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Elicitor induced activation of the methylerythritol phosphate pathway toward phytoalexins biosynthesis in rice

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Abstract Diterpenoid phytoalexins such as momilactones and phytocassanes are produced via geranylgeranyl diphosphate in suspension-cultured rice cells after treatment with a chitin elicitor. We have previously shown that the production of diterpene hydrocarbons leading to phytoalexins and the expression of related biosynthetic genes are activated in suspension-cultured rice cells upon elicitor treatment. To better understand the elicitor-induced activation of phytoalexin biosynthesis, we conducted microarray analysis using suspension-cultured rice cells collected at various times after treatment with chitin elicitor. Hierarchical cluster analysis revealed two types of early-induced expression (EIE-1, EIE-2) nodes and a late-induced expression (LIE) node that includes genes involved in phytoalexins biosynthesis. The LIE node contains genes that may be responsible for the methylerythritol phosphate (MEP) pathway, a plastidic biosynthetic pathway for isopentenyl diphosphate, an early precursor of phytoalexins. The elicitor-induced expression of these putative MEP pathway genes was confirmed by quantitative reverse-transcription PCR. 1-Deoxy-D-xylulose 5-phosphate synthase (DXS), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), and 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol synthase (CMS), which catalyze the first three committed steps in the MEP pathway, were further shown to have enzymatic activities that complement the growth of *E. coli* mutants disrupted in the corresponding genes. Application of ketoclomazone and fosmidomycin, inhibitors of DXS and DXR, respectively, repressed the accumulation of diterpene-type phytoalexins in suspension cells treated with chitin elicitor. These results suggest that activation of the MEP pathway is required to supply sufficient terpenoid precursors for the production of phytoalexins in infected rice plants.

Keywords Disease resistance · Diterpenoid phytoalexins · Elicitor · MEP pathway · *Oryza sativa* · Rice

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Introduction

Upon pathogen attack, plants activate a complex series of responses leading to the local and systemic induction of broad-spectrum antimicrobial defenses. The activation of defense responses is associated with the increased expression of a large number of genes involved in rapid and localized cell death, the production of pathogenesis-related proteins, and the accumulation of antimicrobial compounds called phytoalexins (Stoessl 1980; Subba and Strange 1994; VanEtten et al. 1994). Of the 15 phytoalexins that have been isolated from rice, 14 are polycyclic diterpenoid phytoalexins and the other is a flavonoid-type phytoalexin named sakuranetin. The diterpenoid phytoalexins have been

classified into four groups according to their carbon skeletons: phytocassanes A–E (Koga et al. 1995, 1997; Yajima and Mori 2000), oryzalexins A–F (Akatsuka et al. 1985; Kato et al. 1993, 1994), momilactones A and B (Kato et al. 1973; Cartwright et al. 1981), and oryzalexin S (Tamogami et al. 1993). The antimicrobial activity of phytoalexins has been evaluated in a spore germination experiment using the blast fungus *Magnaporthe grisea* (Peters 2006).

Proposed biosynthetic pathways of the above phytoalexins are shown in Fig. 1. The biosynthetic pathways for diterpenoid phytoalexins have been well established from the cyclization steps from geranylgeranyl diphosphate (GGDP) to the diterpene hydrocarbons *ent*-cassa-12,15-diene, *ent*-sandaracopimaradiene, 9βH-pimara-7,15-diene, and stemar-13-ene, precursors of the four types of diterpenoid phytoalexins. Six diterpene cyclase genes (*OsCyc1*, *OsCyc2*, *OsDTC1*, *OsDTC2*, *OsKS4*, and *OsKS10*) have been identified and shown to cause the two successive cyclizations of GGDP into the above diterpene hydrocarbons (Cho et al. 2004; Nemoto et al. 2004; Otomo et al. 2004a, b). However, the genes responsible for the biosynthetic steps after the diterpene hydrocarbons have not been identified, although the participation of cytochrome P450 monooxygenases and a dehydrogenase in these steps has been suggested (Kato et al. 1995; Atawong et al. 2002; Peters 2006) (Fig. 1).

In contrast, the biosynthetic steps before GGDP are understood as early terpenoid biosynthesis in plants. This process includes the steps for supplying the basic C5 precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), as well as the step that produces GGDP by the action of GGDP synthase on IPP and DMAPP (Scolnik and Bartley 1994; Okada et al. 2000). In plants, two distinct pathways produce IPP and DMAPP: the mevalonate (MVA) pathway and the methylerythritol phosphate (MEP) pathway (Lichtenthaler et al. 1997). Various isoprenoids, including sesquiterpenes, triterpenes, sterols, brassinosteroids, and prenylquinones, are synthesized in the cytoplasm from IPP derived from the MVA pathway (Heintz et al. 1972; Nes and Venkatramesh 1999; Lange and Ghassemian 2003). Many diterpenoids, including gibberellins, abscisic acids, carotenoids, and phytol chains of chlorophylls, are separately synthesized in plastids from IPP and DMAPP, both derived from the MEP pathway (Lichtenthaler 1999; Rohmer 1999). Like the enzymes responsible for the MEP pathway, the diterpene cyclases involved in phytoalexins biosynthesis are thought to be localized in plastids (Cho et al. 2004; Nemoto et al. 2004). Thus, diterpenoid phytoalexins are predicted to be produced through the MEP pathway in plastids. However, there has been no report of the identification of the biological roles of genes that are thought to function in the MEP pathway in rice.

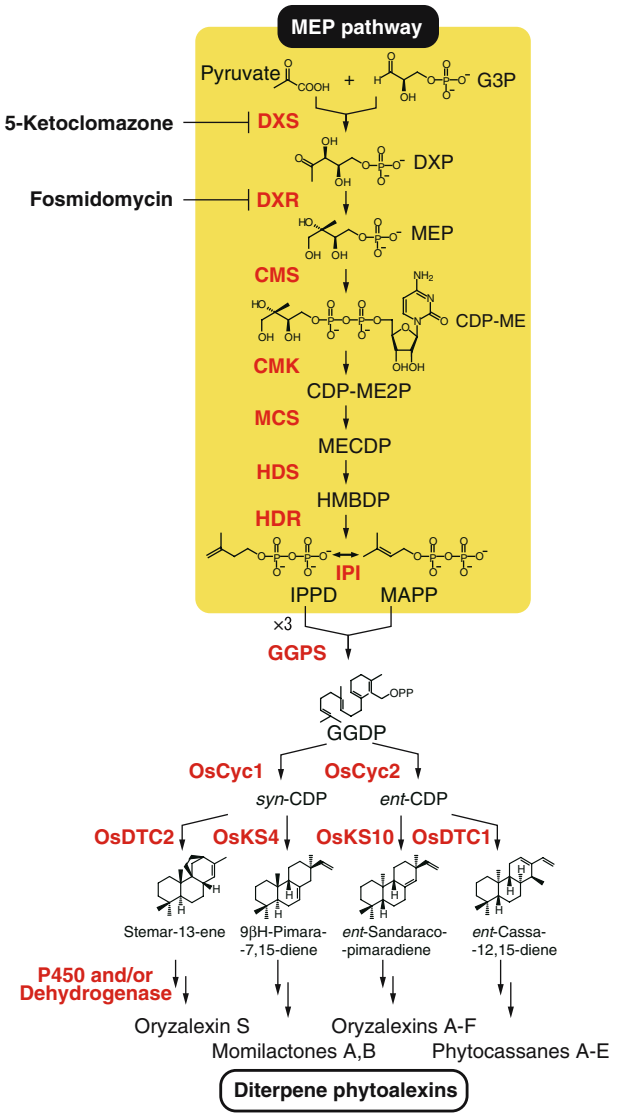


Fig. 1 Diterpenoid biosynthesis pathways in rice. The MEP pathway leading to geranylgeranyl diphosphate biosynthesis and the proposed diterpenoid phytoalexin pathway. G3P, glyceraldehyde-3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; CDP-ME, 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol; CDP-ME2P, 2-phospho-4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol; MECDP, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate; HMBDP, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGDP, geranylgeranyl diphosphate; and CDP, copalyl diphosphate. Enzymes in the MEP pathway and six diterpene cyclases are indicated in bold: **DXS**, DXP synthase; **DXR**, DXP reductoisomerase; **CMS**, CDP-ME synthase; **CMK**, CDP-ME kinase; **MCS**, MECDP synthase; **HDS**, HMBDP synthase; **HDR**, HMBDP reductase; **IPI**, IPP isomerase; **GGPS**, GGDP synthase; **OsCyc1**, *syn*-CDP synthase; **OsCyc2**, *ent*-CDP synthase; **OsDTC1**, *ent*-cassa-12,15-diene synthase; **OsDTC2**, stemar-13-ene synthase; **OsKS4**, 9βH-pimara-7,15-diene synthase; and **OsKS10**, *ent*-sandaracopimaradiene synthase. The steps specifically inhibited by 5-ketoclomazone (KCZ) and fosmidomycin (FOS) are indicated

Large amounts of diterpenoid phytoalexins accumulate in the culture medium of elicitor-treated rice cells. This phytoalexins accumulation occurs because an adequate supply of substrate is present, resulting from the activation of an unknown gene together with those of known diterpene cyclases. To better understand inducible phytoalexins biosynthesis in rice, we used oligomicroarrays to survey the genes whose transcription levels change following elicitor treatment of suspension-cultured rice cells. The microarray analysis revealed that putative MEP-pathway genes exhibit elicitor-induced expression with profiles similar to those of diterpene cyclase genes. Molecular and chemical approaches showed that OsDXS, OsDXR, and OsCMS are functional enzymes that catalyze the first three steps in the MEP pathway, and that the application of ketoclomazone and fosmidomycin, inhibitors of DXS and DXR, respectively, repress the accumulation of diterpene-type phytoalexins in suspension-cultured cells induced by elicitor. These results suggest that activation of the MEP pathway is required for the production of sufficient amounts of phytoalexins to supply terpenoid precursors in plastids.

Materials and methods

Plant materials and chemical treatment

Suspension-cultured rice cells (*Oryza sativa* L. cv. Nipponbare) were maintained as described by Cho et al. (2004). Six days after subculturing, the cells were subjected to treatment with the elicitor *N*-acetylchitooctaose at 1 ppm, and the DXS inhibitor 5-ketoclomazone at 50 μM, the DXR inhibitor fosmidomycin at 100 μM, or the HMG-CoA reductase inhibitor mevastatin at 5 μM (Sauret-Gueto et al. 2006). A small amount of callus was continually harvested at various time points after elicitor treatment for the extraction of total RNA. For phytoalexins measurements, 0.5-ml samples of culture medium were collected from each sample. Leaf discs (6 mm diameter) were punched from latest leaf of 6 weeks after seedling. Discs were treated with JA (500 μM), UV-irradiation (254 nm, 20-cm distance for 20 min), or $CuCl_2$ (500 μM) and incubated for 72 h under light and humid condition at 25°C. H_2O treatment was used as a negative control. After incubation, phytoalexins or total RNA were extracted.

RNA preparation and fluorescent labeling of probes

Total RNA was extracted from elicitor-treated rice cells using Sepasol-RNA I Super (Nacalai Tesque) and purified using an RNeasy Plant Mini Kit (Qiagen K.K., Tokyo, Japan) following the manufacturer's instructions. The total RNA was used as a template to synthesize Cy-3- or Cy-5-conjugated deoxy CTP-labeled cRNA probes using the Low Input Linear Amplification and Labeling kit (Agilent), following the manufacturer's instructions. Cy-3- and Cy-5-labeled cRNAs were purified using an RNeasy Mini Kit.

Microarray data acquisition and cluster analysis

Agilent Technologies custom microarrays (G4138A) were hybridized according to the manufacturer's instructions. cRNA synthesized from an untreated sample (0 h) was used as a reference, and two time-course replicate analyses were performed using RNA prepared from independent cell cultures. Microarray slides were scanned using a microarray scanner (G2565B Agilent), and the resulting output files (tiff images) were imported into the Feature Extraction software (Version 9.1 Agilent). Spot and background intensities from scanned slides were quantified and data files were normalized using the software algorithm. The results were filtered to identify candidate clones at all time points with expression more than 2.5-fold greater than that of the references in both experiments. Data from the selected clones were imported into Multi Experiment Viewer (TMEV4, The Institute of Genomic Research, http://www.TIGR.org) for cluster analysis. A hierarchical clustering analysis based upon the average linkage and cosine correlation was then used to cluster genes on the *y*-axis using TMEV4.

RT-PCR and quantitative real-time PCR

For each sample, 1 μg of total RNAs was treated with 10 units of DNase (Roche Diagnostics) to remove contaminating genomic DNA, and then used for reverse transcription (RT) to synthesize first-strand cDNA using an oligo-dT primer and a Superscript III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA). cDNA samples diluted 10-fold were subjected to polymerase chain reaction (PCR) (*OsCyc1*, *OsCyc2*, *OsDTC1*, *OsDTC2*, *OsKS4*, *OsKS10*, and *UBQ*) or quantitative RT-PCR (*OsDXS3*, *OsDXR*, *OsCMS*, *OsCMK*, *OsMCS*, *OsHDS*, *OsHDR*, and *UBQ*) using SYBR Green technology on an ABI PRISM 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). Raw data of qRT-PCR were analyzed using the ΔCT (difference in threshold cycles) method, and the results were expressed as relative mRNA values normalized to the expression level of *UBQ* (ubiquitin fused to ribosomal protein L40, D12629) as an internal control. For each sample, the mean value from triplicate amplifications was adapted to calculate the transcript abundance. Primers for amplification were designed with the aid of Primer Express software (Applied Biosystems), and sequences of the amplified products were confirmed using an ABI310

Genetic Analyzer (Applied Biosystems). The primers used were as follows: UBQ-F (5′-GGACTGGTTAAATCAATC GTCA-3′) and UBQ-R (5′-CCATATACCACGACCGTCA AAA-3′); OsCyc1-F (5′-TGACGAGGCTGGGCATATC-3′) and OsCyc1-R (5′-TCTGGAGTCCAGTTCCTGAAA-3′); OsCyc2-F (5′-TTAGGAAAATGGTTGACTAC-3′) and Os-Cyc2-R (5′-ATCGACTAAATTCATCTCAC-3′); OsDTC1-F (5′-TTCATCTCTGTCACTTTTTCTTTTT-3′) and OsDTC1-R (5′-ATCCCACGAAGTCATCCAC-3′); OsDTC2-F (5′-CC AGCGTCTTCTACCAGGAG-3′) and OsDTC2-R (5′-AAA ATGTTCAGCGAGGGAGA-3′); OsKS4-F (5′-CGCCTTTG TAACTCTAAGGTA-3′) and OsKS4-R (5′-ACGTAAAAG GCTTGTATATC-3′); OsKS10-F (5′-TGATCGTCCATTTC AGCTCT-3′) and OsKS10-R (5′-TTCTCAATCAGCGCGA TGTA-3′); OsDXS3-F (5′-GGGGGAGGTTCCAGTAAGA A-3′) and OsDXS3-R (5′-TCATTTTGCATTTGGAAGCA-3′); OsDXR-F (5′-GCTCCATGCATAGTCAGCAG-3′) and OsDX R-R (5′-GCACGGACGAACGATTTATT-3′); OsCMS-F (5′-A CGGGATGGACTTGAGGTCA-3′) and OsCMS-R (5′-TTATT TCTCATTCATCAGGCG-3′); OsCMK-F (5′-CCCAAAATTA GCCCATTCAA-3′) and OsCMK-R (5′-GTCGACTTTCTCGT GCGTCT-3′); OsMCS-F (5′-TCGACCTTTATGTTGGCAAG-3′) and OsMCS-R (5′-CATGCTACGGCCTACTCCTC-3′); Os HDS-F (5′-GGAAAAGAACCATTCCACCTT-3′) and OsH DS-R (5′-TGTCACTTGGTATGCCCGTA-3′); and OsHDR-F (5′-CTGATGGCTTGGTGAAGGTT-3′) and OsHDR-R (5′-CA GCACATGCCGTAGTATGC-3′).

Plasmid construction

First-strand cDNA was used as a PCR template to amplify the *OsDXS3d58* cDNA using the primers OsDXS3d58-F (5′-CACCGGATCCATCGACTACTCCGGCGAG-3′) and OsDXS3d58-R (5′-AAGCTTTCAGCTGAGCTGAAGTG CCT-3′). The 1,984-bp amplified fragment was digested with *Bam*HI and *Hind*III and then fused between the same sites in the pQE30 vector to yield the plasmid pQE-Os-DXS3, which expresses a hexahistidine-tagged protein. An *OsDXR* cDNA fragment was prepared from the plasmid pFLC-1-OsDXR (AK099702) obtained from the Rice Genome Resource Center (Rice Genome Project of the National Institute of Agrobiological Sciences, Japan). The 1,490-bp *Sma*I-*Xba*I fragment of *OsDXR* was fused between the same sites in the pUC18 vector to yield the plasmid pUC-OsDXR, which expresses OsDXR fused with the LacZ alpha peptide. To isolate the *OsCMS* cDNA, the Arabidopsis *AtCMS* (At2g26930) gene product sequence was used as a query in a BLASTX search of rice, because the predicted amino acid sequence of OsCMS in the Rice Genome Automated Annotation System (http://www.ricegaas.dna.affrc.go.jp/) (Sakata et al. 2002) did not exhibit high similarity with known CMS proteins. The longest *OsCMS* cDNA that encodes a highly homologous region was amplified by PCR using first-strand cDNA as a template with the primers CMSorf-F (5′-CAGAATT CTATGCCAAAACAATATCTACC-3′) and CMSorf-R (5′-CACTGCAGCTATTTCTCATTCATCAGGCG-3′). The 621-bp amplified fragment was digested with *Eco*RI and *Pst*I and then fused between these sites in the pSTV28 vector to yield the plasmid pST-OsCMSt. The sequence of the truncated *OsCMS* cDNA was deposited in GenBank under the accession number AB296384.

Complementation analysis

The *E. coli dxs* disruptant DXM3 (Kim et al. 2006) and the *dxr* disruptant DYM1 (Takahashi et al. 1998; Kuzuyama et al. 1999) were grown on Luria-Bertani (LB) medium supplemented with 0.01% 2-*C*-methyl-D-erythritol (ME) and 50 μg/ml kanamycin (Km), and were transformed with pQE-OsDXS3 and pUC-OsDXR, respectively. Transformants of each were selected on LB + ME solid medium containing 50 μg/ml ampicillin (Amp) and then transferred to LB + Amp medium lacking ME. The *E. coli ispD* mutant NMW33 (Kuzuyama et al. 2000), harboring the pTMV20km plasmid, which carries the MVA pathway operon consisting of the mevalonate kinase, phosphomevalonate kinase, and diphosphomevalonate decarboxylase genes of *Streptomyces* sp. CL190, was grown on LB + Km supplemented with 0.01% mevalonolactone (MVL) and transformed with pST-OsCMS. The transformant was first selected on LB containing 35 μg/ml chloramphenicol (Cm) and MVL, and then transferred onto LB + Cm medium lacking MVL. The plasmids pQE-DXS, pMEW73, and pMNMW33-1 were used as positive controls harboring the native *E. coli* genes *DXS*, *DXR*, and *CMS*, respectively (Takahashi et al. 1998; Kuzuyama et al. 1998b, 2000).

Phytoalexins measurements

A total of 0.5-ml samples of media from rice cell cultures treated with elicitor and/or inhibitor were extracted three times with 0.5 ml of ethyl acetate at an approximate pH of 5.8. The combined ethyl acetate extracts were evaporated to dryness. The residues were dissolved in 5 ml of 70% aqueous methanol. Leaf discs were immersed in 200 μl of 70% aqueous methanol directly and heated at 95°C twice. About 5-μl samples of the resulting solutions were subjected to HPLC-ESI-MS/MS. An Agilent 1100 separation module (Agilent Technologies, Palo Alto, CA, USA) equipped with a Pegasil C_{18} column (150 × 2.1 mm in diameter; Senshu Scientific, Tokyo, Japan) was used with 70% aqueous acetonitrile containing 0.1% acetic acid as a solvent at a flow rate of 0.2 ml/min. All of the diterpenoid phytoalexins tested by HPLC-ESI-MS/MS were analyzed with a quadrupole tandem mass spectrometer (API-3000,

Applied Biosystems Instruments, Foster City, CA, USA) outfitted with an electrospray ion source in positive-ion mode. Nitrogen was used as the collision gas. The ES capillary was set at 3.0 kV and the source temperature was 400°C. Other parameters were optimized by the spectrometer software (Applied Biosystems Instruments). The diterpenoid phytoalexins was determined with combinations of precursor/product ions; *m/z* 315/271 for momilactone A, *m/z* 331/269 for momilactone B, *m/z* 317/299 for phytocassanes A, D, and E, *m/z* 335/317 for phytocassane B and *m/z* 319/301 for phytocassane C in the MRM mode.

Results

Synchronous expression of diterpene cyclase genes triggered by chitin elicitor treatment

We previously reported that *OsDTC1* and *OsDTC2* are inducibly expressed by elicitor treatment (Cho et al. 2004; Nemoto et al. 2004). To further investigate the expression profiles of diterpene cyclase genes, we performed reverse-transcriptase PCR (RT-PCR) using total RNA prepared from suspension-cultured rice cells treated with chitin elicitor for 0, 2, 4, 6, 8, 10, 12, or 24 h. All six diterpene cyclase genes shown in Fig. 1 exhibited transient expression patterns that peaked at 4–8 h after elicitor treatment (Fig. 2). Given that the accumulation of phytoalexins was previously detected from 8 h after elicitor treatment, the expression profiles of these genes are consistent with roles in phytoalexins biosynthesis. This result prompted us to search for novel genes that are elicitor-inducible and involved in the biosynthesis of phytoalexins.

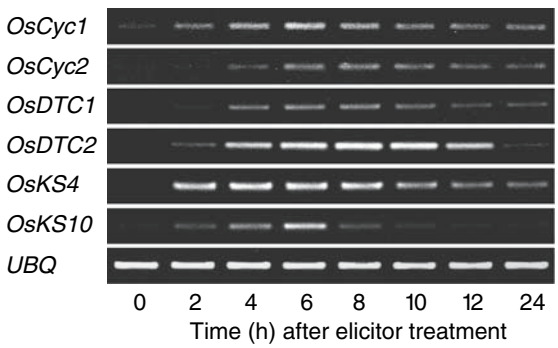
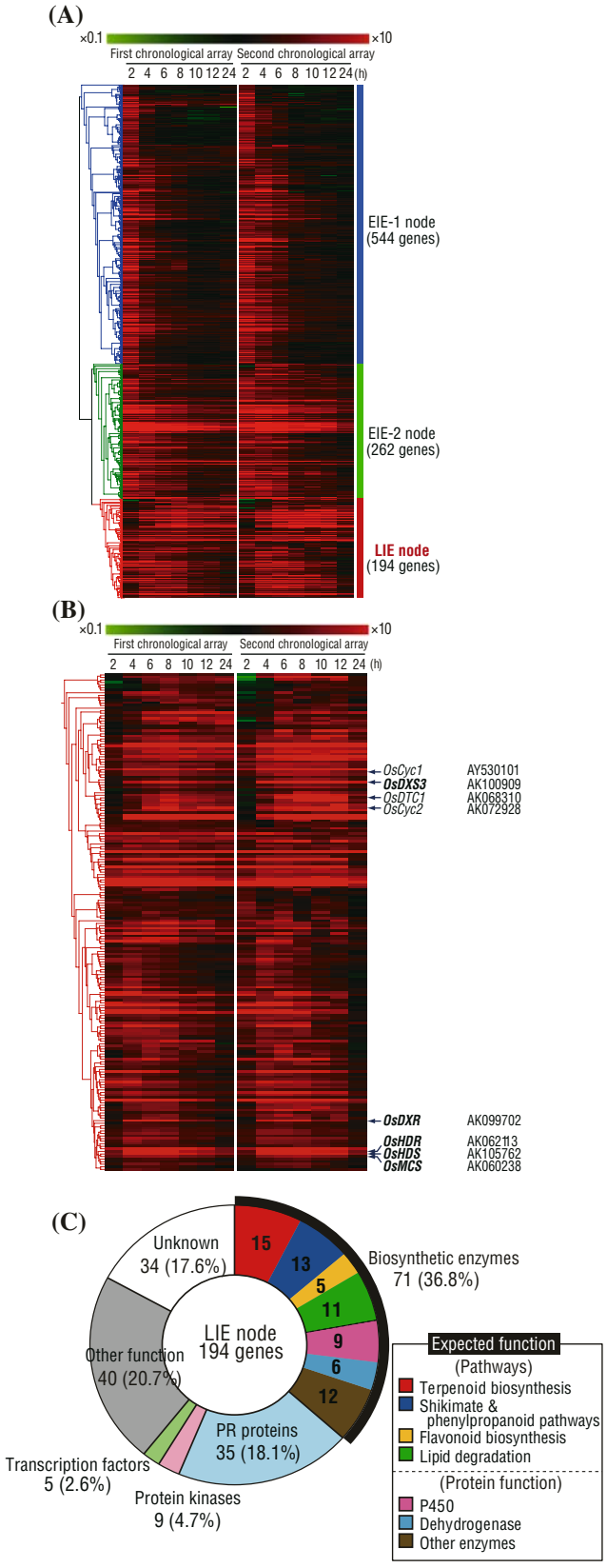


Fig. 2 Expression profiles of diterpene cyclase genes analyzed by RT-PCR. The total RNAs used in RT-PCR were isolated from rice cells exposed to 1 ppm chitin elicitor for the indicated times and a pair of the gene-specific primers. As an internal standard, the rice ubiquitin fused to ribosomal protein gene *UBQ* was amplified by RT-PCR using the gene-specific primers

Identification of genes involved in phytoalexins biosynthesis using a 22-k rice oligomicroarray based on full-length rice cDNAs

We screened for elicitor-responsive genes that may be involved in phytoalexins biosynthesis using a highly efficient comprehensive analysis with a rice oligo microarray. cRNA probes for the microarray analysis were synthesized from the total RNA used in the RT-PCR analysis of diterpene cyclase genes described above. The Cy-3 labeled cRNA probe from the 0-h time point sample was used as a reference, and the Cy-5 labeled cRNA probes from seven other time points (2, 4, 6, 8, 10, 12, and 24 h) were compared against the 0-h reference. As described in the "Materials and Methods," the genes that were upregulated greater than 2.5-fold at each of the time points were identified. Of 21,495 genes on the microarray, 1,000 genes were upregulated by the elicitor treatment, and the diterpene cyclase genes *OsCyc1*, *OsCyc2*, and *OsDTC1* were among the 1,000 genes. The *OsDTC2* gene was excluded during the sorting because one of the microarray analyses exhibited less than 2.5-fold upregulation of the gene, and the *OsKS4* and *OsKS10* genes are not included in probes on rice 22-k microarray. The upregulated genes were subjected to hierarchical clustering using the cosine correlation and average linkage methods to be sorted into groups of genes with similar expression patterns following elicitor treatment. As shown in Fig. 3A, the genes were categorized into three expression pattern groups: a group of 544 genes whose expression quickly increased and then decreased rapidly after elicitor treatment, named the early-induced expression-1 (EIE-1) node; a group of 262 genes whose expression quickly increased and then decreased slowly after the treatment, named the early-induced expression-2 (EIE-2) node; and a group of 194 relatively late-induced genes, named the late-induced expression (LIE) node. The complete lists of these genes are available in the supplemental data (Tables S1–S3). Because the three diterpene cyclase genes (*OsCyc1*, *OsCyc2*, and *OsDTC1*) were among the 194 genes in the LIE node, other candidate genes responsible for phytoalexins biosynthesis were also expected to be in the LIE node. Therefore, focusing on the remaining 191 genes in the LIE node, we found that five genes annotated to encode the MEP pathway enzymes 1-deoxy-D-xylulose 5-phosphate synthase (DXS), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase (CMK), 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase (HDS), and 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase (HDR) were present in the LIE node (Fig. 3B). The genes of two related proteins involved in the MEP pathway, 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-



◀**Fig. 3** Hierarchical clustering of expression profiles of genes induced by the elicitor. (**A**) Hierarchical clustering sort of the 1,000 elicitor-induced genes, such that similar genes appear near each other. Shades of red indicate increased relative expression; shades of green indicate decreased relative expression. The EIE-1 and -2 nodes (blue and green bars) and the LIE node (red bar) are indicated. (**B**) Hierarchical clustering sort of the LIE node. Elicitor-induced genes possibly involved in the MEP pathway are indicated by arrows with accession numbers. (**C**) Number of clones involved in different metabolic pathways

erythritol synthase gene (*CMS*) and 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase gene (*MCS*), have also been annotated in the rice genome, but were not in the LIE node because *MCS* was excluded during the sorting as a gene activated less than 2.5-fold, and *CMS* was not spotted on the microarray. We tentatively named these seven genes *OsDXS*, *OsDXR*, *OsCMS*, *OsCMK*, *OsMCS*, *OsHDS*, and *OsHDR*. The genes for HMG-CoA synthase, HMG-CoA reductase, and mevalonate kinase, all of which are involved in the mevalonate pathway, an alternative IPP biosynthesis pathway, were not induced by elicitor treatment in our microarray analysis (Table S4).

Figure 3C shows the expected functions of the 194 genes in the LIE node. The LIE node includes 71 genes (about 36% of the total) that encode enzymes of several biosynthesis pathways: genes that encode enzymes involved in terpenoid synthesis, amino acid synthesis and metabolism (shikimate and phenylpropanoid pathways), flavonoid synthesis, and lipid catabolism, and genes for P450s and dehydrogenases. Since P450s and dehydrogenases have been suggested to be involved in the downstream oxidation of diterpene hydrocarbons leading to phytoalexins, the P450s and dehydrogenases in the LIE node could be promising candidates for phytoalexins biosynthetic genes (Kato et al. 1995; Atawong et al. 2002; Peters 2006). The remaining 123 genes in the LIE node appear to be unrelated to biosynthetic pathways. Approximately 18% of the genes in the LIE node have known functions or had previously been identified as pathogenesis-related proteins. Only five genes encoding transcription factors are included in the LIE node.

Expression profiles of putative MEP-pathway genes

The changes in the transcript levels of the MEP-pathway-gene homologs that exhibited late-induced expression in the microarray analysis were confirmed by qRT-PCR analysis. Gene-specific primers were designed for all seven genes of the MEP pathway using sequences in the 3′ untranslated regions. Including the *OsCMS* gene, whose cDNA was not present in the database, all of the genes were clearly induced by elicitor treatment (Fig. 4) and showed expression

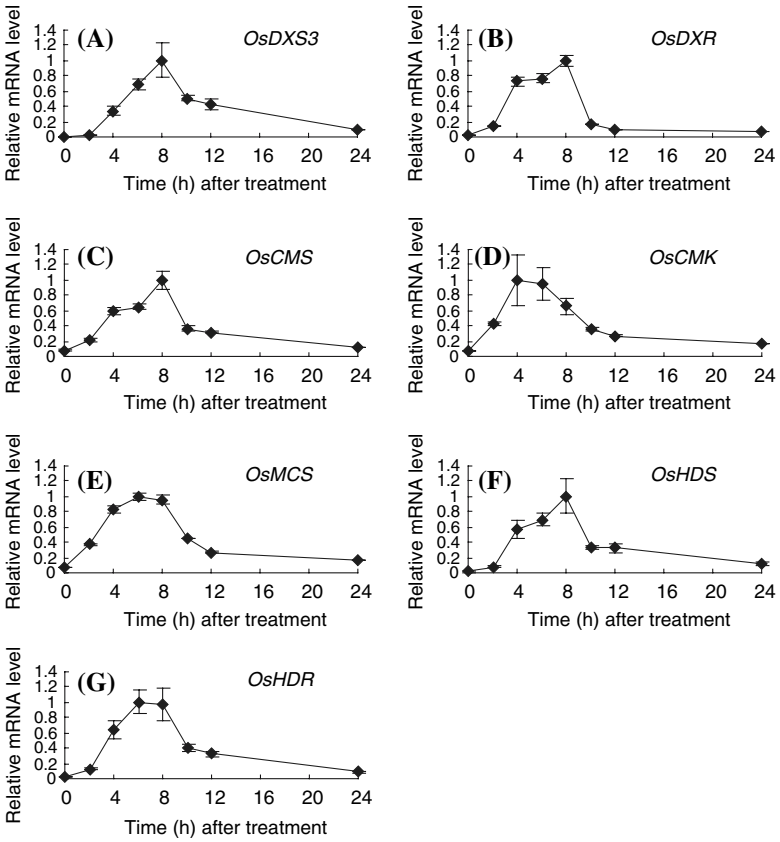


Fig. 4 Expression profiles of possible genes in the MEP pathway in cultured-cells. The total RNAs used for expression profiling were isolated from rice cells exposed to 1 ppm chitin elicitor for the indicated times. Values indicate relative mRNA levels normalized to the expression of the *UBQ* gene, and the maximal value in each experiment with different primers was arbitrarily set to 1.0. **A**, *OsDXS3*; **B**, *OsDXR*; **C**, *OsCMS*; **D**, *OsCMK*; **E**, *OsMCS*; **F**, *OsHDS*; **G**, *OsHDR*. The results are the average of at least three independent experiments; bars indicate the standard deviation of the mean

profiles similar to those of diterpene cyclase genes (Fig. 2). Kim et al. reported that the rice genome contains three *OsDXS*-related sequences (*OsDXS1-3*), and the expression of *OsDXS3* is induced in rice plants by UV irradiation (Kim et al. 2005). This result is consistent with our result that expression of the *OsDXS* gene (AK100909), which is identical to *OsDXS3*, is specifically induced by the chitin elicitor. In contrast, the expression of two other homologs *OsDXS1* (AK104851) and *OsDXS2* (AK064944) remains at a basal level or less after elicitor treatment in our microarray analysis (Table S5). Based on the designation of Kim et al. we named the elicitor-induced *OsDXS* gene *OsDXS3*. We also found that, in addition to *OsDXS3*, all of the possible genes in the MEP pathway in rice are upregulated upon elicitor treatment. This result also suggests that the MEP pathway is coordinately activated along with the genes responsible for diterpenoid phytoalexins production.

Diterpenoid phytoalexins production is also induced by jasmonic acid (JA), UV-irradiation, or $CuCl_2$ treatment in rice leaves (Tamogami et al. 1997; Kodama et al. 1988a, b). To investigate whether the coordinate expression of the MEP-pathway-gene observed in suspension cultured rice cells is seen in rice plants as well, we analyzed mRNA levels of these genes in rice leaves treated with JA, UV-irradiation, and $CuCl_2$ by qRT-PCR. As a result, expressions of all MEP-pathway-genes were found to be induced by any elicitation similar to that of *OsKS4* in rice plants. Phytoalexins production was also confirmed in leaves treated by these elicitations (Fig. 5). These results suggest that the involvement of the MEP pathway in the phytoalexins production in planta was inferred from the synchronous expressions of genes in the MEP pathway in rice plants.

Functional analysis of OsDXS3, OsDXR, and OsCMS

The qRT-PCR analysis revealed the synchronous upregulation of all of the putative MEP-pathway genes by elicitor treatment. However, evidence of the functions of these genes in rice was lacking. To examine the functions of these genes, we performed complementation analysis using *E. coli* mutants defective in the MEP-pathway genes. The *E. coli* DXM3 and DYM1 strains, which are disrupted in the *DXS* and *DXR* genes, can only survive in culture medium supplemented with methylerythritol (ME) because the MEP pathway is essential for the growth of *E. coli*. The *E. coli* CMS-disrupted strain NMW33, harboring the plasmid pTMV20Km, which carries the mevalonate pathway gene cluster, can only grow in the presence of supplementary mevalonolactone (MVL) (Fig. 6). These mutants were transformed with the

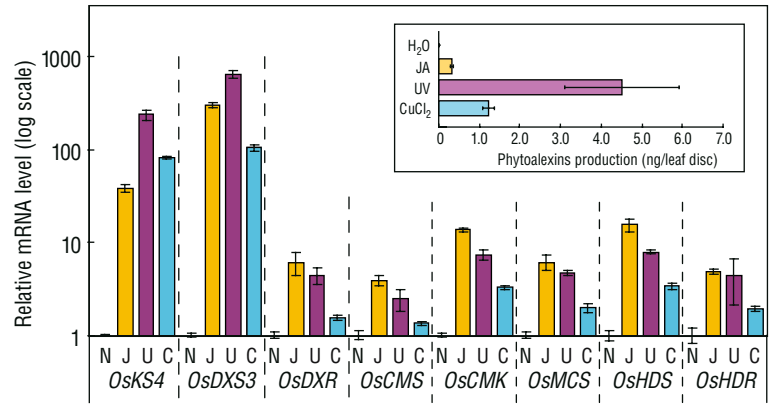


Fig. 5 Expression analysis of possible genes in the MEP pathway in rice leaves. The total RNAs used for expression profiling were isolated from rice leaf discs exposed with H_2O (N), JA (J), UV (U), or $CuCl_2$ (C) for 72 h. Expression of the *OsKS4* gene was shown as a positive control for these treatments. Values indicate relative mRNA levels normalized to the expression of the *UBQ* gene. Inset shows levels of phytoalexins production in the treated leaf discs

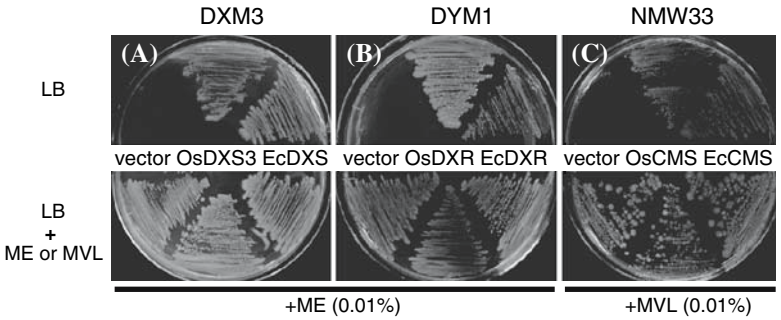


Fig. 6 Complementation of the *E. coli* DXM3, DYM1, and NMW33 mutants. The DXM3, DYM1, and NMW33 mutants cannot grow on LB without supplemented 2-*C*-methyl-D-erythritol (ME), which is used by *E. coli* in place of MEP (Duvold et al. 1997), or mevalonolactone (MVL). The DXM3, DYM1, and NMW33 mutants transformed with plasmids harboring *OsDXS3* (**A**), *OsDXR* (**B**), or *OsCMS* (**C**), respectively, were grown for three days at 37°C on LB plates containing (below) or lacking (top) 0.01% (V/V) ME or MVL. Plasmids harboring the *EcDXS*, *EcDXR*, or *EcCMS* genes were used as positive controls and empty vectors (pQE30, pUC18, or pSTV28) were used as negative controls, respectively

plasmids pQE-OsDXS3, pUC-OsDXR, or pST-OsCMS, which harbor the *OsDXS3*, *OsDXR*, and *OsCMS* cDNAs, to generate the DXM3/OsDXS3, DYM1/OsDXR, and NMW33/OsCMS strains, respectively. As shown in Fig. 6, when these transformants were spread on LB medium without supplemental ME or MVL, all transformants carrying the rice homologs of the MEP-pathway genes showed normal growth, similar to that of the control strains carrying the native genes for the *E. coli* MEP pathway. These results indicate that *OsDXS3*, *OsDXR*, and *OsCMS* encode functional enzymes that are involved in the MEP pathway, and that this complementation system is a powerful tool for confirming that genes from various organisms function in the MEP pathway. Since these genes, thought to encode the rice MEP pathway enzymes, exist as single copies in the rice genome, except for *OsDXS*, the remaining genes (*OsCMK*, *OsMCS*, *OsHDS*, and *OsHDR*) probably also encode functional enzymes. However, these functions also need to be experimentally confirmed.

Decreased accumulation of phytoalexins in rice cells treated with inhibitors of the DXS and DXR proteins

The synthesis of diterpenoids in plants is thought to begin in plastids from the common precursor GGDP. Diterpenoid-type phytoalexins such as momilactones and phytocassanes are also thought to be synthesized in plastids from GGDP derived from the MEP pathway. To investigate the contribution of the MEP pathway to the production of diterpenoid-type phytoalexins in rice, the chemical inhibitors KCZ and FOS, which suppress the metabolic steps catalyzed by OsDXS and OsDXR, respectively (Sakakibara et al. 2005, Kuzuyama et al. 1998a), were added to suspension-cell culture media, and then phytoalexins levels were measured after elicitor treatment. When either

100 μM FOS or 50 μM KCZ were added to the culture media, the production of momilactones and phytocassanes that normally occurs in untreated cells was strongly suppressed in inhibitor-treated cells. In contrast, treatment of 5 μM mevastatin (MEV), an inhibitor for HMG-CoA reductase in the MVA pathway, was shown to be less effective than FOS and KCZ treatments to the phyntoalexins production; the phytoalexins levels in the FOS-treated and the MEV-treated cells at 48 h after elicitation were about 30 and 90% of those in rice cells treated with the elicitor alone, respectively (Fig. 7). These results strongly suggest that in plastids, the MEP pathway is responsible for the biosynthesis of diterpene-type phytoalexins in rice.

Discussion

By analyzing the gene expression in elicitor-treated suspension-cultured rice cells using a 22-k rice oligomicroarray, we narrowed down the genes responsible for the early stage of phytoalexins biosynthesis, the MEP pathway, which supplies GGDP. Hierarchical clustering sorted the genes that were differentially expressed in response to elicitor treatment into three groups with similar expression patterns. Almost 80% of the elicitor-induced genes were clustered into the early-induced expression-1 (EIE-1) and EIE-2 nodes. The remaining genes were clustered into the late-induced expression (LIE) node, which contains many

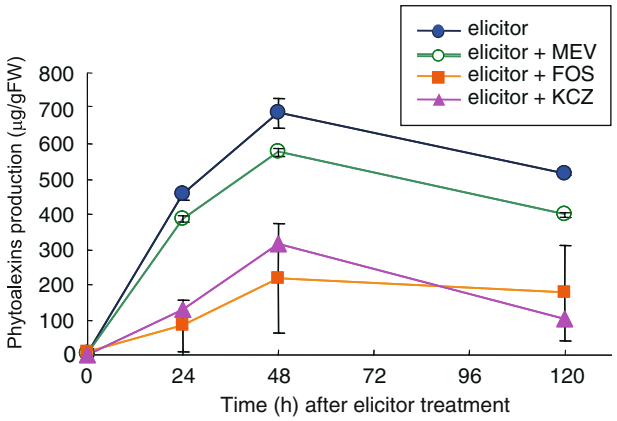


Fig. 7 Effects of inhibitors of the MEP pathway on the production of diterpenoid phytoalexins. Diterpenoid phytoalexins (momilactones A and B and phytocassanes A–E) were extracted from media collected at various time points (0, 24, 48, and 120 h) after treatment with elicitor and inhibitors, and the phytoalexins levels were determined by HPLC-ESI-MS/MS. Solid circles indicate treatment with 1 ppm *N*-acetylchitooctaose (elicitor), solid squares indicate treatment with elicitor and 100 μM fosmidomycin (FOS), solid triangles indicate treatment with elicitor and 50 μM 5-ketoclomazone (KCZ), and open circles indicate treatment with elicitor and 5 μM mevastatin (MEV). The results are the averages of at least three independent experiments; bars indicate the standard deviation of the mean

genes involved in terpenoid biosynthesis, including the phytoalexins biosynthetic genes. The LIE node contained the genes *OsCyc1*, *OsCyc2*, and *OsDTC1*, all of which are maximally expressed at around 8 h after elicitor treatment, suggesting that narrowing down phytoalexins biosynthetic genes using microarrays is a reliable approach. In fact, in addition to the MEP-pathway genes, genes for cytochrome P450 monooxygenases and dehydrogenases, both of which have been suggested as being involved in phytoalexin biosynthesis (Atawong et al. 2002; Peters 2006), are also present in the LIE node. Since these genes are strong candidates for involvement in phytoalexins biosynthesis, their biochemical functions are currently being analyzed by our group.

Kim et al. have reported that *OsDXS3*, one of the three *DXS* homologs in rice, is inducibly expressed by the UV irradiation of rice leaves (Kim et al. 2005). Expression of the *MtDXS2* gene, a *DXS* homolog of the legume *Medicago truncatula*, has also been reported to be strongly stimulated in roots upon their colonization by mycorrhizal fungi, correlated with the accumulation of carotenoids (Walter et al. 2002). This evidence suggests that the activation of the first committed step in the MEP pathway catalyzed by DXS is a common response in plants under both biotic and abiotic stresses. Our results further indicate that, in addition to the *DXS* gene, six more genes possibly involved in the MEP pathway are synchronously expressed upon treatment with chitin elicitor. Moreover, these seven genes of the MEP pathway exhibit expression patterns very similar to those of diterpene cyclase genes responsible for phytoalexins biosynthesis. This suggests that the activation of the MEP pathway and downstream phytoalexins biosynthetic pathways is coordinately controlled by an unidentified transcriptional regulation mechanism. Guevara-Garcia et al. have reported that coordinated regulation at the transcript level of all the genes in the MEP pathway in *Arabidopsis* is observed during development (Guevara-Garcia et al. 2005). However, the synchronous regulation of MEP-pathway gene expression in rice we demonstrate here must be different from the already reported regulation in *Arabidopsis*, because the elicitor-induced expression of the MEP pathway genes in rice is a transient event that recovers from an activated state to a basal state within 12 h. On the other hand, the composition of the MEP-pathway genes appears to be similar among plant species. For example, the genomes of both rice and Arabidopsis contain three highly homologous *DXS* genes and single copies of the six other MEP-pathway genes. Furthermore, in these plants, the basal expression levels of the *CMS* genes, which encode 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol synthase, are very low, because neither the ESTs nor the full-length cDNA sequences for the rice *OsCMS* genes have been deposited in the database, and the

Arabidopsis *CMS* gene (*AtMECT*) has not been able to be detected by northern blot analysis (Rohdich et al. 2000; Okada et al. 2002).

There have been some reports that the MEP pathway is post-transcriptionally modulated in *Arabidopsis*; Guevara-Garcia et al. have reported that the MEP pathway enzyme levels are regulated at translational or post-translational levels in response to developmental cues and changes in the MEP pathway flux (Guevara-Garcia et al. 2005), and Sauret-Güeto et al. have reported that a loss of function of the chloroplast-targeted exoribonuclease polyribonucleotide phosphorylase (PNPase) resulted in the post-transcriptional up-regulation of DXR and three other enzymes of the MEP pathway (DXS, HDS, and HDR) (Sauret-Güeto et al. 2006). On the other hand, we have demonstrated in this study that synchronous and transient expression at mRNA level of all the genes in the MEP pathway in rice upon elicitor treatment. Therefore, although it might be expected that post-transcriptional modulation also occurs during development in rice plant as a common regulatory system similar to that in *Arabidopsis*, we consider that the MEP pathway is likely to be regulated at transcription level upon elicitor-induced phytoalexins biosynthesis in rice. Further experiments will be needed to prove regulation of the MEP pathway in rice at protein level.

GGDP is a common precursor in the branch point of the diterpenoid metabolic pathway. In addition to phytoalexins, many important diterpene compounds are synthesized using the same GGDP pool in plastids, including the plant hormone gibberellin and photosynthetic pigments. Therefore, it is highly possible that the MEP pathway is an important origin of the GGDP used in the synthesis of all plastidial diterpenoids, including phytoalexins. Our phytoalexins measurement data indicated that, following elicitor treatment, more than 700 μg/g FW of phytoalexins accumulated in the culture medium (Fig. 7). This productivity is much greater than that of the plant hormone gibberellin, which is produced in an organ- and time-specific manner at a level of around several ng/g FW using the same GGDP pool as diterpene phytoalexins synthesis. Therefore, it appears that the activation of the MEP pathway in infected rice is essential for the production of sufficient secondary metabolites, including phytoalexins.

In this study, we demonstrated that the inducible production of phytoalexins in rice occurs as a consequence of the coordinate expression of genes responsible for both the early and downstream stages of phytoalexins biosynthesis. This coordinate expression also suggests that the same or similar mechanisms elicit the transcription of these genes. However, the mechanism of the synchronous regulation of the expression of this series of genes is still unknown. One potential approach would be a search for a factor that regulates the coordinate expression of elicitor-inducible genes in the rice oligomicroarray analysis.

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