Transcriptional regulation of the biosynthesis of phytoalexin: A lesson from specialized metabolites in rice

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Abstract Plants have the ability to produce vast amounts of secondary metabolites, including terpenoids, flavonoids, alkaloids, and their derivatives. These metabolites sometimes affect the growth of other organisms, either positively or negatively. Production of defensive compounds, for example phytoalexins, is a well-known strategy used by plants to defend against and withstand invading pathogens. As phytoalexins serve an obvious function in plant defense, they are known as specialized metabolites, substances with a specific biological role that function during specific circumstances. In rice, 15 diterpenoid-type phytoalexins and one flavonoid-type phytoalexin have been identified. Extensive studies have explored the biochemistry, biosynthesis, and biological functions of phytoalexins in rice, specifically the mechanism by which they function in disease resistance. This review focuses on our current knowledge of the transcriptional and hormonal regulation of the production of phytoalexins in rice.

Key words: Flavonoid, jasmonate, rice, terpenoid, transcription factor.

Phytoalexins as defensive compounds

The disease resistance of rice, a staple crop, has been extensively investigated. The production of secondary metabolites called phytoalexins, which function in defense, was discovered in rice leaves that had been exposed to the rice blast *Magnaporthe oryzae* over 50 years ago. An important event triggering phytoalexin production is the perception of a chitin oligosaccharide, one of the microbe-associated molecular patterns (MAMPs) from the blast fungus, by specific receptors (CEBiP and CERK) (Shimizu et al. 2010). The production of phytoalexins has been induced not only by treating rice cells with chitin elicitor but also by exposing rice to ultraviolet (UV) irradiation, phytohormones, cholic acid, and heavy metals such as CuCl₂ (Daw et al. 2008; Ko et al. 2010; Koga et al. 2006; Nakazato et al. 2000; Rakwal et al. 1996b; Shimizu et al. 2008).

The rice phytoalexins that have been identified fall into two major groups: the first group includes 15 diterpenoid-type phytoalexins: momilactones A and

B (Cartwright et al. 1981), oryzalexin A–F (Akatsuka et al. 1983; Akatsuka et al. 1985; Kato et al. 1993; Kato et al. 1994; Kono et al. 1984; Kono et al. 1985; Sekido et al. 1986), oryzalexin S (Kodama et al. 1992b), phytocassanes A–E (Koga et al. 1995; Koga et al. 1997; Yajima and Mori 2000), which are collectively known as labdane-related diterpenoids, and *ent*-10-oxodepressin, a macrocyclic diterpenoid (Inoue et al. 2013). The second group includes a single phytoalexin, the flavonoid-type phytoalexin sakuranetin (Kodama et al. 1992a). Among these, momilactones, phytocassanes, *ent*-10-oxodepressin and sakuranetin are considered biologically important phytoalexins because they show high antimicrobial activity in *in vitro* assays, demonstrating potent inhibitory activity on germ tube elongation of *M. oryzae*, with an ED₅₀ value below 1–10 mg·l⁻¹ (Cartwright et al. 1977; Inoue et al. 2013). They also show high accumulation in rice leaves infected by *M. oryzae*. It has recently been shown that momilactone A actually inhibits the growth of rice blast fungus at the site of infection in rice leaves (Hasegawa et al. 2010). In

Abbreviations: BTH, benzothiadiazole; bZIP, basic leucine zipper; CK, cytokinin; COMT, caffeic acid 3-O-methyltransferase; CYP, cytochrome P450; DMAPP, dimethylallyl diphosphate; ET, ethylene; GGDP, geranylgeranyl diphosphate; IPP, isopentenyl diphosphate; JA, jasmonic acid; JA-Ile, jasmonoyl-isoleucine; MAMPs, microbe-associated molecular patterns; MEP, methylerythritol phosphate; MVA, mevalonate; NOMT, naringenin 7-O-methyltransferase; PAL, phenylalanine ammonia lyase; RNAi, RNA interference; SA, salicylic acid; UV, ultraviolet.

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Figure 1. Known phytoalexins in rice. Of the 16 phytoalexins identified in rice, all are diterpenoids except for the flavonoid sakuranetin. Diterpenoid phytoalexins are classified into five groups according to the structure of their hydrocarbon precursors: phytocassanes A through E, momilactones A and B, oryzaalexin A through F, oryzaalexin S, and *ent*-10-oxodepressin.

contrast, there is no direct evidence that phytocassanes, sakuranetin, and other types of phytoalexins prevent fungal growth at the site of infection in rice.

Rice also constitutively produces phytoalexins in roots and exudes them into the rhizosphere (Toyomasu et al. 2008). Momilactone B is an allelopathic substance exuded from the roots of rice that inhibits the growth of neighboring plants (Kato-Noguchi and Ino 2003). Phytocassanes are also constitutively exuded with momilactones; they are believed to be defensive substances, but not phytotoxic substances (Toyomasu et al. 2008). Given that the production of phytoalexins is constitutive or induced and that they play defensive roles, it appears that rice has evolved the production of phytoalexins as specialized metabolites. Their characteristic biosynthetic routes rely on the expression of relevant biosynthetic genes, some of which are located close together on chromosomes in genomic clusters.

Biosynthetic pathways and genes for diterpenoid phytoalexins

In plants, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are the basic C5 precursors for terpenoid biosynthesis, are produced via two distinct pathways: the mevalonate (MVA) pathway and the methylerythritol phosphate (MEP) pathway (Lichtenthaler et al. 1997). Sesquiterpenes and triterpenes are synthesized in the cytoplasm from IPP that is derived from the MVA pathway (Vranová et al. 2013). In contrast, diterpenes, including diterpenoid phytoalexins, are synthesized in plastids from IPP and DMAPP that are derived from the MEP pathway (Estévez et al. 2001; Vranová et al. 2013) (Figure 2). In fact, the elicitor-induced accumulation of diterpenoid

phytoalexin in rice cells is suppressed by treatment with 5-ketoclofazone and fosmidomycin, chemical inhibitors of MEP pathway, but not by treatment with mevastatin, an inhibitor of the MVA pathway (Okada et al. 2007). In addition, the expression of seven MEP pathway genes (*OsDXS3*, *OsDXR*, *OsCMS*, *OsCMK*, *OsMCS*, *OsHDS*, and *OsHDR*) is synchronously induced in elicited rice cells and rice leaves treated with jasmonic acid (JA), UV, or CuCl_2 . This effect provides further evidence that the MEP pathway is involved in inducible production of diterpene phytoalexins (Okada et al. 2007).

In the phytoalexin biosynthesis, geranylgeranyl diphosphate (GGDP), a common precursor of diterpenoids, is converted into diterpene hydrocarbons in two sequential cyclization steps. Six diterpene cyclase involved in these steps have been identified (Peters 2006; Toyomasu 2008). GGDP is first cyclized by *OsCPS2/OsCyc2* and *OsCSP4/OsCyc1* to form *ent*-copalyl diphosphate and *syn*-copalyl diphosphate (Otomo et al. 2004b; Prisic et al. 2004; Sakamoto et al. 2004; Xu et al. 2004). Next, *OsKSL7/OsDTC1*, *OsKSL10*, *OsKSL4*, and *OsKSL8/OsDTC2* catalyze the second cyclization of *ent*-copalyl diphosphate or *syn*-copalyl diphosphate to four distinct diterpene hydrocarbons: *ent*-cassa-12,15-diene, *ent*-sandaracopimaradiene, $9\beta\text{H}$ -pimara-7,15-diene, and stemar-13-ene (Cho et al. 2004; Nemoto et al. 2004; Otomo et al. 2004a; Wilderman et al. 2004) (Figure 2). In contrast, cDNA encoding a diterpene cyclase responsible for biosynthesis of *ent*-10-oxodepressin, a macrocyclic diterpenoid, has not been identified yet.

After the diterpene cyclases involved in diterpenoid phytoalexin biosynthesis were identified, it was shown that *OsCPS4* and *OsKSL4*, which encode diterpene cyclases involved in momilactone biosynthesis, are located in a narrow region on rice chromosome 4.

Figure 2. Established biosynthetic routes of diterpenoid phytoalexins. Diterpenoid phytoalexins are synthesized from geranylgeranyl diphosphate (GGDP). GGDP is cyclized by terpene cyclase (OsCPSs and OsKSLs) and then converted to each phytoalexin by P450 monooxygenase and dehydrogenase. Activation of the methylerythritol phosphate (MEP) pathway leading to GGDP synthesis may be required to supply sufficient amounts of terpenoid precursors for the production of phytoalexins.

Two cytochrome P450 (CYP) genes (*CYP99A2* and *CYP99A3*) and a dehydrogenase gene are located in the same region, forming a gene cluster (Sakamoto et al. 2004). Early work with cell-free extracts suggested that CYPs and dehydrogenases are involved in the downstream oxidation steps of diterpene hydrocarbon precursors (Atawong et al. 2002; Kato et al. 1995). The following line of evidence clearly proved that assumption. Double knockdown of *CYP99A2* and *CYP99A3* by

RNA interference (RNAi) caused the reduction of momilactone accumulation in elicitor-treated rice cells (Shimura et al. 2007). Moreover, *CYP99A3* catalyzed C19-oxidation of 9βH-pimara-7,15-diene in vitro, and 9βH-pimara-7,15-diene-19-oic acid was detected in rice plants, suggesting that *CYP99A2* and/or *CYP99A3* are involved in the downstream oxidation steps of the diterpene hydrocarbons (Wang et al. 2011). The function of the dehydrogenase gene as a momilactone A synthase

(*OsMAS*) was also investigated by in vitro enzyme assay, resulting in the catalytic conversion of 3 β -hydroxy-9 β H-pimara-7,15-diene-19,6 β -olide into momilactone A (Shimura et al. 2007). Taken together, these reports indicate the presence of a momilactone biosynthetic gene cluster, although further research is required to reveal the complete pathway for momilactone biosynthesis.

The phytocassane biosynthetic genes *OsCPS2* and *OsKSL7* are located in a narrow region on rice chromosome 2, along with two diterpene cyclase genes (*OsKSL5* and *OsKSL6*) and six CYP genes (*CYP71Z6*, *CYP71Z7*, and *CYP76M5–8*), creating another gene cluster. These five CYP genes, with the exception of *CYP71Z6*, show an elicitor-inducible expression pattern similar to that of *OsCPS2* and *OsKSL7* (Okada et al. 2007), suggesting that these CYP genes are involved in the biosynthesis of phytocassane.

In recent years, Reuben Peters' group has extensively studied and summarized the catalytic functions of these CYPs using in vitro enzyme assay, as described in their recent review (Schmelz et al. 2014). It was reported that *CYP76M7* catalyzes the 11 α -hydroxylation of *ent*-cassa-12,15-diene (Swaminathan et al. 2009). In addition, *CYP71Z7* can catalyze the C2-hydroxylation of *ent*-cassa-12,15-diene in vitro. However, *ent*-cassa-12,15-diene is a poorly recognized substrate for *CYP71Z7* ($K_m=200\mu\text{M}$) in in vitro assays, suggesting that the true substrate of *CYP71Z7* in planta has not been identified (Wu et al. 2011).

We used a transgenic approach to investigate the involvement of these CYPs in the biosynthesis of phytocassane. Double knockdown of *CYP76M7* and *CYP76M8* by RNAi causes a decrease in phytocassane accumulation in elicitor-treated rice cells. Collectively, these biochemical and genetic data indicate that *CYP76M7* and/or *CYP76M8* are involved in phytocassane biosynthesis (Wang et al. 2012a). We also found that the accumulation of C2-oxygenated phytocassanes is specifically suppressed in *CYP71Z7* knockdown lines (Okada 2011). Taken together, these reports indicate the presence of the phytocassane biosynthetic gene cluster. However, it has also been reported that another CYP, which is encoded out of the gene cluster, is involved in phytocassane biosynthesis. *OsKOL4/CYP701A8*, an *ent*-kaurene oxidase paralog encoded on rice chromosome 6, catalyzes the 3 α -hydroxylation of *ent*-cassa-12,15-diene and *ent*-sandaracopimaradiene, suggesting that this enzyme functions in the biosynthesis of both phytocassane and oryzalexin (Wang et al. 2012b).

In vitro conversion assays of diterpene hydrocarbons other than *ent*-cassa-12,15-diene with *CYP76M5*, *CYP76M6*, and *CYP76M8* exhibited promiscuous catalytic activities, resulting in the 6 β -hydroxylation of *syn*-pimara-7,15-diene and 7 β -hydroxylation of

ent-sandaracopimaradiene (Wang et al. 2012a). The findings suggest that they serve potential functions in momilactone and oryzalexin biosynthesis (Figure 2).

Two genes (*OsKSL6* and *CYP71Z6*) on the chromosome 2 gene cluster may be relevant to the biosynthesis of oryzalide-related diterpenoids, which are phytoanticipins produced by rice (Kono et al. 1991; Kono et al. 2004; Watanabe et al. 1990; Watanabe et al. 1992). *OsKSL6* catalyzes the cyclization of *ent*-copalyl diphosphate to *ent*-kaur-15-ene, which is putative precursor of oryzalide-related compounds (Kanno et al. 2006). *CYP71Z6* also catalyzes the C2-hydroxylation of *ent*-kaur-15-ene (Wu et al. 2011). These facts suggest that the chromosome 2 gene cluster may be associated not only with phytocassane biosynthesis but also with the biosynthesis of oryzalide-related compounds. It remains to be investigated whether the biosynthetic enzymes from the chromosome 2 gene cluster are involved in the biosynthesis of oryzalide-related compounds, momilactones, and oryzalexins in vivo.

Gene clusters for the biosynthesis of specialized metabolites

The momilactone and phytocassane biosynthetic genes are clustered as described, whereas the oryzalexin biosynthetic genes are not clustered. To date, over 10 examples of plant metabolic gene clusters for the biosynthesis of defense compounds have been reported (Nützmann and Osbourn 2014). It is known that plant metabolic gene clusters exhibit tissue- or site-specific co-expression patterns (Nützmann and Osbourn 2014). Therefore, it has been hypothesized that the formation of a gene cluster allows the coordinated regulation of clustered genes and the efficient biosynthesis of specialized metabolites (Chu et al. 2011). Indeed, the momilactone and phytocassane biosynthetic genes exhibit a root-specific expression pattern (Toyomasu et al. 2008). Moreover, these genes show the temporally coordinated expression of mRNAs after treatment with an elicitor in rice cells, resulting in synchronized production of momilactones and phytocassanes (Okada et al. 2007; Shimura et al. 2007).

It is possible that the clustering of the momilactone and phytocassane biosynthetic genes contributes to the effective production of these phytoalexins. Consistent with this idea, momilactones and phytocassanes accumulate at higher levels than does oryzalexin S in rice leaves infected with *M. oryzae* (Toyomasu et al. 2014). It has also been hypothesized that the formation of gene clusters facilitates the co-inheritance of functionally related biosynthetic genes. Deficits in biosynthetic genes for specialized metabolites can lead not only to a loss of the end product but also to the accumulation of toxic bioactive intermediates. The co-inheritance of

functionally related biosynthetic genes may prevent the loss of parts of these genes. Further research to investigate the complete biosynthetic pathways of the diterpenoid phytoalexin and the transcriptional regulatory mechanism(s) of the momilactone and phytocassane biosynthetic gene clusters may reveal the significance of clustering of biosynthetic genes.

Biosynthetic genes involved in sakuranetin production

The flavonoid sakuranetin was first identified from *Prunus* bark cortex as an aglycone of sakuranin (Asahina 1908). After that, sakuranetin was found in rice and several other plant species, including *Artemisia campestris*, *Prunus* spp., *Baccharis* spp., *Betula* spp., and *Juglans* spp. (Ahuja et al. 2012). The final step in sakuranetin biosynthesis is 7-*O*-methylation of naringenin, a key biosynthetic intermediate in isoflavone and a variety of flavones, which is catalyzed by *S*-adenosyl-*L*-methionine dependent naringenin 7-*O*-methyltransferase (NOMT) (Rakwal et al. 1996a). Therefore, NOMT plays a key role in sakuranetin biosynthesis at the point at which it branches from a common flavonoid biosynthetic pathway.

The first purification of NOMT to apparent homogeneity from crude extracts of UV-treated wild-type rice leaves was likely accomplished by Rakwal et al. (2000). The purified protein, however, was actually highly homologous to a caffeic acid 3-*O*-methyltransferase (COMT) from maize, and in vitro enzyme assay consistently showed that it had COMT activity, but not NOMT activity. Thus, this enzyme was named OsCOMT1 (Lin et al. 1996; Rakwal et al. 2000).

As a loss-of-function mutant of *OsCOMT1* was able to confer NOMT enzymatic activity and accumulation of sakuranetin after elicitation, *OsCOMT1* apparently is not essential for sakuranetin production in rice. Therefore, we took advantage of the *OsCOMT1* mutant to purify the *OsNOMT* enzyme and successfully obtained active *OsNOMT* protein, thereby identifying the *OsNOMT* gene in rice (Shimizu et al. 2012). An in vitro enzyme assay using a recombinant *OsNOMT* protein tagged with glutathione-*S*-transferase exhibited the highest substrate preference to naringenin with a reasonable K_m value ($1.9 \mu\text{M}$), compared with other related substrates, the flavanone liquiritigenin and flavones. The protein showed no methylation activity on phenolic compounds, including isoflavonoids. As *OsNOMT* mRNA was induced prior to sakuranetin accumulation in elicited rice leaves, these results strongly suggested that *OsNOMT* is a major sakuranetin synthase (Shimizu et al. 2012).

Naringenin is commonly biosynthesized from phenylalanine via the phenylpropanoid and flavonoid

pathways. According to the Rice Annotation Project database (RAP-DB: <http://rapdb.dna.affrc.go.jp/>), there are a number of homologous genes involved in the phenylpropanoid and flavonoid pathways, including 10 phenylalanine ammonia lyases (PAL), 11 4-coumarate CoA ligases, 13 chalcone synthases, and seven chalcone isomerases in the rice genome. Among these, *OsPAL06* knock-down plant shows a lower accumulation of sakuranetin and naringenin in the roots, suggesting that *OsPAL06* is involved in sakuranetin production in rice roots (Duan et al. 2014). However, the contribution of other genes in the phenylpropanoid and flavonoid pathway to stress-inducible sakuranetin production has not been demonstrated. Some of these genes show a co-expression pattern with *OsNOMT* in response to JA treatment or UV-irradiation (Miyamoto et al. 2012; Park et al. 2013). Such information might help to identify biosynthetic genes involved in the production of sakuranetin.

Signaling pathways leading to the phytoalexin biosynthesis mediated by plant hormones

An important topic of study is endogenous signaling leading to the induction of phytoalexin production after the perception of biotic and abiotic elicitors. It has been assumed that similar signaling pathways may induce both diterpenoid and flavonoid phytoalexin production in rice. However, current studies demonstrate that the signaling pathways for diterpenoid and flavonoid phytoalexin production have important differences.

One of the best studied signaling pathways involved in stress-induced phytoalexin production in rice is mediated by jasmonate, a plant hormone that is inductively synthesized in response to wounding and pathogen infections. A component of jasmonate, jasmonoyl-isoleucine (JA-Ile), is biosynthesized by conjugating isoleucine to JA by JAR1 protein. It is a major active compound perceived by the co-receptor complex of COI1, an F-box component of the SCF-type E3-ubiquitin ligase complex (SCF^{COI1}), which triggers ubiquitination and subsequent degradation of JAZ repressor proteins by 26S proteasome. Release of a transcriptional activator such as MYC2 from repression by the JAZ protein interaction accelerates the expression of downstream genes, such as JA-inducible defense-related genes (Wasternack and Hause 2013). These core components in JA signaling are conserved in rice as well (Cai et al. 2014; Taniguchi et al. 2014a; Ye et al. 2012).

The production of rice diterpenoid phytoalexins and the flavonoid sakuranetin is induced not only by biotic and abiotic stresses, as mentioned above, but also by JA treatment in rice leaves (Rakwal et al. 1996a; Rakwal et al. 1996b). Jasmonate also induces expression of

phytoalexin biosynthetic genes in rice leaves (Miyamoto et al. 2012). Furthermore, jasmonate accumulates in response to *M. oryzae* infection and CuCl₂ treatment in rice leaves (Rakwal et al. 1996b; Riemann et al. 2013). In suspension-cultured rice cells, chitin elicitor treatment induces accumulation of JA, followed by accumulation of momilactone A (Nojiri et al. 1996). Hence, jasmonate is thought to be an important secondary signaling molecule for the production of the diterpenoid phytoalexins and sakuranetin in rice.

Recently, several rice jasmonate mutants were identified. Using them, researchers have investigated the contribution of endogenous jasmonate to phytoalexin production in detail. In two loss-of-function mutants of *allene oxide cyclase* of rice, *cpm2* and *hebiba*, constitutive and wound-induced levels of JA and JA-Ile were extremely low. When leaves were inoculated with *M. oryzae*, the accumulation levels of momilactone B and phytocassanes were comparable in *cpm2*, *hebiba*, and the wild type, although accumulation of momilactone A was lower in *cpm2* and *hebiba* than in the wild type. On the other hand, the accumulation of sakuranetin in leaves of *cpm2* and *hebiba* that were inoculated with *M. oryzae* was severely suppressed compared with that of the wild type (Riemann et al. 2013). These results are inconsistent with the original understanding that jasmonate is an important secondary signaling molecule for the production of both diterpenoid phytoalexins and sakuranetin in rice. Of course, jasmonate has some impacts on the production of diterpenoid phytoalexins under specific conditions, but it is clearly not essential, whereas in sakuranetin production it is an absolute requirement.

Further investigation of phytoalexin production focusing on JA-Ile, an active jasmonate, was performed by using the rice *JAR1* mutant *osjar1-2*, which contains remarkably lower levels of JA-Ile than the wild type after wounding. Several experiments have demonstrated the significance of JA-Ile. When leaves of *osjar1-2* were treated with JA, accumulation levels of both sakuranetin and diterpenoid phytoalexins were severely suppressed compared with those of the wild type, but the response of *osjar1-2* was restored by treatment with JA-Ile. On the other hand, when *osjar1-2* was inoculated with *M. oryzae* or treated with CuCl₂, the accumulation levels of sakuranetin were far lower than those of the wild type, whereas the levels of diterpenoid phytoalexins were comparable to those of the wild type, as has been observed in the *cpm2* and *hebiba* mutants (Shimizu et al. 2013). These results suggested the existence of a JA-Ile-dependent pathway for production of both diterpenoid phytoalexins and sakuranetin, but a jasmonate-independent signaling pathway is also involved in stress-induced production of diterpenoid phytoalexins. Further analysis is necessary to find an endogenous signaling

molecule capable of the production of diterpenoid phytoalexins in the rice jasmonate mutants, which in turn can provide evidence for a jasmonate-independent pathway for phytoalexin production.

Regarding core signaling components of JA-Ile, the rice genome encodes three copies of COI1 and 14 copies of JAZ protein genes. Among these, *OsCOI1a* and *OsJAZ8* were individually reported to be involved in jasmonate-mediated rice defense responses (Taniguchi et al. 2014a; Taniguchi et al. 2014b; Ye et al. 2012). However, their roles in the production of rice phytoalexins have not been investigated. On the other hand, *OsPAD4*, a lipase-like protein, was suggested to be involved in JA accumulation, JA-mediated systemic defense responses, and the accumulation of momilactone A in rice. By comparison, *Arabidopsis* AtPAD4 functions in SA-dependent defense signaling by interacting with another lipase-like protein and is involved in the production of camalexin (Ke et al. 2014). How *OsPAD4* functions in rice is still unknown, but these results suggested that *OsPAD4* may have a specific function for JA-mediated signaling in rice and may be involved in rice phytoalexin production (Figure 3A).

Treatment with natural and synthetic cytokinins (CKs), as well as with jasmonate, induces the production of diterpenoid phytoalexins in rice leaves and suspension-cultured cells (Ko et al. 2010). However, CK treatment inhibits JA-inducible sakuranetin production in rice leaves (Tamogami et al. 1997). On the other hand, exogenous root applications of salicylic acid (SA) promote the accumulation of oryzalexins and momilactone A in leaves (Daw et al. 2008). Recent reports presented the role of CKs and SA in plant defense responses (Choi et al. 2010; Großkinsky et al. 2011; Jiang et al. 2013). A synergistic crosstalk of CK/SA signaling was also reported showing that 0.1 mM CKs treated together with benzothiadiazole (BTH), a plant activator that enhances SA signaling pathway, had several-fold enhancement of the induction of momilactone and phytocassane biosynthetic genes (Akagi et al. 2014). Besides, SA-dependent and JA-dependent pathways frequently interact either synergistically or antagonistically in plant-pathogen interaction (De Vleeschauwer et al. 2013) (Figure 3B, C).

In addition, it was reported that treatment of wounded rice leaves with methionine, the precursor of ethylene (ET), could induce the accumulation of momilactone A and sakuranetin. However, ET treatment of wounded leaves can induce sakuranetin but not momilactone A, suggesting that the induction of diterpenoid phytoalexin production by methionine is not regulated by ET alone (Nakazato et al. 2000; Xu et al. 2012) (Figure 3D). As mentioned, there are different views of the effects of plant hormone treatment on phytoalexin production in rice. The endogenous roles of each hormone remain

Figure 3. Hormonal regulation of phytoalexin production. (A) Role of jasmonate in phytoalexin production of rice. Endogenous jasmonoyl-isoleucine (JA-Ile) induced by biotic and abiotic stress is involved in the production of both flavonoid and diterpenoid phytoalexins. JA-Ile-mediated signaling is indispensable to the production of flavonoid phytoalexin. Production of diterpenoid phytoalexins also may be induced by a jasmonate-independent pathway. (B) Crosstalk among cytokinins (CKs), salicylic acid (SA), and jasmonate on phytoalexin production. Treatment with CKs induces production of diterpenoid phytoalexins in suspension-cultured rice cells and rice leaves. In response to *Magnaporthe oryzae* infection, CKs and SA synergistically induce the production of diterpenoid phytoalexins. Benzothiadiazole (BTH) acts as a plant activator that enhances SA signaling. On the other hand, treatment of CKs inhibits the production of JA-induced production of flavonoid phytoalexin in rice leaves. (C) Effect of exogenously applied salicylic acid (SA) on phytoalexin production in rice roots. SA treatment induces the production of diterpenoid phytoalexins in rice roots. (D) Effect of exogenously applied ethylene (ET) and methionine on phytoalexin production. Treatment with methionine, the precursor of ET, induces production of both flavonoid and diterpenoid phytoalexins in rice leaves, whereas treatment with ET alone induces only the production of flavonoid phytoalexin in rice leaves.

to be elucidated. In the future, investigations of the roles not only of endogenous jasmonate but also of the other endogenous hormones, such as SA, ET, and CKs, are necessary in order to fully understand regulation of stress-induced phytoalexin production in rice.

Transcriptional networks regulating the gene expression for phytoalexin production

Much work has been done in an effort to identify the transcription factors involved in the regulation of diterpenoid phytoalexin production. Transcription factors that are inductively expressed during the blast infection or simply treated with chitin elicitor have been screened in an attempt to find candidate factors by using transcriptomes. Some of these were not responsive to blast infection, probably due to the infectious agent's

organ-specific effects, whereas the chitin elicitor treatment of rice cells can trigger the expression of all factors identified. The transcriptional network comprised of such transcription factors involved in the phytoalexin biosynthesis is summarized in Figure 4.

Okada et al. (2009) reported that the chitin elicitor-induced basic leucine zipper (bZIP) transcription factor OsTGAP1 is involved in the regulation of the production of momilactones and phytocassanes in elicitor-treated rice cells. *OsTGAP1*-overexpressing (*OsTGAP1ox*) rice cells exhibit hyperaccumulation of momilactones and phytocassanes, as well as enhanced expression of all momilactone biosynthetic genes, the phytocassane biosynthetic gene *OsKSL7*, and the MEP pathway gene *OsDXS3*, after being treated with an elicitor (Okada et al. 2009). We sought to investigate the mechanisms of the elicitor-stimulated coordinated hyperinduction of these phytoalexin biosynthetic genes

Figure 4. Transcriptional networks leading to phytoalexin production. Solid arrows represent established relationships, while dotted arrows indicate gaps in knowledge. The expression of *OsTGAP1* and *OsbZIP79* are induced by chitin elicitor treatment in rice cells (Okada et al. 2009). JA treatment also induces the expression of *OsTGAP1*. Overexpression of *OsTGAP1* causes the hyperaccumulation of momilactones and phytocassanes, as well as the enhanced expression of diterpenoid phytoalexin biosynthetic genes, after treatment with an elicitor in rice cells (Miyamoto et al. 2014; Okada et al. 2009). By comparison, overexpression of *OsbZIP79* suppressed the chitin elicitor-inducible expression of diterpenoid phytoalexin biosynthetic genes, causing a decrease in the accumulation of momilactones and phytocassanes in elicitor-treated rice cells (Miyamoto et al. 2015). A direct physical interaction between *OsTGAP1* and *OsbZIP79* has been observed (Miyamoto et al. 2015). The expression of *OsWRKY76* is also induced by chitin elicitor and JA treatments in rice cells. In *OsTGAP1*-overexpressing rice cells, *OsTGAP1* binds to the upstream region of *OsWRKY76* and the chitin elicitor-induced expression of *OsWRKY76* is suppressed (Miyamoto et al. 2014). The expression of *OsWRKY76* is also induced by *Magnaporthe oryzae* infection in rice leaves. The overexpression of *OsWRKY76* suppresses the accumulation of both diterpenoid phytoalexins and flavonoid phytoalexin in *M. oryzae*-infected rice leaves (Yokotani et al. 2013). *OsWRKY53* expression is induced by *M. oryzae*, and *OsWRKY53* protein is phosphorylated and activated by the *OsMKK4-OsMPK3/OsMPK6* cascade. Overexpression of phosphomimic mutated *OsWRKY53* causes the upregulation of momilactone biosynthetic genes and the slight accumulation of momilactones in the absence of pathogen attack (Chujo et al. 2014). *OsWRKY45* is involved in the regulation of diterpenoid phytoalexin production in salicylic acid (SA) and cytokinin (CK) signaling pathway (Akagi et al. 2014). Benzothiadiazole (BTH) acts as a plant activator that enhances SA signaling pathway.

in *OsTGAP1ox* cells. We performed transcriptome analysis and chromatin immunoprecipitation with next-generation sequencing using *OsTGAP1ox* cells and identified *OsTGAP1* target genes. None of the clustered momilactone and phytocassane biosynthetic genes was included among the *OsTGAP1* target genes, suggesting that *OsTGAP1* did not directly regulate the expression of these biosynthetic genes through binding to each promoter region. Interestingly, however, several *OsTGAP1*-binding regions were found in the intergenic regions among and near the cluster regions. Although the detailed regulatory mechanism of these clustered genes remains unknown, the binding of *OsTGAP1* to the intergenic regions of the gene clusters might play a particular role in transcriptional regulation. Concerning the MEP pathway genes, only *OsDXS3* possessed an *OsTGAP1*-binding region in its upstream region. A subsequent transactivation assay further confirmed the direct regulation of *OsDXS3* expression by *OsTGAP1*

(Miyamoto et al. 2014).

The screening of *OsTGAP1*-interacting proteins using the yeast two-hybrid technique resulted in the identification of 10 candidates. Among the *OsTGAP1*-interacting protein candidates, a TGA factor, *OsbZIP79*, was investigated to verify its involvement in the regulation of phytoalexin production. Intriguingly, whereas *OsbZIP79* transactivation activity was observed in a transient reporter assay, the overexpression of *OsbZIP79* resulted in suppression of the chitin elicitor-inducible expression of diterpenoid phytoalexin biosynthetic genes, which reduced the accumulation of phytoalexin in elicitor-treated rice cells. These results suggest that *OsbZIP79* functions as a negative regulator of phytoalexin production triggered by the chitin elicitor in rice cells (Miyamoto et al. 2015). The mRNA level of *OsTGAP1* is induced slightly earlier than that of *OsKSL4* in rice cells after chitin elicitor treatment. In contrast, the induction of the *OsbZIP79* mRNA level is synchronous

with that of the *OsKSL4* mRNA level (Okada et al. 2009). Therefore, it is possible that *OsbZIP79* suppresses the action of *OsTGAP1* in upregulating the transcription levels of diterpenoid phytoalexin biosynthetic genes. *OsTGAP1* and *OsbZIP79* may act competitively to modulate the expression of diterpenoid phytoalexin biosynthetic genes, allowing fine-tuning of phytoalexin production (Miyamoto et al. 2015). Further studies should examine how the interaction of *OsTGAP1* and *OsbZIP79* affects elicitor-inducible phytoalexin production in rice cells.

The plant-specific zinc finger WRKY family transcription factors are also involved in phytoalexin biosynthesis. Yokotani et al. (2013) reported that in *OsWRKY76*-overexpressing rice plants, the inducible expression of the genes that are relevant to phytoalexin biosynthesis is suppressed, causing a decrease in the accumulation of momilactones, phytocassanes, and sakuranetin in *M. oryzae*-infected rice leaves. These results suggest that *OsWRKY76* functions as a negative regulator of the production of both the diterpenoid phytoalexins and the flavonoid phytoalexin (Yokotani et al. 2013). It is also reported that *OsWRKY76* and *OsTGAP1* interact in the signaling pathway for diterpenoid phytoalexin production. The elicitor-induced expression of *OsWRKY76* was suppressed in *OsTGAP1ox* cells. Taken together with the fact that *OsTGAP1* was bound to the upstream region of *OsWRKY76*, it appears that *OsTGAP1* directly downregulates the expression of *OsWRKY76*. The downregulation of *OsWRKY76* expression is among the mechanisms explaining the enhanced expression of diterpenoid phytoalexin biosynthetic genes in *OsTGAP1ox* cells (Miyamoto et al. 2014). On the other hand, overexpression of *OsbZIP79* did not greatly affect elicitor-induced expression of *OsWRKY76*, suggesting that *OsbZIP79* and *OsWRKY76* occupy different signaling pathways and suppress phytoalexin production in different ways (Miyamoto et al. 2015).

Another rice WRKY transcription factor, *OsWRKY53*, was found to be involved in the regulation of momilactone biosynthetic genes. Chujo et al. (2014) reported that *OsWRKY53* was phosphorylated and activated by the *OsMKK4*-*OsMPK3*/*OsMPK6* cascade (Kishi-Kaboshi et al. 2010), a fungal elicitor-responsive MAPK cascade in rice. Transgenic rice plants overexpressing a phosphomimic mutated *OsWRKY53* (*OsWRKY53SD*) showed further enhanced disease resistance to the blast fungus, compared with native *OsWRKY53*-overexpressing rice plants. In addition, a substantial number of defense-related genes, including momilactone biosynthetic genes, were upregulated in the *OsWRKY53SD*-overexpressing plants compared with the *OsWRKY53*-overexpressing plants. *OsWRKY53SD*-overexpressing plants also exhibit a slight accumulation

of momilactones even in the absence of a pathogen attack (Chujo et al. 2014). Considering that activation of the *OsMKK4*-*OsMPK3*/*OsMPK6* cascade leads to the accumulation of diterpenoid phytoalexins (Kishi-Kaboshi et al. 2010), *OsWRKY53* may be one of the components of downstream signaling of this cascade.

Recently, Akagi et al. (2014) reported that the rice WRKY transcription factor, *OsWRKY45*, is involved in the regulation of diterpenoid phytoalexin production in SA/CK signaling pathway. *OsWRKY45* plays a central role in the priming of diterpenoid phytoalexin biosynthetic genes in BTH-treated rice leaves. They also showed that the diterpenoid phytoalexin biosynthetic genes are up-regulated by the treatment of both SA and CK in a *OsWRKY45*-dependent manner (Akagi et al. 2014).

Conclusion and perspective

Concentrated efforts have revealed the relevant factors and signaling pathways that are involved in the regulation of phytoalexin production in rice. It is still unknown, however, how these transcription factors regulate phytoalexin biosynthetic genes in concert with one another. Further research to investigate the molecular mechanisms of transcriptional regulation of phytoalexin biosynthetic genes and to reveal how upstream signals activate each transcription factor in the signaling cascade is essential. Such work will elucidate the full picture of endogenous signaling that leads to the biosynthesis of phytoalexins.

Our current understanding, based on the evidence showing the defensive function of phytoalexins in rice, does not fully explain why rice produces this specialized metabolite. The biological roles of phytoalexins, should be investigated in more detail by characterizing mutants that are defective in the production of phytoalexin. Recently, loss-of-function mutants for *OsCPS2* and *OsKSL4*, which are defective in momilactone, were analyzed to assess the endogenous function of momilactones (Toyomasu et al. 2014; Xu et al. 2012). In a study, the *OsCPS2* mutant exhibited increased susceptibility to the blast fungus, but this effect was not seen in the case of the *OsKSL4* mutant. With regard to allelopathic activity, neither of the mutants displayed an allelopathic effect on nearby weeds (Toyomasu et al. 2014; Xu et al. 2012).

So far, there has been no attempt to examine the effect of overproduction of the phytoalexins on the above biological responses in rice. The development of transgenic overexpression lines would enable the study of plants that are resistant to pathogens and suppress of the growth of weeds and would represent progress toward a chemical-free model of agriculture. We hope that the knowledge of the transcriptional regulation

of phytoalexins synthesis will be applied to beneficial strategies in an environmentally friendly agricultural system.

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