Biosynthetic Pathways of Rice Diterpenoids.

Most diterpene cyclases had alternative names, for example OsDTC1 for OsKSL7,[12] OsKS4 and OsDTS2 for OsKSL4,[22,30] OsDTC2 for OsKSL8,[20] OsKS10 for OsKSL10,[22] and OsCyc1 and 2 for OsCPS4 and 2.[21] To avoid confusion, Peters (2006) proposed that non-kaurene-producing family members be termed kaurene synthase-like (KSL),[31] with the corresponding number from Sakamoto *et al.* (2004).[23] This naming, along with OsCPS1, 2, and 4 for type B diterpene cyclases, is followed throughout this review.

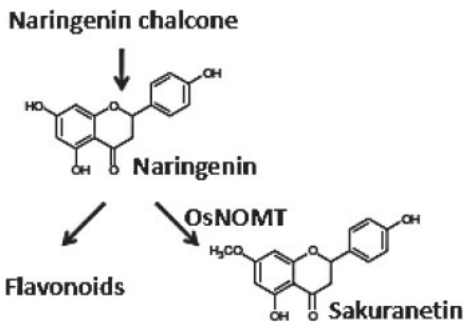

Fig. 3. Biosynthesis of Sakuranetin in Rice.

transduction pathways for chitin oligosaccharide elicitor-induced production of diterpenoid phytoalexins in suspension-cultured rice cells. This review discusses the functional identification of rice phytoalexin biosynthetic enzyme genes and the regulatory mechanisms of the expression of those genes in rice.

I. Identification and Functional Analysis of Diterpene Cyclase Genes Involved in the Biosynthesis of Diterpenoid Phytoalexins in Rice

Diterpene cyclases are involved in the biosynthesis of diterpenoid phytoalexins. The first cyclization step, GGDP to *ent*- or *syn*-CDP, is initiated by the addition

of a proton to the terminal olefin bond of GGDP (type B cyclases), and the second cyclization step, *ent*-CDP or *syn*-CDP to their hydrocarbon precursors, is initiated by elimination of the diphosphate (type A cyclases). Diterpene cyclase cDNAs were isolated by reverse-transcription-polymerase chain reaction (RT-PCR) using primers based on conserved motifs of known diterpene cyclases, followed by 3′- and 5′-rapid amplification of cDNA ends. We isolated full-length cDNAs encoding two type A diterpene cyclases from suspension-cultured rice cells treated with a chitin elicitor, *ent*-cassa-12,15-diene synthase (OsKSL7)[12] and stemar-13-ene synthase (OsKSL8).[20] Otomo *et al.* (2004) isolated full-length cDNAs encoding two type B diterpene cyclases, *ent*-CDP synthase (OsCPS2) and *syn*-CDP synthase (OsCPS4), from UV-irradiated rice leaves.[21] We confirmed that *OsKSL7*and *OsKSL8* are induced both in suspension-cultured rice cells after chitin-elicitor treatment and in rice leaves after UV irradiation.[12,20] Otomo *et al.* (2004) also confirmed that *OsCPS2* and *OsCPS4* are induced in rice leaves after UV irradiation.[21] Thus it can be concluded that these four genes are involved in phytoalexin biosynthesis in rice (Fig. 2). During the course of this study, the rice genome database became publicly available (http://RiceBlAST.dna.affrc.go.jp/), and it indicated that the rice genome contains three and seven genes encoding type B and type A diterpene cyclases respectively.[22,23]

The three rice type B diterpene cyclases are OsCPS1, OsCPS2, and OsCPS4. The fact that an *OsCPS1* loss-of-function mutation causes a severe gibberellin (GA)-deficient dwarf phenotype suggests that OsCPS1 functions in GA biosynthesis.[23] Otomo *et al.* (2004) also found that OsCPS1 functions as an *ent*-CPS, but that it is not induced in rice leaves after UV irradiation. They concluded that both OsCPS1 and OsCPS2 catalyze the conversion of GGDP to *ent*-CDP, but that the former is involved in GA biosynthesis, and the latter in phytoalexin biosynthesis.[21]

Of the seven rice type A diterpene cyclases (OsKS1, and OsKSL4, 5, 6, 7, 8, and 10), OsKS1 is thought to be involved in GA biosynthesis because a loss-of-function mutation causes a severe GA-deficient dwarf phenotype similar to the *OsCPS1* mutant.[23] To characterize the enzymatic activities of OsKSL4, 5, 6, and 10, their cDNAs were isolated by RT-PCR and the translated gene products were subjected to *in vitro* enzymatic assays using GGDP, *ent*-CDP, or *syn*-CDP as substrates, confirming that OsKSL4, 5, 6, and 10 function as 9βH-pimara-7,15-diene synthase, *ent*-pimara-8(14),15-diene synthase, *ent*-kaur-15-ene synthase, and *ent*-sandaracopimaradiene synthase respectively (Fig. 2).[22,24,25] The *OsKSL4* and *OsKSL10* genes are induced in rice leaves by UV irradiation, but *OsKSL5* and *OsKSL6* are not induced under this condition.[24,26] Although Morrone *et al.* (2006) functionally identified OsKSL11 as stemod-13(17)-ene synthase in the two *Oryza sativa* L. subspecies *indica* and *japonica* (Fig. 2),[27] the corresponding sequence is not present in the rice genome database.[25] In parallel with our studies, Peters and his colleagues also reported extensive identification and functional characterization of diterpene cyclase genes in rice.[25,28–30]

II. Involvement of the Methylerythritol Phosphate (MEP) Pathway in the Biosynthesis of Diterpenoid Phytoalexins in Rice

Because we found that the production of diterpenoid phytoalexins and the expression of the associated biosynthetic genes were enhanced in suspension-cultured rice cells by chitin elicitor treatment, we performed a time-course experiment and microarray analysis better to characterize the genes involved in diterpenoid phytoalexin biosynthesis.[26] Hierarchical clustering of the expression profiles of the genes induced by the chitin elicitor showed two types of early-induced expression (EIE-I and EIE-II) nodes and a late-induced expression (LIE) node. Expression of the genes in the EIE-I node peaked within 2 h of elicitor treatment and rapidly decreased and that of the genes in the EIE-II node peaked within 2 h after elicitor treatment and slowly decreased. Expression of the genes in the LIE node peaked 4 h to 8 h after elicitor treatment. Because the diterpene cyclase genes involved in phytoalexin biosynthesis were present in the LIE node, other genes that might be responsible for phytoalexin biosynthesis were expected to be in the LIE node. The biosynthetic steps before GGDP in plants consist of two distinct pathways supplying C5 precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the mevalonate (MVA) and MEP pathways.[32] Some isoprenoids such as GAs, abscisic acid, and carotenoids are synthesized in the plastids from IPP and DMAPP derived from the MEP pathway, whereas others, such as sesquiterpenes, triterpenes, sterols, and brassinosteroids, are synthesized in the cytoplasm from IPP derived from the MVA pathway.[33,34] Diterpenoid phytoalexins are thought to fall within the former category. In fact, diterpene cyclases involved in phytoalexin biosynthesis contain N-terminal transit-peptide-like sequences that probably target the proteins to plastids.[12,20–22] In addition, MEP pathway genes—1-deoxy-D-xylulose 5-phophate synthase (*OsDXS3*), 1-deoxy-D-xylulose 5-phophate reductoisomerase (*OsDXR*), 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol synthase (*OsCMS*), 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase (*OsCMK*), 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (*OsMCS*), 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase (*OsHDS*), and 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase (*OsHDR*)—were present in the LIE node. Quantitative RT-PCR (qRT-PCR) confirmed that the expression of these MEP pathway genes was synchronized in the elicited suspension-cultured rice cell samples, peaking 4–8 h after elicitor treatment. Of these MEP pathway genes, the functions of *OsDXS3, OsDXR*, and *OsCMS* were confirmed by complementation analysis using *Escherichia coli* mutants defective in MEP pathway genes. Elicitor-induced diterpenoid phytoalexin accumulation was strongly suppressed by treatment with fosmidomycin and 5-ketoclomazone, chemical inhibitors of the DXS and DXR proteins, but not by treatment with mevastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase in the MVA pathway.[26] It was hence concluded that the MEP pathway enzymes present in plastids are involved in the early biosynthetic steps of diterpenoid phytoalexins.

III. Identification of Biosynthetic Gene Clusters for Major Diterpenoid Phytoalexins in Rice

In the biosynthetic pathway of the diterpenoid plant hormone GA, P450s are involved in the oxidation of the diterpene hydrocarbon precursor *ent*-kaurene. It has been suggested that by analogy P450s are involved in the oxidation of diterpene hydrocarbons in the bioactivation of phytoalexins. In fact, *ent*-sandaracopimaradiene-3β-ol was converted into oryzalexins D and E in the microsome fraction from UV-irradiated rice leaves in the presence of oxygen and NADPH, and this conversion was repressed by P450 inhibitors.[16] The accumulation of momilactones and phytocassanes in suspension-cultured rice cells was also suppressed by treatment with a P450 inhibitor,[35] suggesting the involvement of P450s in the biosynthesis of both major diterpenoid phytoalexins. In addition, 3β-hydroxy-9βH-pimara-7,15-diene-19,6β-olide was converted by a soluble protein fraction from UV-irradiated rice leaves into momilactone A in the presence of NAD^+,[17] strongly suggesting that a dehydrogenase is involved in momilactone biosynthesis.

After identifying the diterpene cyclase genes involved in phytoalexin biosynthesis, we found that *OsKSL4* was located near *OsCPS4* on rice chromosome 4 and near three chitin- and UV-inducible genes, two P450 genes (*CYP99A2* and *CYP99A3*), and a putative dehydrogenase gene (*AK103462*). Because *OsKSL4* and *OsCPS4* are involved in momilactone biosynthesis, these results suggest that rice momilactone biosynthesis genes are clustered in a 168-kb region on chromosome 4.[22,35] The function of the putative dehydrogenase gene was confirmed by heterologous expression in *E. coli* followed by *in vitro* enzyme assays, confirming that the gene product converts 3β-hydroxy-9βH-pimara-7,15-diene-19,6β-olide to momilactone A. To investigate the involvement of *CYP99A2* and *CYP99A3* in chitin elicitor-inducible production of diterpenoid phytoalexins in rice cells, we attempted to deplete these proteins individually by RNA interference, but because the *CYP99A2* and *CYP99A3* open reading frames are highly homologous to each other, this attempt resulted in simultaneous knockdown of the two genes. The accumulation of momilactones A and B in the culture medium of chitin-treated double knockdown cells was much lower than in the *CYP99A2*- and *CYP99A3*-expressing control, but the accumulation of phytocassanes was similar to the control, suggesting that *CYP99A2*, *CYP99A3*, or both are involved in momilactone biosynthesis.[35] Recently Wang *et al.* (2010) reported that CYP99A3 catalyzes three oxidation steps on the C19 methyl group of the momilactone precursor 9βH-pimara-7,15-diene, and they detected 9βH-pimara-7,15-diene-19-oic acid in rice plants,[36] although it remains unknown whether 9βH-pimara-7,15-dien-19-oic acid is a true biosynthetic intermediate of momilactones. Taken together, these results strongly suggest the presence of a momilactone biosynthetic gene cluster on rice chromosome 4.

We also found that *OsKSL7* is located near *OsCPS2* on rice chromosome 2 and near five chitin-inducible P450 genes (*CYP71Z7*, *CYP76M5*, *CYP76M6*, *CYP76M7*, and *CYP76M8*). Because *OsKSL7* and *OsCPS2* are involved in phytocassane biosynthesis, these seven genes may constitute a phytocassane biosynthetic gene cluster.[22,26] We analyzed the function of the P450s in this gene cluster by RNA interference. The accumulation of phytocassanes in the culture media of the *CYP76M7* and *CYP76M8* double knockdown cells was significantly lower than in the control, suggesting that *CYP76M7*, *CYP76M8*, or both are involved in phytocassane biosynthesis, whereas no consistent changes in the momilactone levels were observed.[37] In fact, both CYP76M7 and CYP76M8 catalyze 11β-hydroxylation of *ent*-cassa-12,15-diene, an early step in phytocassane biosynthesis that presumably produces the keto group found in all phytocassanes, in *in vitro* enzymatic assays.[37,38] CYP76M8 can also catalyze the 6β-hydroxylation of 9βH-pimara-7,15-diene and the 7β-hydroxylation of *ent*-sandaracopimaradiene,[37] but it remains to be investigated whether CYP76M8 is involved in the biosynthesis of momilactones or oryzalexins A to D. Okada (2011) reported unpublished results indicating that the accumulation of C-2 oxygenated phytocassanes such as phytocassanes A, B, and D was specifically suppressed in *CYP71Z7*-knockdown cell lines,[39] suggesting that *CYP71Z7* is involved in C-2 oxygenation in phytocassane biosynthesis. Wu *et al.* (2011) also reported that CYP71Z7 can catalyze the C-2 hydroxylation of *ent*-cassa-12,15-diene in *in vitro* enzymatic assays, but that *ent*-cassa-12,15-diene is a poorly recognized substrate for CYP71Z7 ($K_m = 200$ μM), suggesting that CYP71Z7 functions as C-2 hydroxylase in phytocassane biosynthesis, but that its true substrate *in planta* has not been identified.[40]

It thus became evident that the genes responsible for the biosynthesis of the major rice diterpenoid phytoalexins, phytocassanes and momilactones, are clustered on chromosomes 2 and 4 respectively, and that their elicitor-induced expression is coordinately regulated. The momilactone and phytocassane gene clusters appear to be unique in plants in that their expression is coordinated and stress-inducible, resulting in efficient high-level production of diterpenoid phytoalexins. Besides the rice phytoalexin biosynthetic gene clusters, only a few other examples of gene clusters for secondary metabolites in higher plants, including benzoxazinoids in maize, avenacins in oat, and thalianol and marneral in *Arabidopsis*, have been reported,[41–44] but the expression of these gene clusters is unlikely to be stress-induced.

IV. Identification of a Key Enzyme in Sakuranetin Biosynthesis

Sakuranetin is not only one of the major phytoalexins in rice, it is also a potential pharmaceutical agent that induces the adipogenesis of 3T3-L1 cells[45] and exhibits anti-inflammatory,[46] anti-mutagenic,[47] anti-*Helicobacter pylori*,[48] anti-leishmanial, and anti-trypanosomal activities.[49] NOMT, which catalyzes the conversion of naringenin to sakuranetin, is a key enzyme in sakuranetin biosynthesis. Rakwal *et al.* (2000) attempted to purify a putative NOMT from crude extracts of UV-irradiated wild-type rice,[18] but the purified protein proved highly homologous to maize caffeic acid 3-*O*-methytransferase (COMT), and was named OsCOMT1. The recombinant

OsCOMT1 expressed in *E. coli* exhibits COMT but not NOMT activity.[18,19] In addition, *OsCOMT1* expression was consititutive in rice plants, and the transcript levels were not affected by treatment with $CuCl_2$, jasmonic acid, or UV irradiation,[19] although accumulation of sakuranetin was induced by these treatments. These results suggest that the major component, OsCOMT1, exhibits behavior similar to the minor component, OsNOMT, in the purification steps and masks the presence of OsNOMT. Hence we decided to purify OsNOMT from the *oscomt1* mutant.[50] A crude protein preparation from UV-irradiated *oscomt1* leaves was ammonium sulfate-precipitated and then subjected to anion exchange and affinity chromatography, resulting in >400-fold enrichment of the enzyme. A minor band at an apparent molecular mass of 40 kDa was detected by SDS-polyacrylamide gel electrophoresis. It was excised from the gel, digested with trypsin, and subjected to MALDI-TOF/TOF mass spectrometry fingerprinting analysis, leading to the identification of two *O*-methyltransferase-like proteins encoded by Os04g0175900 and Os12g0240900. Glutathione-*S*-transferase-fused recombinant Os04g0175900 and Os12g0240900 proteins were prepared in *E. coli* and subjected to *in vitro* NOMT assay. Only the latter showed NOMT activity. Os12g0240900 transcription was induced by treatment with jasmonic acid in rice plants prior to the accumulation of sakuranetin, and its kinetic properties, such as K_m and k_{cat}, fit reasonably well with the function of OsNOMT in sakuranetin production in rice leaves. Hence we concluded that Os12g0240900 encodes OsNOMT (Fig. 3).

V. Regulatory Mechanisms in Phytoalexin Biosynthesis

The chitin elicitor induces the biosynthesis of diterpenoid phytoalexins such as phytocassanes and momilactones in rice cells.[26] Shimizu *et al.* (2010) reported that rice cells require two types of LysM-containing plasma membrane glycoproteins, CEBiP (chitin elicitor binding protein) and OsCERK1 (chitin elicitor receptor kinase) for perception of the chitin elicitor.[51] In the absence of the chitin elicitor, CEBiP and OsCERK1 mostly are present separately from each other, but once the system is activated, a receptor complex including both CEBiP and OsCERK1 is transiently formed, resulting in the activation of downstream defense responses.[51] Elicitor-induced accumulation of diterpenoid phytoalexins is severely suppressed in rice *OsCERK1* knockdown cell lines, indicating that OsCERK1 plays a pivotal role in the perception of the chitin elicitor, leading to the production of diterpenoid phytoalexins in rice.[51]

Mitogen-activated protein kinase (MAPK) cascades also play a critical role in responding to microbial-associated molecular pattern molecules such as chitin oligosaccharides.[52,53] Kishi-Kaboshi *et al.* (2010) found that the chitin elicitor activates two rice MAPKs (OsMPK3 and OsMPK6) and one MAPK kinase (OsMKK4), and that OsMPK6 is essential for the chitin elicitor-inducible biosynthesis of diterpenoid phytoalexins in rice cells.[54] Conditional expression of the constitutively active form of OsMKK4 ($OsMKK4^{DD}$) induces the activation of OsMPK3 and OsMPK6 and accumulation of diterpenoid phytoalexins. $OsMKK4^{DD}$-induced expression of diterpenoid phytoalexin biosynthetic genes, including MEP pathway genes, is dependent on OsMPK6. Hence Kishi-KaboshiIt *et al.* concluded that the OsMKK4-OsMPK6 cascade plays a crucial role in the chitin elicitor-inducible biosynthesis of diterpenoid phytoalexins in rice.[54]

To determine how coordinated expression of the phytocassane and momilactone gene clusters is achieved, first we looked for *cis*-acting elements responsive to chitin elicitor treatment within a 2-kb region upstream of *OsKSL4* by a luciferase reporter assay.[55] Deletion and mutation analysis of the *OsKSL4* promoter region identified a chitin elicitor-responsive *cis*-acting TGACG motif 1,040 bp upstream of the translation start site, suggesting that a basic leucine zipper (bZIP) transcription factor is involved in the chitin-inducible expression of *OsKSL4*. Hence we searched for chitin-inducible bZIP transcription factors with expression profiles similar to that of *OsKSL4* or those expressed prior to *OsKSL4* by microarray analysis of suspension-cultured rice cells after chitin elicitor treatment[26] followed by qRT-PCR. This search identified two candidate genes, *AK073715* and *AK102690*.[55] The nuclear localization of both gene products was confirmed using a green fluorescent protein reporter. Loss-of-function mutations created by *Tos17* retrotransposon insertion in *AK073715* (H0155) and *AK102690* (NC0005) were used to examine the biological functions of these two genes. The levels of momilactones in the culture media of H0155 mutant cells at 48 h and 72 h after chitin elicitor treatment were much lower than those in the wild-type cells. In addition, induced expression of the five momilactone biosynthetic genes on chromosome 4, including *OsKSL4*, which was observed in the wild-type cells 6 h after elicitation, was strongly suppressed in the H0155 mutant. On the other hand, the levels of phytocassanes were nearly identical between the H0155 mutant and the wild-type cells at 48 h, but the induced expression of the phytocassane biosynthetic gene *OsKSL7* observed in the wild-type cells 6 h after elicitation was weakened in the H0155 mutant, and the expression levels at 0 and 24 h were slightly higher than those of the wild-type cells. Although the involvement of AK073715 in phytocassane biosynthesis remains uncertain, the protein binds directly to the elicitor-responsive *cis*-acting element in the promoter region of *OsKSL4* and has transactivation activity. This strongly suggests that AK073715 is involved in chitin elicitor-inducible momilactone biosynthesis through coordinated activation of momilactone biosynthetic genes. Hence we designated the *AK073715* gene as *OsTGAP1* (*Oryza sativa* TGA factor for phytoalexin production 1). In the NC0005 mutant cells, production of diterpenoid phytoalexins and biosynthetic gene expression (*OsKSL4*) after elicitation were comparable to those in the wild-type cells, suggesting that AK102690 is unlikely to be involved in the biosynthesis of diterpenoid phytoalexins in rice.[55]

To characterize further the biological functions of OsTGAP1, we generated an OsTGAP1-overexpressing rice cell line.[55] Only low levels of momilactones and phytocassanes were detected in the rice cells overexpressing OsTGAP1 prior to elicitation, but hyper-accumulation of both phytoalexins and the preceding

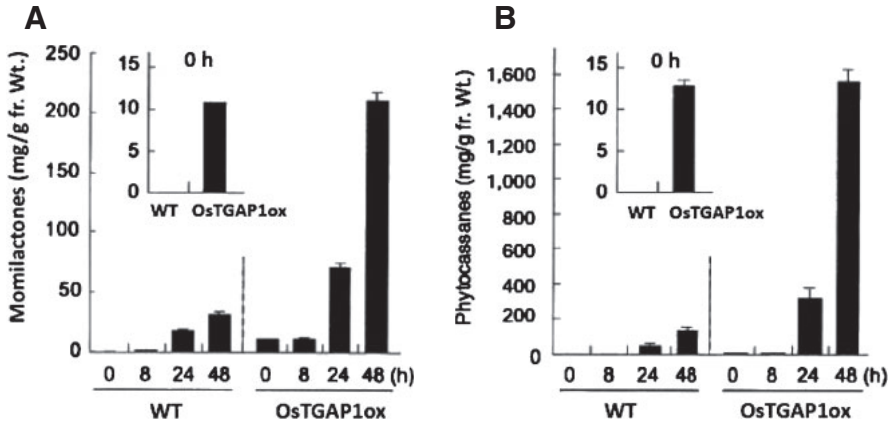

Fig. 4. Hyperaccumulation of Diterpenoid Phytoalexins in Elicited OsTGAP1-Overexpressing Rice Cells.
The amounts of momilactones (A) and phytocassanes (B) in the culture media of OsTGAP1-overexpressing (OsTGAP1ox) and wild-type (WT) rice cells 0 to 48 h after elicitation. The levels of accumulated phytoalexins before elicitation are indicated in the *inset*.

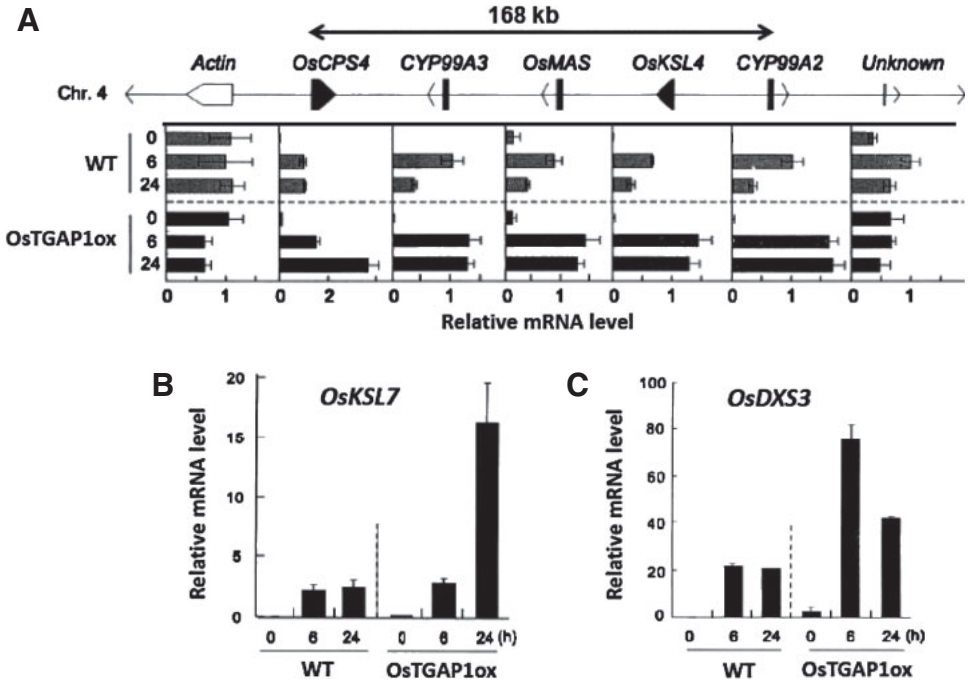

Fig. 5. Hyperexpression of Diterpenoid Phytoalexin Biosynthetic Genes in Elicited OsTGAP1-Overexpressing Rice Cells.
Relative mRNA levels as determined by qRT-PCR of the momilactone biosynthetic genes clustered on chromosome 4 (A), the phytocassane biosynthetic gene *OsKSL7* (B), and the MEP pathway gene *OsDXS3* (C) in OsTGAP1ox and wild-type rice cells 0 to 24 h after elicitation. Values have been normalized to expression of the *UBQ* gene.

coordinated hyperinduction of momilactone biosynthetic genes, the phytocassane biosynthetic gene *OsKSL7*, and the MEP pathway gene *OsDXS3* were observed after elicitation (Figs. 4 and 5).[55] Although these results indicate that OsTGAP1 is involved in regulating the expression of most diterpenoid phytoalexin biosynthetic genes, overexpression of OsTGAP1 itself is not sufficient for full transcriptional activation. This suggests that post-translational modification of OsTGAP1 is required for maximum activity and/or that an unknown factor operates synergistically with OsTGAP1 to respond to elicitation.

In rice plants, diterpenoid phytoalexins and the flavonoid phytoalexin sakuranetin are induced not only by biotic and abiotic stressors such as the pathogen *M. oryzae*, $CuCl_2$, and UV irradiation, but also by the plant hormone jasmonate. Furthermore, treatment with *M. oryzae* and $CuCl_2$ induces the biosynthesis of jasmonates in rice plants.[13,56] This suggests that *M. oryzae* and $CuCl_2$ induction of phytoalexin production results from increased production of jasmonates. However, Riemann *et al.* (in press) found that diterpenoid phytoalexins accumulate in response to *M. oryzae* in the jasmonate-deficient rice mutant *cpm2* but that sakuranetin does not accumulate in *cpm2*.[56] These results suggest that jasmonate-dependent and -independent pathways function in the pathogen-induced production of diterpenoid phytoalexins, and that only the jasmonate-dependent pathway functions in the pathogen-induced production of sakuranetin. The involvement of jasmonates in the UV-induced production of diterpenoid phytoalexins and sakuranetin in rice plants remains to be clarified. In *Nicotiana attenuata*, UV-irradiation fails to elicit the accumulation of jasmonates, but amplifies the response of jasmonate-inducible defense-related genes.[57]

VI. Future Prospects

This review focuses on functional identification of the genes involved in the biosynthesis of diterpenoid phytoalexins and the flavonoid phytoalexin sakuranetin and on the regulatory mechanisms of phytoalexin biosynthesis.

We have identified many of the genes necessary for the biosynthesis of rice diterpenoid phytoalexins, and we have identified two momilactone and phytocassane biosynthetic gene clusters. In addition, we identified the transcriptional activator OsTGAP1, which regulates the expression of clustered diterpenoid phytoalexin biosynthetic genes and upstream MEP pathway genes, and we found that OsTGAP1-overexpressing rice cells hyperaccumulate diterpenoid phytoalexins. The mechanism by which OsTGAP1 coordinately transactivates all of the diterpenoid phytoalexin biosynthetic genes is currently unknown, but our results provide evidence of the presence of an effective coordinated regulation system controlled by OsTGAP1 that makes possible the production of high levels of diterpenoid phytoalexins in rice. To understand better how OsTGAP1 coordinates the expression of the phytoalexin biosynthetic gene cluster in rice, efforts are now underway to determine the *in vivo* DNA binding sites of OsTGAP1 by chromatin immunoprecipitation coupled with next-generation sequencing technologies, and to identify the proteins that interact with OsTGAP1 *in vivo*.

We have also succeeded in cloning the key sakuranetin biosynthetic enzyme OsNOMT. Our success should make possible the regulation of endogenous sakuranetin in rice to enhance pathogen resistance and should also allow the production of large amounts of sakuranetin in microorganisms for medical research.

As for signal transduction pathways leading to the production of diterpenoid phytoalexins, we suggested that the jasmonate-independent pathway functions in jasmonate-deficient rice mutant *cpm2*. Efforts are in progress to identify the specific factors involved in this jasmonate-independent pathway.

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