Award Review
The Biosynthesis of Isoprenoids and the Mechanisms Regulating It in Plants

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Plants synthesize isopentenyl diphosphate (IPP) via the mevalonate pathway and the methylerythritol phosphate (MEP) pathway. IPP is condensed to its allylic isomer, dimethylallyl diphosphate, to yield geranylgeranyl diphosphate, a common precursor for the production of cyclic diterpenoids. Studies of subcellular localization and of transgenic plants defective in the enzymes involved in the pathway have revealed that the synthesis processes of plastidic diterpenoids are metabolically separated in the plastids. Diterpenoid phytoalexins are synthesized through the plastidic MEP pathway in rice. The biosynthetic genes responsible for diterpenoid phytoalexins are clustered on the rice chromosomes, and the expression of them was coordinately regulated under stress conditions. Furthermore, a basic leucine zipper transcription factor, OsTGAP1, which is induced by the fungal chitin oligosaccharide elicitor, was identified as a key regulator of coordinated expression of the clustered biosynthetic genes for phytoalexin production in rice.

Key words: isoprenoids; diterpenoid phytoalexins; rice; methylerythritol phosphate (MEP) pathway; gene cluster

Isoprenoids, the largest group of natural products in living organisms, are derived from a basic five-carbon unit, isopentenyl diphosphate (IPP), and its allylic isomer, dimethylallyl diphosphate (DMAPP). IPP is sequentially condensed to DMAPP to yield short-chain isoprenoid precursors, geranyl diphosphate, farnesyl diphosphate, and geranylgeranyl diphosphate (GGPP), which often are further metabolized to monoterpene (C10), sesquiterpenes (C15), and diterpenes (C20) after they are cyclized by terpene cyclasses and modified by other oxidases. IPP biosynthesis occurs via two pathways in living organisms: the mevalonate (MVA) pathway, first reported in yeast and mammals, and the methylerythritol phosphate (MEP) pathway, identified afterwards in eubacteria and plants. In plants, GGPP is a key compound converted into a variety of diterpenes and photosynthetic pigments, which play important roles in plant growth, physiology, and defense against pathogens. These include many bioactive cyclic diterpenoids, including plant hormone gibberellins, the anti-cancer drug Taxol, and antimicrobial compound phytoalexins, including monomethylated monooxygenase compounds, all of which are synthesized through the MEP pathway (Fig. 1). The production of these isoprenoids is precisely regulated in plants. Therefore, from the viewpoint of agrochemistry, an understanding of the regulatory mechanism of plant isoprenoid biosynthesis is key to the efficient production of useful substances in plants.

This review highlights the biosynthetic pathway of isoprenoid production in plants with a special focus on the pathway of diterpenoid production, and also the coordinated regulation of gene expression, which is responsible for the production of diterpene phytoalexins in rice.

I. The Early Stage of the Biosynthetic Pathway for Diterpene Production in Plastids

It is now widely accepted that IPP and DMAPP in plants are formed through two distinct pathways: the MVA pathway and the MEP pathway, but before the discovery of the MEP pathway, it was thought that isoprenoids are biosynthesized through the cytosolic MVA pathway in all living organisms. In 1996, Rohmer et al. discovered the first reaction step of the MEP pathway, catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS) in E. coli. Then the Arabidopsis ortholog of DXS gene was discovered in studies of an Arabidopsis clal mutant that exhibits decreased chlorophyll accumulation, suggesting that the MEP pathway in plastids functions as the main route of diterpenoid biosynthesis in plants (Fig. 2).

Transgenic plants of Arabidopsis expressing the antisense AtMECT gene, encoding 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (catalyzing the second step in the pathway), were generated to elucidate the physiological role of the MEP pathway in plants. In the transgenic plants, the accumulation of photosynthetic pigments such as carotenoids and chlorophylls was reduced as compared to wild-type plants. Fosmidomycin, an inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) (catalyzing the third step in the pathway), caused similar depletion of the pigments in the transgenic plants. We also found that production of ent-kaurene, a plastidic precursor of gibberellins, fell in plants in which the MEP pathway was genetically or chemically repressed. These observations suggest that...
both AtMECT and DXR are important in the synthesis of IPP and DMAPP, and that 13-kaurene is produced mainly through the MEP pathway in the plastid.

IPP isomerase (IPI) catalyzes the interconversion of IPP to DMAPP, an essential starter moiety for the condensation reactions. It has been reported that the S. cerevisiae IPI gene, IDI1, is an essential single-copy gene, and that disruption of the IDI1 gene results in a lethal phenotype.3) Based on information as to other kingdoms, the conversion of IPP to DMAPP by IPI in plants is thought to be necessary for isoprenoid biosynthesis via the cytosolic MVA pathway.14–16) In contrast, DMAPP is synthesized directly along with IPP without IPI activity by 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase via the MEP pathway in plastids.4,5,17) Thus, the contribution of plant IPI to DMAPP synthesis downstream of the MEP pathway was ambiguous. To determine the critical role of IPI in isoprenoid biosynthesis in plants, we characterized knockout mutants of Arabidopsis IPI and the subcellular localization of this enzyme.

Most plants have two IPI paralogs that show distinct subcellular localizations. Like other plant species, Arabidopsis has two IPI genes, AtIPI1 and AtIPI2, which have been isolated by complementation of the yeast idi mutant.18) Stable transformants carrying IPI-green fluorescent protein (GFP) fusion constructs under the control of the native promoter. The plasmid constructs used in these experiments were designated AtIPI1g-GFP and AtIPI2g-GFP. They con-
tained genomic fragments of the loci of the IPIs fused to the GFP gene. Whereas AtIPI12-GFP was detected in the mitochondria of the shoots and roots, AtIPI11-GFP was observed mainly in the cytosol, not in the plastids in the shoots and roots. This suggests that IPIs are present mainly in the cytosol and mitochondria in *Arabidopsis*.

The *atipi1* and *atipi2* single mutants grown for 2 weeks in soil under continuous light were phenotypically normal and indistinguishable from wild-type plants in appearance throughout the life cycle. In contrast, the *atipi1atipi2* double mutant showed pleiotropic phenotypes, including dwarfism and male sterility under long-day conditions and decreased pigmentation under continuous light. We also found that the sterol and ubiquinone levels in the *atipi1atipi2* double mutant were 50% of those in the wild-type plants. The male-sterile phenotype was chemically complemented by squalene, a sterol precursor. These findings are in good agreement with previous reports that plants defective in MVA pathway enzymes display dwarfism, early senescence, and male sterility; whereas a block in the MEP pathway results in an albino phenotype.

**In vivo** isotope labeling experiments using the *atipi1atipi2* double mutant revealed a decrease in the incorporation of MVA (in its lactone form) into sterols, with no decrease in the incorporation of MEP pathway intermediates into tocopherol. These lines of evidence indicate a critical role of IPI in isoprenoid biosynthesis via the MVA pathway in plants. Thus, in plants, the MVA and MEP pathways are metabolically separated and provide IPP and DMAPP for the biosynthesis of distinct sets of isoprenoids in the various organs, whereas some evidence suggests crosstalk of a small fraction of isoprenoid precursors, including IPP, DMAPP, and other prenyl diphosphates, between the cytosolic MVA pathway and the plastidial MEP pathway.

After the production of IPP and DMAPP in the plastids, GGPP synthase functions as a crucial branchpoint enzyme responsible for the biosynthesis of various diterpenoid compounds in plants. There is a small gene family for GGPP synthases encoding 11 possible homologs in *Arabidopsis*, and all the homologs have a putative localization signal to translocate into specific subcellular compartments. Using GFP as reporter, we studied the subcellular localization of six GGPP synthases whose enzymatic activities were confirmed. When these fusion proteins were expressed by the CaMV 35S promoter in *Arabidopsis*, the GGPS1-GFP, GGPS3-GFP, and GGPS9-GFP proteins were translocated into the chloroplast, the GGPS2-GFP and GGPS4-GFP proteins were localized in the endoplasmic reticulum, and the GGPS6-GFP protein was localized in the mitochondria. This suggests that GGPP is synthesized by the organelles themselves rather than being transported into the organelles.

**II. Inductive Biosynthetic Gene Clusters for Diterpene Phytoalexin in Rice**

Plants attacked by pathogenic microorganisms respond with a variety of defensive reactions, including the production of antimicrobial secondary metabolites known as phytoalexins. These compounds are transiently generated in response to signal molecules called elicitors, which are usually derived from pathogens, such as the rice blast fungus *Magnaporthe oryzae*. Phytoalexins have been classified into four structurally distinct types of polycyclic diterpenoids based on the structures of their diterpene hydrocarbon precursors: phytocassanes A to E, oryzalexins A to F, momilactones A and B, and oryzalexin S. Upon synthesis of these phytoalexins, the common substrate GGPP is converted into diterpene hydrocarbon precursors via two-step sequential cyclization. Toyomasu and our group identified and characterized all six diterpene cyclases involved in phytoalexins biosynthesis. Subsequent chemical modifications, including oxidation and hydroxylation, are thought to function in the production of bioactive phytoalexins. (Fig. 3). It has been suggested that microsomal cytochrome P450 monoxygenases (P450s) are involved in the downstream oxidation of diterpene hydrocarbons leading to phytoalexins, and that a dehydrogenase is involved in momilactone biosynthesis. However, none of the enzymes involved in the downstream oxidation of diterpene hydrocarbons have been identified. We have found that the production of diterpene phytoalexins and the expression of related biosynthetic genes are activated in suspension-cultured rice cells upon elicitor treatment. In order to identify biosynthetic genes for phytoalexin production, we conducted microarray analysis using suspension-cultured rice cells collected at various durations after treatment with a chitin elicitor. Hierarchical cluster analysis revealed two types of early-induced expression nodes (EIE-1 and EIE-2) and a late-induced expression node (LIE) that included known genes involved in phytoalexin biosynthesis. Hence we paid close attention to the genes classified into this group. As for the results, putative dehydrogenase genes and several functionally unknown P450 genes were identified as chitin oligosaccharide elicitor- and UV-inducible genes. Of these genes, a putative dehydrogenase gene (*AKI03462*) and putative P450 genes (*CYP99A2* and *CYP99A3*) were found to form a gene cluster together with *OsKSL4* and *OsCPS4*, the diterpene cyclase genes involved in momilactone biosynthesis. Hence we first focused on the analysis of these three genes to determine whether the clustered genes compose a functional biosynthetic gene cluster for momilactone production.

Functional analysis by heterologous expression in *E. coli* followed by enzyme assays indicated that the AKI03462 protein catalyzes the conversion of 3β-hydroxy-9βH-pimar-7,15-dien-19,6β-olide into momilactone A (Fig. 3). The double knockdown of *CYP99A2* and *CYP99A3* specifically suppressed the elicitor-inducible production of momilactones, strongly suggesting that *CYP99A2*, *CYP99A3*, or both are involved in momilactone biosynthesis. Wang *et al.* recently reported that *CYP99A3* catalyzes consecutive oxidation of the C19 methyl group of the momilactone precursor *syn*-pimara-7,15-diene to form *syn*-pimaradien-19-ol, *syn*-pimaradien-19-al, and *syn*-pimaradien-19-oic acid, sequentially. These results provide strong evidence of the presence on chromosome 4 of a gene cluster involved in momilactone biosynthesis (see Fig. 4, bottom). In addition, a phytocassane biosynthetic gene cluster consisting of two diterpene cyclase genes (*OsCPS2*, *OsKSL7*) and six P450 genes (*CYP71Z6*, *CYP71Z7*, *CYP76M5*, *CYP76M6*, *CYP76M7*, and *CYP76M8*) were identified.
CYP76M6, CYP76M7, and CYP76M8) was perhaps present on chromosome 2 (see Fig. 4, top).\textsuperscript{36} By the RNAi method, we investigated functions of these P450 genes in the cluster in phytocassane biosynthesis. Our unpublished results indicate that knockdown of the CYP71Z7 gene specifically suppressed elicitor-inducible accumulation of phytocassanes A, B, and D (C-2 oxygenated phytocassanes), whereas the levels of phytocassanes C and E, and 1-deoxyphytocassane C (C-2 non-oxygenated phytocassanes) accumulated in the knockdown line were more than 10 times higher than those of the wild-type plants. This suggests that the CYP71Z7 is probably catalyzes the C-2 hydroxylation of phytocassanes. Recently, Swaminathane et al. reported that one of these co-clustered, co-regulated P450s, CYP76M7, is an \textit{ent-cassadiene}-specific C11\alpha-hydroxylase that appears to catalyze an early step in phytocassane biosynthesis, and thus defines a second diterpenoid biosynthetic gene cluster in rice.\textsuperscript{42} We found that some RNAi lines, which were generated to knock down CYP76M7 and CYP76M8, exhibited impaired production of phytocassanes. This also suggests that the involvement of CYP76M7 and/or CYP76M8 in phytocassane biosynthesis (unpublished results).

The genes for most of the metabolic pathways in plants are not organized in gene clusters, but there are a few examples of gene clusters for the biosynthesis of isoprenoids and plant defense compounds other than monilactones in rice. For example, the genes responsible for the biosynthesis of thalianol in \textit{Arabidopsis},
benzoazinoids in maize, andavenacins in oat are all organized in clusters. Spatial expression of the avenacin biosynthetic genes is tightly regulated, and occurs only in the root epidermis, the site of accumulation of the end product. In contrast, the rice momilactone biosynthetic gene cluster exhibits a temporally coordinated expression pattern of mRNAs, peaking at 6–12 h after elicitor treatment of rice cell suspension cultures. Such coordinated, stress-inducible clustered gene expression, responsible for the biosynthesis of one particular compound, has not previously been reported for plants.

III. A Regulatory Factor in the Biosynthesis of Diterpene Phytoalexin in Rice

To elucidate the regulatory mechanisms of coordinate expression of the momilactone biosynthetic gene cluster, promoter analyses of clustered genes were carried out, followed by identification of the transcription factors involved in the expression of the respective genes. First we examined chitin oligosaccharide elicitor-inducible promoter activity within a 2-kb region upstream of the OsKSL4 gene, which is responsible for the first committed step specific to momilactone biosynthesis, by LUC reporter assay. Deletion and mutation analyses of the promoter identified a TGACG motif in the region −1,045 to −1,040 bp, upstream of the translation start site of OsKSL4, as a cis-acting element required for the elicitor-inducible expression of OsKSL4. This suggests that a bZIP transcription factor (specifically a TGA factor) is probably involved in the regulatory process. Then we focused on elicitor-inducible bZIP transcription factors with expression profiles similar to that of OsKSL4 and those expressed prior to the OsKSL4 gene by examining time course microarray data following elicitor treatment.

Of more than 100 transcription factors having features of bZIP transcription factors, the AK073715, AK102690, and AK106988 proteins were TGA-type bZIP transcription factors, having inductive expression profiles with the typical conserved motifs of TGA factors. Expression analyses of these genes by qRT-PCR revealed that the AK073715 gene was maximally induced 4 h after elicitor treatment (slightly earlier than OsKSL4), that expression returned to basal levels by 8 h, and that the AK102690 gene was induced for 4–8 h after treatment, similarly to OsKSL4. We did not clearly detect inducible expression of the AK106988 gene. Hence, we focused on the AK073715 and AK102690 genes to determine whether they are responsible for the inducible expression of OsKSL4.

Rice Tos17 insertion mutants H0155, for AK073715, Hitomebore background, and NC0005, for AK102690, Nipponbare background, were used to examine physiological functions. LC-MS/MS of the phytoalexins accumulating in the culture medium 0, 48, and 72 h after elicitor treatment revealed that the level of momilactones in the H0155 mutant cells tended to be lower than in the parental wild-type cells, whereas the phytocassane levels were almost the same as between the mutant and wild-type cells at 48 h. Thereafter, the phytocassane levels were somewhat higher than in the wild-type cells at 72 h in H0155 mutant. In the NC0005 mutant, diterpenoid phytoalexin production was comparable to that in the wild-type cells, suggesting that AK102690 is unlikely to be involved in the regulation of phytoalexin biosynthesis. Hence the AK073715 gene was designated OsTGAP1 (Oryza sativa TGA factor for phytoalexin production). We further analyzed the function of this gene product. Gel mobility shift assays revealed that OsTGAP1 regulates momilactone biosynthesis through positive control of OsKSL4 expression, apparently by binding directly to the cis-element in the OsKSL4 promoter.

To address the manner in which OsTGAP1 regulates the expression of phytoalexin biosynthetic genes, transformed rice plants constitutively expressing OsTGAP1 were generated. In calli overexpressing OsTGAP1, low but significant accumulation of momilactones was observed by LC-MS/MS analysis, without any elicitation. Furthermore, OsTGAP1-overexpressing lines showed enhanced accumulation of momilactones as compared with the wild-type cells after treatment with an elicitor. Similarly, accumulation of phytocassanes in the overexpressing line was detected without elicitation. When the overexpressing line was treated with the elicitor, enhanced accumulation of phytocassanes was also evident in the overexpressing lines as was hyperaccumulation of momilactones. The level of accumulated phytoalexins correlated well with the expression level of the OsTGAP1 protein. The expression of OsKSL4 and OsKSL7 (involved in the MEP pathway) in the OsTGAP1-overexpressing lines was assessed by qRTPCR. Although the OsTGAP1-overexpressing lines exhibited slight expression of these genes without elicitation, striking induction of expression was detected in the overexpressing cells as compared with the wild-type cells 6 h after elicitation, consistently with the hyperaccumulation of momilactones and phytocassanes in the elicitor-treated OsTGAP1-overexpressing lines. High levels of mRNA expression of these genes were maintained for at least 24 h after elicitation in the overexpressing lines. This result suggests that OsTGAP1 can influence the biosynthetic pathway for diterpenoid phytoalexins, including the upstream MEP pathway, at the transcriptional level, leading eventually to the production of large amounts of elicitor-inductive diterpenoid phytoalexins.

As described above, momilactone biosynthetic genes are organized in a gene cluster and exhibit coordinated, inductive expression upon elicitor treatment. OsTGAP1 overexpression led to constitutive production of momilactones even without elicitation, indicating that the five momilactone biosynthetic genes, including OsKSL4, might be coordinately regulated by OsTGAP1. To verify this, we analyzed the expression of the genes in the momilactone biosynthetic gene cluster (with neighboring genes). Expression of the five momilactone biosynthetic genes (OsCPS4, OsKSL4, CYP99A2, CYP99A3, and OsMAS), but not of the neighboring genes (encoding actin and hypothetical proteins), was hyperinductively achieved in the overexpressing cells stimulated by an elicitor. This indicates that clustered genes for momilactone biosynthesis are coordinately regulated by OsTGAP1 (Fig. 5).
IV. Conclusions and Future Prospects

We described the early biosynthetic pathway for diterpenoid production in plastids, in which the key compound GGPP is synthesized via the MEP pathway. A critical role of IPP isomerase in isoprenoid biosynthesis via the MVA pathway in plants was suggested, and distinct plastidic GGPP synthases were found to be localized in the plastids and to be responsible for diterpenoid synthesis. In plants, the MVA and MEP pathways are metabolically separated and provide IPP and DMAPP for the biosynthesis of distinct sets of isoprenoids in each organ. The precise bioengineering approach to target responsive biosynthetic enzymes into appropriate locations in the cells should be important for efficient production of bioactive isoprenoid substances in plants. We detected the presence of biosynthetic gene clusters for diterpene phytoalexins in rice, and found a regulating factor, OsTGAP1, that coordinately regulates clustered genes for inductive production of phytoalexins in rice. Our findings include the capability of OsTGAP1-overexpressing rice cells, which exhibited hyperaccumulation of momilactone, for the production of bioactive diterpenoid compounds. Determining the in vivo binding sites of OsTGAP1 on rice chromosomes is our next aim in order to understand how this key transcription factor regulates the coordinated expression of the gene cluster for phytoalexin production in rice.

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References

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