The potential bioproduction of the pharmaceutical agent sakuranetin, a flavonoid phytoalexin in rice

Takahumi Shimizu,1,† Fengqiu Lin,1 Motifumi Hasegawa,1 Hideaki Nojiri,1 Hisakazu Yamane1 and Kazunori Okada1,*
1Biotechnology Research Center; The University of Tokyo; Bunkyo-ku, Tokyo, Japan; 2College of Agriculture; Ibaraki University; Ami, Ibaraki, Japan
Current affiliations: †Botanical Gardens; Graduate School of Science; Osaka City University; Katano-shi, Osaka Japan; ‡Department of Biosciences; Teikyo University; Utsunomiya, Tochigi Japan

Sakuranetin, the major flavonoid phytoalexin in rice, can be induced by ultraviolet (UV) irradiation, treatment with CuCl2 or jasmonic acid (JA), or phytopathogenic infection. In addition to sakuranetin’s biological significance on disease resistance in rice, its broad bioactivities have recently been described. Results from these studies have shown that sakuranetin is a useful compound as a plant antibiotic and a potential pharmaceutical agent. Sakuranetin is biosynthesized from naringenin, a precursor of sakuranetin, by naringenin 7-O-methyltransferase (NOMT), but the relevant gene has not yet been identified in rice. Recently, we identified the OsNOMT gene, which is involved in the final step of sakuranetin biosynthesis in rice. In previous studies, OsNOMT was purified to apparent homogeneity from UV-treated wild-type rice leaves; however, the purified protein, termed OsCOMT1, exhibited caffeic acid 3-O-methyltransferase (COMT) activity, but not NOMT activity. Based on the analysis of an oscomt1 T-DNA tagged mutant, we determined that OsCOMT1 did not contribute to sakuranetin production in rice in vivo. Therefore, we took advantage of the oscomt1 mutant to purify OsNOMT. A crude protein preparation from UV-treated oscomt1 leaves was subjected to three sequential purification steps resulting in a 400-fold purification from the crude enzyme preparation with a minor band at an apparent molecular mass of 40 kDa in the purest enzyme preparation. Matrix-assisted laser desorption/ionization time of flight/time of flight analysis showed that the 40 kDa protein band included two O-methyltransferase-like proteins, but one of the proteins encoded by Os12g0240900 exhibited clear NOMT activity; thus, this gene was designated OsNOMT. Gene expression was induced by treatment with jasmonic acid in rice leaves prior to sakuranetin accumulation, and the recombinant protein showed reasonable kinetic properties to NOMT. Identification of the OsNOMT gene enables the production of large amounts of sakuranetin through transgenic rice and microorganisms. This finding also allows for the generation of disease-resistant and sakuranetin biofortified rice in the future.

Introduction

When plants are attacked by pathogenic microorganisms, they respond with a variety of defense reactions, including the production of secondary metabolites called phytoalexins,1,2 which serve as plant antibiotics. In rice, 15 phytoalexins have been isolated and characterized, including 14 diterpenes: momilactones A and B, phytocassanes A through E, oryzalexins A through F,3-11 and one flavonoid phytoalexin, sakuranetin (Fig. 1). Among them, sakuranetin is considered one of the most biologically important phytoalexins in terms of its high antimicrobial activity and high accumulation...
in rice leaves infected by *Magnaporthe oryzae*, a major phytopathogenic fungi. Recently, we reported that momilactone A, a diterpenoid-type phytoalexin, displayed an inhibitory effect on the growth of rice blast fungus at the site of the infection in rice leaves. In contrast, no direct evidence has shown that sakuranetin contributes to fungal growth prevention and spore germination at the infection site in rice. Addressing whether sakuranetin also plays a role in the inhibition of fungal growth at the site of infection will be important.

Sakuranetin was recently shown to display anti-inflammatory activity by inhibiting 5-lipoxygenase, which is involved in arachidonic acid metabolism and is important.

Sakuranetin has been implicated in antitrypanosomal activities. In addition, sakuranetin was able to enhance adipogenesis to contribute to the maintenance of glucose homeostasis in animals. Sakuranetin is involved in downregulation of transcription factors, especially GATA-2, which plays a critical role in adipogenesis, and through abrogation of a suppressive pathway mediated by the GATA family of transcription factors, especially GATA-2, which is involved in downregulation of adipogenesis to contribute to the maintenance of glucose homeostasis in animals. This finding suggests that sakuranetin may be used to alleviate diabetes by sensitizing adipocytes to insulin. Thus, sakuranetin may be a useful compound as a plant antibiotic and a potential pharmaceutical agent.

Sakuranetin was first identified from the cortex of cherry tree bark (*Prunus* spp) as an aglycone of sakuranin, and later found in rice and several other plant species, including *Artemisia campestris*, *Baccharis spp*, *Betula spp* and *Juglans spp*, as well as several plant species, the NOMT plays an important role in the biosynthesis of isoflavonoids, flavones and a flavanone, and its biological function remains unknown. Although sakuranetin is not found in healthy rice leaves, its biosynthesis is rapidly induced by both biotic and abiotic stresses, including infection with phytopathogens, such as *M. oryzae*, *Xanthomonas oryzae* and *Diaporthe oryzae*, infection with *Sogatella furcifera*, UV irradiation and treatment with *CaCl*₂ or jasmonic acid (JA). Purification of NOMT from crude extracts of UV-treated wild-type rice leaves has been attempted and was believed to be purified in apparent homogeneity (~985-fold). However, the amino acid sequence of the purified protein was highly homologous to that of caffeic acid 3-O-methyltransferase (COMT) from maize. In fact, the recombinant protein expressed in *Escherichia coli* showed COMT activity but not NOMT activity, and this enzyme was termed OsCOMT1. We also determined that the OsCOMT1 protein was predominantly localized in the cortex of cherry tree bark (*Prunus* spp) as an OMT with a high homology (~985-fold). However, despite many reports detecting sakuranetin production in several plant species, the NOMT gene has not yet been identified. Although an OMT from barley (F1-OMT) that catalyzes the reaction, however, SaOMT-2 has broad substrate specificity against isoflavonoids, flavonols and flavones, and its biological function remains unknown. Although sakuranetin is not found in healthy rice leaves, its biosynthesis is rapidly induced by both biotic and abiotic stresses, including infection with phytopathogens, such as *M. oryzae*, *Xanthomonas oryzae* and *Diaporthe oryzae*, infection with *Sogatella furcifera*, UV irradiation and treatment with *CaCl*₂ or jasmonic acid (JA). Purification of NOMT from crude extracts of UV-treated wild-type rice leaves has been attempted and was believed to be purified in apparent homogeneity (~985-fold). However, the amino acid sequence of the purified protein was highly homologous to that of caffeic acid 3-O-methyltransferase (COMT) from maize. In fact, the recombinant protein expressed in *Escherichia coli* showed COMT activity but not NOMT activity, and this enzyme was termed OsCOMT1. We also determined that the OsCOMT1 protein was predominantly purified from UV-treated wild-type rice leaves instead of OsNOMT using the same strategy. Given the limited success of OsNOMT purification, we hypothesized at least two possibilities: OsCOMT1, a major component in rice leaves, may mask rice NOMT (OsNOMT), a minor protein in the purified fraction; thus making it difficult to purify OsNOMT; and/or that OsCOMT1 is involved in NOMT enzymatic activity in rice in vivo, but a posttranslational modification and/or the presence of an interacting factor is needed for NOMT activity. In the former case, use of an OsCOMT1-defective mutant as plant material may successfully purify OsNOMT quite efficiently without masking by OsCOMT1. In the latter case, NOMT activity would not be induced in an OsCOMT1-defective mutant even after elicitation.

Molecular Identification of the OsNOMT Gene in Rice

We obtained an OsCOMT1-defective mutant from the Rice Functional Genomics Express Database (FPG-2B-50240 [cv Dongjin]) as a T-DNA insertion line in the first exon of Os08g0157500. We
first confirmed whether NOMT activity was induced by elicitor treatment in oscomt1 mutant leaves. After confirmation of impaired transcriptional expression of the OsCOMT1 gene in leaves of the oscomt homologous mutant by reverse transcription-polymerase chain reaction (RT-PCR), we examined the ability of the oscomt mutant leaves to produce sakuranetin. The analysis revealed that sakuranetin accumulation and NOMT enzymatic activity were similarly induced after elicitation in the oscomt mutant and wild-type rice leaves, strongly suggesting that OsCOMT1 is not involved in sakuranetin production in rice. One possible reason as to why OsNOMT was not detected in the purified fraction with NOMT enzymatic activity from crude extracts of UV-treated wild-type rice leaves in the previous study may be that the abundantly generated OsCOMT1 exhibited similar behavior to OsNOMT in a series of purification steps and masked the presence of OsNOMT.

Next, we sought to purify OsNOMT from UV-irradiated leaves of the oscomt1 mutant. In this trial, purification of OsNOMT was successfully performed without masking by OsCOMT1 using eluted oscomt leaves. Among the three OsNOMT purification steps, adenosine agarose chromatography was the most effective, leading to more than a 400-fold enrichment of the enzyme. This type of chromatography is very effective at purifying SAM-dependent methyltransferases. Following this purification step, a 40-kDa band was detected by sodium dodecyl sulfate-PAGE (SDS-PAGE). The molecular masses of type 1 OMTs, whose substrates are caffeic acid, flavonoids, coumarin and alkaloids, were ~38 to 43 kDa, whereas OsNOMT and OsCOMT1 have been suggested to have molecular masses of ~41 kDa. The 40-kDa band was excised from the gel and subjected to matrix-assisted laser desorption/ionization time of flight/time of flight (MALDI-TOF/TOF) mass spectrometry (MS) analysis after treatment with trypsin, resulting in the identification of the two OMTs (Os04g0575900 [AK104764] and Os12g0249090 [AB692949]) as candidate proteins for OsNOMT. Alignment between these two candidates and OsCOMT1 indicated that the OMTs have high similarity; thus, we were unable to distinguish these candidates from OsCOMT1 regarding their possible function as a NOMT without examining their enzymatic activities (Fig. 2).

Preparation of glutathione S-transferase (GST)-tagged gene products of Os04g0575900 (AK104764) and Os12g0249090 (AB692949) in E. coli followed by in vitro NOMT enzymatic assays clearly demonstrated that Os12g0249090 encoded OsNOMT. Kinetic analysis of recombinant GST-OsNOMT revealed that the $K_m$ of GST-OsNOMT for naringenin was ~1.9 μM. In contrast, the endogenous level of naringenin accumulation was ~1.8 μM in rice leaves 48 h after JA treatment. The $K_m$ value was reasonable considering the function of OsNOMT in sakuranetin production in rice leaves. Substrate specificity of OsNOMT was also determined as described by Christensen et al. The results showed that GST-OsNOMT displayed higher methylation activity on naringenin than the flavanone isorhamnetin and flavones, and showed no methylation activity on other phenolics, including isoflavonoids. Regarding F1-OMT in barley, the methylation activity on apigenin was 3-fold higher than that on naringenin. In contrast, SaOMT-2 possessed broad substrate specificity and...
catalyzed the methylation of isoflavonoids and naringenin. Sakuranetin has not yet been identified in either barley or *S. avermiltis.* The results from this study indicate that the specific biological functions of F1-OMT and SaOMT-2 remain unknown; this is the first report on the cloning and characterization of rice OsNOMT as a sakuranetin synthase in plant.

The expression profile of *OsNOMT,* accumulation of sakuranetin and its direct precursor, naringenin, in rice leaves treated with JA indicated that *OsNOMT* expression was transiently induced prior to accumulation of sakuranetin, and that the accumulation of naringenin was also transiently induced slightly prior to that of sakuranetin. Uptimization of *OsNOMT* was also confirmed in *M. oryzae*-infected rice leaves in which the accumulation of sakuranetin and its precursor increased. These results further support the hypothesis that *OsNOMT* functions as a sakuranetin synthase and is involved in defense responses through elicitor-induced production of sakuranetin in rice.

In addition to our effort to purify the OsNOMT protein by biochemical methods, we screened candidate genes for *OsNOMT* using transcriptome data. Time-course microarray analyses of JA-treated rice leaves and cultured cells in suspension were performed, and JA-inducible OMT family genes were selected. In this approach, we focused on the genes that were predominantly induced in rice leaves, but not in cultured cells, since neither sakuranetin production nor NOMT activity was detected in JA-treated cultured cells in suspension. Why cultured cells in suspension cannot produce sakuranetin even under elicited conditions is unclear, but we were able to confirm that the cells exhibited no NOMT activity in our NOMT activity assay. Therefore, we hypothesized that expression of the *OsNOMT* gene is silenced in cultured cells in suspension. As a result, seven OMT-like genes (*OMTL1-OMTL7*) were selected for analysis. Among the seven OMTL genes, *OMTL5* and *OMTL7* clearly showed rice leaf-specific expression of the relevant genes (Table 1). Consequently, we first succeeded in identifying the *OsNOMT* gene using a biochemical approach, but it was just a matter of time before we identified that the *OMTL7* gene clearly encodes NOMT in rice.

**Table 1. JA-inducible expression of OMTL family genes in rice leaves and calli**

| Accession number | Name | JA-treated leaf disc* | JA-treated calli** |
|------------------|------|-----------------------|--------------------|
| AR069950         | OMTL1| 3.0 5.4 29.3 43.0 1.7 | 1.5 2.3 3.0 3.1 |
| AX072740         | OMTL2| 34.1 39.3 281.0 359.0 5.1 | 3.4 3.2 3.5 3.3 |
| AR069721         | OMTL3| 31.2 8.7 22.8 20.4 4.3 | 3.1 2.9 2.9 2.8 |
| AR069738         | OMTL4| 94.7 24.0 30.6 13.1 1.0 | 1.7 2.2 2.3 3.0 |
| OX11 g05359050   | OMTL5| 67.3 26.7 56.3 25.5 1.0 | 1.0 1.0 1.0 1.0 |
| OX12 g0202700    | OMTL6| 0.9 2.0 5.3 6.6 1.7 | 1.5 2.5 3.2 3.0 |
| OX12 g0240900 (OsNOMT)| OMTL7| 4.3 8.7 15.2 11.9 1.0 | 1.0 1.0 1.0 1.0 |

*Os12 g0240900 (OsNOMT)* gene is indicated by bold text. *Each value represents the ratios of signal intensity of JA-treated leaf disk to that of H2O-treated leaf disk. **Each value represents the ratios of signal intensity of each time point to that of 0 h after treatment.

Potential Applications of the *OsNOMT* Gene for Production of the Pharmaceutical Agent, Sakuranetin

The successful identification of *OsNOMT* as a sakuranetin synthase in our study will enable enhanced pathogen resistance through regulation of the endogenous content of sakuranetin in rice. We are currently generating transgenic rice plants whose expression level of the *OsNOMT* gene has been modified (Fig. 3). We expect to see a biological significance of sakuranetin on disease resistance in rice by examining *OsNOMT*-overexpressing and -repressing plants against the rice blast fungus infection. We also plan on introducing the *OsNOMT* gene into other plant species, such as tobacco and wheat, to produce sakuranetin heterologously. Since naringenin appears to exist in various plant species as a flavonoid precursor, the production of sakuranetin in plants other than rice may be accessible by expressing the *OsNOMT* gene in host plants. In addition, sakuranetin is a useful compound showing various pharmaceutical activities, including glucose homostasis, which would be highly important for patients with diabetes. Since the fermentative production of naringenin by microorganisms carrying an artificially assembled phenylpropanoid pathway has been established, our successful cloning of *OsNOMT* will enable the production of large amounts of sakuranetin by microorganisms for medical research (Fig. 3). Another potential approach for *OsNOMT* utilization in human nutritional prospects is the generation of sakuranetin biofortified rice grains. Golden rice, which produces provitamin A-enriched grains, has already been developed and is ready for cultivation in Asian countries, including the Philippines, Vietnam and India. Also, reports have described the production of coenzyme Q10 and gamma-aminobutyric acid (GABA) in rice grains. These biofortified rice are dependent on the supply of essential nutrients required for a healthy life. Similar to these applications, our findings may not only allow for the generation of a disease-resistant plant, but also sakuranetin biofortified rice, which may alleviate the response to insulin for patients with diabetes in the future.
Acknowledgments
We thank Dr H. Nagasawa, Dr S. Nagata, M. Kurouga (Graduate School of Agricultural and Life Sciences, The University of Tokyo) and Dr T. Umeda (Biotechnology Research Center, The University of Tokyo) for their support with the purification and identification of OsNOMT. We thank Dr T. Kiyosawa and T. Ozaki (Biotechnology Research Center, The University of Tokyo) for providing the OsNOMT. We thank Dr T. Kuzuyama and Dr E. Minami (National Institute of Agrobiological Sciences) for sharing the culture of M. oryzae used in this study. We thank Dr Gynheung An (Institution and Pohang University of Science and Technology) for providing the ascom mutant. This work was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences.

References
1. Yoshitake HD, Manafud WD, Reddy JA, Farmer SE. "Two classes of plant antibiotics. Phytalexins versus "Phytoanticipins." Plant Cell 1994; 6:1191-2; PMID:12244206.
2. Ahuja J, Kusum B, Braoj AM. Phytalexins in foliar- defend. Trends Plant Sci 2002; 7:73- 90; PMID:12229208; http://dx.doi.org/10.1016/ s1360-1385(01)11102.
3. Kojo J, Minami M, Onikina K, Ogura N, Yamazaki T, Ogawara N, et al. B, C, and D, novel diterpene phytoalexins from rice. Oyae sativa L. Tetrahedron 1993; 51:7867-8; http://dx.doi.org/10.1016/0040- 4020(93)90443-4.
4. Kojo J, Ogura N, Yamazaki T, Kikuchi K, Ogawara N, Shimizu M. Functional entity for the arylating activity of phytoalexin E, a diterpene phytoalexin from rice. Phytomchimica 1997; 44:245-51; http://dx.doi.org/10.1016/S0031-9422(96)00485-4.
5. Yamao A, Mori K. Synthesis and Absolute Configuration of Cyptophyacin D, a diterpene phytoalexin isolated from the rice plant. Oyae sativa. J Org Chem 2008; 73:11879-91; http://dx.doi.org/10.1021/jo802154f; http://dx.doi.org/10.1021/jo802154f 12.04.09.1; 2.4.4.0.9; 1. AID-JOC4797.1; JOC9323.
6. Akamatsu R, Kadono O, Sekido H, Kato Y, Tsukino S, Nishino phytoalexin (narin and B, C) isolated from rice blast leaves infected with Pyricularia oryzae. Part I: Isolation, characterization, and biological activity of oryzyalin. Agric Biol Chem 1985; 49:1689-94; http://dx.doi.org/10.1271/ bbac.49.1689.
7. Kato H, Kodama O, Akahata T, Oyamada E. A diterpene phytoalexin from UV-irradiated rice leaves. Phytochemistry 1993; 35:78-81; http://dx.doi.org/10.1016/0021-9939(93)85056-P.
8. Kato H, Kodama O, Akahata T, Oyamada E. A diterpene phytoalexin from UV-irradiated rice leaves. Phytochemistry 1996; 38:289-92; http://dx.doi.org/10.1016/S0021-9939(95)00484-X.
9. Carvajal PF, Langape P, Pierce RJ, Letcher EF. Kale PE. Isolation and characterization of two phytoalexins from rice as mandelsonone A and B. Phytochemistry 1986; 26:597-9; http://dx.doi.org/10.1016/S0031-9422(00)18495-9.
10. Kato T, Kihara Y, Tanaka N. Three cyanogenic rice cultivars and their anaerobic metabolism. Holocellulolysis of glicophosphatyl starch biosynthesis with scilomylsine inhibition. Protein Sci 2006; 15:1879-81; http://dx.doi.org/10.1110/ ps.036186.108.
11. Tamagawa S, Minami M, Kodama O, Akahata T, Oyamada E. A new white rice phytoalexin and its biosynthesis. Phytochemistry 1993; 49:295-32; http://dx.doi.org/10.1016/S0031-9422(00)9000-3C.
12. Kodama O, Miyake K, Akahata T, Kiyosawa S, Sakurakadu A. Phytalexin from ultraviolet-irradiated rice leaves. Phytochemistry 1992; 31:907-9; http://dx.doi.org/10.1016/S0031-9422(00)9000-3C.
13. Miyazawa M, Minami K, Akahata T, Kiyosawa S. sakuranetin induces apoptosis in human gastric adenocarcinoma cells through enhanced expression of P53 and BCL-2. Cancer Res 2008; 68:85-9; PMID:18232158; http://dx.doi.org/10.1158/0008-5472.CAN-07-1349.
14. Akhmedov Y, Uesaka S, Saito T, Abe D, Sekiya K. Sakuranetin induces apoptosis of human gastric cancer cells through enhanced expression of P53 and BCL-2. Cancer Res 2008; 68:85-9; PMID:18232158; http://dx.doi.org/10.1158/0008-5472.CAN-07-1349.
15. Miyazawa M, Minami K, Okano T. Antimutagenic activity of sakuranetin from Prunus serotina J. Food Sci 2005; 68:52-6; http://dx.doi.org/10.1111/j.1365-2028.1998.tb09032.x.
16. Zhang L, Yang Z, Wu Z. Zhang H, Wu J, Chen J, et al. Three flavonoids targeting the β-Hexosaminidase: protein diperefase from Helicobacter pylori gastric fungi structure. Function. Sci 2006; 15:1879- 81; http://dx.doi.org/10.1110/ ps.036186.108.
17. Greco B, Emonet J, Taponecchi M, Sartori E, Cazzola R, Bernetti E, et al. Siciliadin and antifungal caldum and antitrypanosomal activities of flavanones from Flavonoidosina DCL (Antennariina). Exp Parasitol 2012; 130:169-75; PMID:22249099; http://dx.doi.org/10.1016/j.exppara.2011.10.002.
18. Saito T, Abe D, Sekiya K. sakuranetin induces apoptosis of human gastric cancer cells through enhanced expression of P53 and BCL-2. Cancer Res 2008; 68:85-9; PMID:18232158; http://dx.doi.org/10.1158/0008-5472.CAN-07-1349.
19. Akhmedov Y, Uesaka S, Saito T, Abe D, Sekiya K. sakuranetin induces apoptosis in human gastric adenocarcinoma cells through enhanced expression of P53 and BCL-2. Cancer Res 2008; 68:85-9; PMID:18232158; http://dx.doi.org/10.1158/0008-5472.CAN-07-1349.

Figure 3. Possible application for sakuranetin bioproduction. (A) Generation of transgenic plants expressing the OsNOMT gene. Sakuranetin accumulation could be expected in transgenic plants with enhanced disease resistance. (B) Fermentative sakuranetin production from tyrosine by recombinant E. coli harboring four biosynthetic pathway genes for naringenin and OsNOMT. PAL, phenylalanine ammonia-lyase; 4CL, 4-coumarate-CoA ligase; CSH, chalcone synthase; CHL, chalcone isomerase.
21. Rakwal R, Hongoa M, Kodanac O. A methyltransferase for synthesis of the flavonone phytoalexin sakuranetin in rice leaves. Biochimie 1996; 78:761-74; PMID:8921285; http://dx.doi.org/10.1016/0300-9084(96)00014-2.

22. Christensen AB, Groger LN, Olson CE, Collinge DR. A flavonoid 7-OMethyltransferase is expressed in barley lines in response to pathogens attack. Plant Mol Biol 1998; 37:297-304; PMID:9448416; http://dx.doi.org/10.1023/A:1006509005303.

23. Kim BG, Jeong BR, Lee Y, Hsu HG, Lim Y, Ahn JH. Regiospecific flavonoid 7-O-methyltransferase with Stepwise sequential O-methyltransferase expressed in Zinnia elegans. J Agric Food Chem 2004; 52:623-8; PMID:1446189; http://dx.doi.org/10.1021/jf034381x.

24. Rakwal R, Agarwal GK, Yonekura M, Kodama O. Naringenin 7-O-methyltransferase involved in the biosynthesis of the flavonone phytoalexin sakuranetin from rice (Oryza sativa L.). Plant Sci 2000; 155:213-20; PMID:10814825; http://dx.doi.org/10.1016/S0168-9452(00)00223-5.

25. Ditylenchus angustus.

26. Pounds in rice after infection by the stem nematode Harborne JB. The induction of phenolic compounds in rice by the white-backed planthopper, Sogatella furcifera (Horváth). J Appl Entomol 2012; 8:2; PMID:22243810; http://dx.doi.org/10.1163/004625996X00063.

27. Kanno H, Hanagavao M, Kodana O. Accumulation of salicylic acid, jasmonic acid, and phytoalexins in rice. (Oryza sativa), infested by the white-backed planthopper, Sogatella furcifera (Horváth). Jpn J Breed 2000; 50:823-8; PMID:16448189; http://dx.doi.org/10.1111/j.1365-313X.2000.00767.x.

28. Lin P, Yarzata G, Hanagavga M, Amia H, Kawasakia S, Kodana O. Cloning and functional analysis of caffeic acid 3-O-methyltransferases from rice (Oryza sativa). J Plant Biol 2006; 49:47-55; http://dx.doi.org/10.1006/jpbi.2005.6467.

29. Jeong DH, Jeo S, Park S, Kang HG, Park GG, Kim SB, et al. Generation of a flanking sequence-variation database for activation-tagging lines in japonica rice. Plant J 2006; 45:123-32; PMID:16367959; http://dx.doi.org/10.1111/j.1365-313X.2005.02610.x.

30. Lin F, Lee S, Jung KH, Jun SH, Jeong DH, Lee Y, Hur HG, Lim Y, Ahn JH, et al. T-DNA insertional mutagenesis for functional analysis of an E-box motif responsible for the expression of jasmonic acid-induced chitinase genes OsChia4a in rice. J Plant Physiol 2012; 169:621-7; PMID:22266099; http://dx.doi.org/10.1016/j.jplph.2011.12.008.

31. Horzinski S. Combinatorial biosynthesis of plant medicinal polyketides by microorganisms. Curr Opin Chem Biol 2009; 13:297-298; PMID:19549534; http://dx.doi.org/10.1016/j.cbpa.2009.02.004.

32. Beyer P. Golden rice and ‘Golden’ crops for human nutrition. New Biotech 2010; 27:478-81; http://dx.doi.org/10.1016/j.nbt.2010.05.010.

33. Takahashi, Ohtani T, Saitoh H, Nakayama Y, Kocimi M, Kodakarvo K. Development of centeze QTL-enchanced rice using nesy 5 and shrunken mutants. Braci Biochem Biotechnol Vech 2010; 76:320-4; PMID:20875152; http://dx.doi.org/10.1016/j.bbvr.2010.06.008.

34. Nakamura K, Kawanabe J, Inoue S, Sakauchi K, Tsujiichi S. Takahashi T. Seed-specific expression of truncated OsGAD2 produces GA3-enriched rice grains that reduce in vitro glucose level in spontaneously hypertensive rats. Transgenic Res 2006; 15:845-56; PMID:16494589; http://dx.doi.org/10.1007/s11248-009-9272-1.