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Analysis of Flavonoids and Characterization of the *OsFNS* Gene Involved in Flavone Biosynthesis in Rice

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The major flavonoids in rice leaves were analyzed via LC-MS/MS after their total flavonoid extracts were hydrolyzed. The most abundant flavones were apigenin, luteolin, and tricetin. Of these, tricetin was methylated at its 3′ and 5′-hydroxyl group to form tricin, which was probably *O*-glycosylated. Both 3′-*O*-methylated luteolin and luteolin were found in the *C*-glycosylated form while apigenin was *C*-glycosylated. We also cloned and characterized *OsFNS*, which catalyzes the reaction from flavanone (naringenin) to flavone (apigenin). Analysis of the reaction product with recombinant OsFNS showed that it indeed converts naringenin to apigenin.

Key words: flavones, flavone synthase, flavonoid analysis, *Oryza sativa*

Flavonoids are diverse secondary metabolites in plants; more than 10,000 compounds have already been reported (Tahara, 2007). All are synthesized from two basic metabolites -- malonyl-CoA and *p*-coumaroyl-CoA -- to 15-carbon skeletons, at a 3:1 ratio. The derived chalcone intermediates comprise two phenolic groups connected by an open three-carbon bridge. That linkage is part of an additional heterocyclic six-member ring that involves one of the phenolic groups on the adjacent ring. Derived from chalcone, various classes of flavonoids -- flavanones, isoflavones, flavanols, anthocyanins, flavonols, and flavones -- can be synthesized (Croteau et al., 2000). This diversity of flavonoids arises from several modification reactions that are mediated by oxygenases, methyltransferases, and glycosyltransferases (Tahara, 2007).

Hydroxylation and desaturation are the common modification reactions for flavonoids after the synthesis of naringenin. Two different classes of oxygenases -- cytochrome P450-dependent mono-oxygenase (P450) and 2-oxoglutarate-dependent dioxygenase (2-ODDs) -- control these reactions (Feldmann, 2001; Hausinger, 2004). Both groups need iron for their activities, with 2-ODDs using the Fe^{2+} ion while P450 mono-oxygenases require iron as a cofactor of heme. Flavone synthase type I (FNSI), flavanone 3-hydroxylase (F3H), flavonol synthase (FLS), and anthocyanin synthase (ANS) are the 2-ODDs involved in flavonoid biosynthesis. FNSI catalyzes the reaction from naringenin to apigenin and F3H transfers a hydroxyl group to naringenin to form dihydroxykaempferol, which is eventually converted into kaempferol by FLS. ANS mediates the reaction to form a double bond between oxygen and the second carbon in ring C. Of these four 2-ODDs, F3H shows high amino acid similarity with FNSI while ANS is more similar to FLS at the amino acid level (Martens and Mithöfer, 2005). Therefore, one cannot predict the actual substrate only by the amino acid sequences of those genes.

The rice genome sequencing project has provided valuable information about flavonoid biosynthetic genes, and we have previously conducted *in vitro* characterizations (Kim et al., 2006a, 2007; Ko et al., 2006). Based on their *in vitro* substrates, we have been able to predict the *in vivo* substrate of each. However, it would be better to rely on flavonoid content when searching for the genes involved in the biosynthesis of a particular compound. Now that analytical tools such as LC-MS/MS have been developed, it is feasible to analyze metabolites from only a small sample size. Here, we studied the flavonoids from rice leaves and characterized the gene involved in their biosynthesis.

MATERIALS AND METHODS

Extraction of Flavonoids from Rice Leaves

Total phenolic compounds were isolated from 90-day-old rice leaves that were ground to a fine powder with pestle and mortar in liquid nitrogen and extracted with 80% methanol. This extract was filtered through a 0.45 μm PTFE membrane (Whatman, Brentford, UK). The resulting fraction was evaporated and then dissolved in 1:1 2N HCl and 50% methanol (v:v). The extract was heated in a sealed vial at 100°C for 1 h before cooling to room temperature. Finally, the hydrolyzed sample was purified through a C-18 Sep-pak cartridge (Waters, Milford, MA, USA).

Identification of Flavonoids by LC-MS/MS

Our LC-MS/MS system comprised a Varian 212 HPLC (Varian Inc., USA), photodiode array detector (Varian 355), and a Varian 500-MS ion trap spectrometer. A Chromsep HPLC column (150.0 × 2.0 mm) was used at a flow rate of 0.2 mL min^{-1}. The mobile phase consisted of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). Samples were run with the gradient varying linearly from 0% to 10% B in 2 min, to 40% B in 10 min, and to 70% B in 20 min.

This system was operated with MS workstation software (version 6.9; Varian, Inc.). Mass spectra were acquired using an electrospray ionization (ESI) source in the negative ionization mode. Full-scan mass spectra were recorded over a

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range of 50 to 2000 *m/z*. Operating parameters for samples in the negative ion mode included a spray needle voltage of 5 kV, capillary voltage of 80 V, drying temperature of 220°C, drying gas pressure of 10 psi nitrogen, nebulizer gas pressure of 35 psi air, and helium as the neutral collision gas. Tandem mass spectrometry analysis was conducted with scan-type turbo data-dependent scanning (DDS). The collision-induced dissociation (CID) voltage was automatically set by DDS, and the mass width was 3 *m/z*.

Cloning and Expression of *OsFNS*

The full-length cDNA of *OsFNS* was cloned by reverse

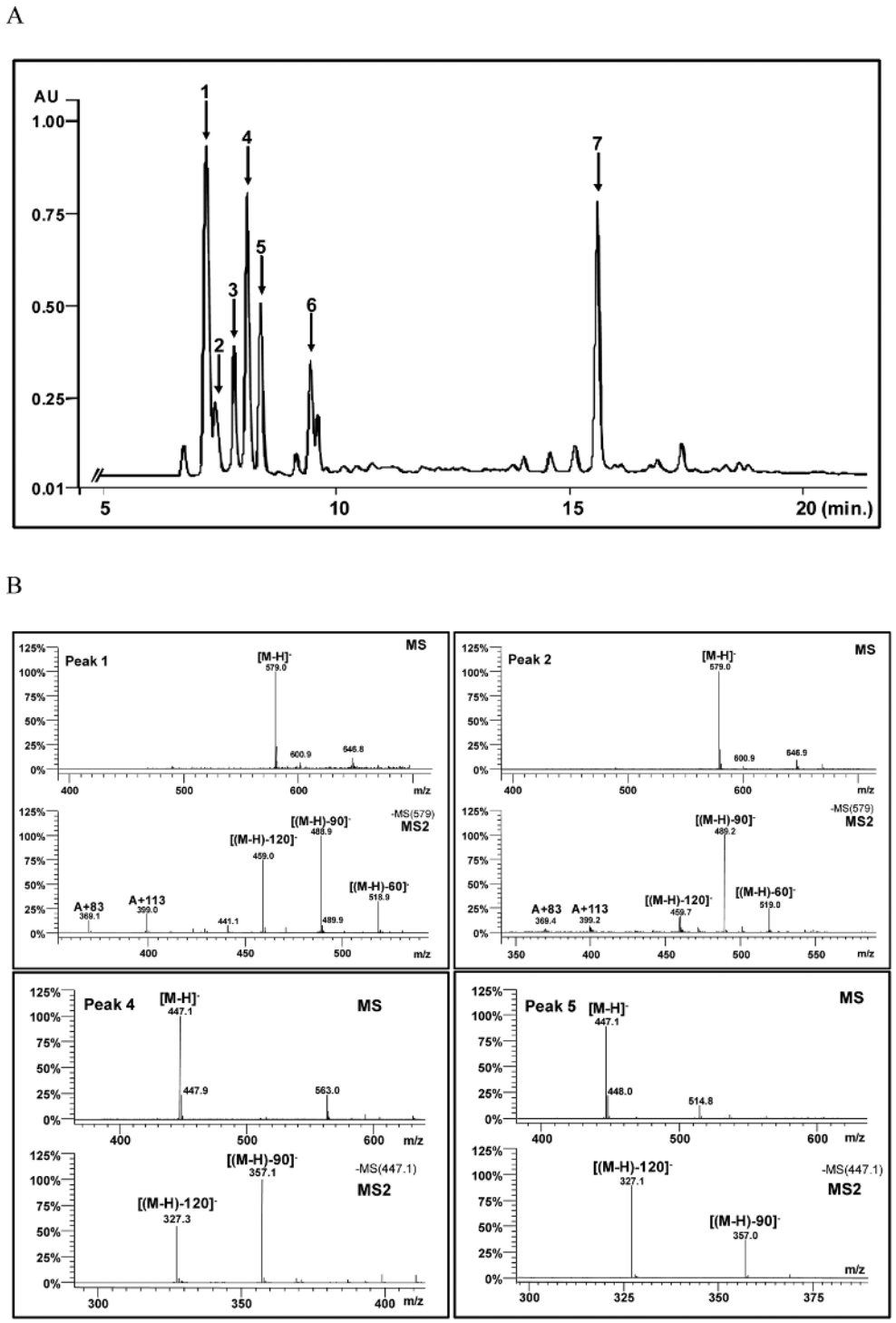

Figure 1. HPLC analysis of flavonoid hydrolysates extracted from rice leaves (**A**), and MS/MS analysis of Peaks 1, 2, 4, and 5 (**B**). Polaris 5 C-18 A HPLC column (250.0 X 4.6 mm) was used for separation, and eluents were monitored at 340 nm. Individual Peaks 1 to 7 were collected for MS/MS analysis.

transcription polymerase chain reaction (RT-PCR). cDNA was synthesized with total RNA from one-month-old whole rice, using Omniscript reverse transcriptase (Qiagen, Germany). A forward primer 5′-ATGGCGGACCAGCTCATCTC-3′ and reverse primer 5′-CAAACCAGTTCTCGACTAGATCA-3′ were designed based on the GenBank accession No. NM_001055334. PCR was carried out under the following conditions: 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min. The PCR product was sub-cloned into the pGEM-T easy vector (Promega, WI, USA) for sequencing. OsFNS was sub-cloned into pGEX 5X-2 (Amersham Biotech, USA) for expression in *Escherichia coli*. Induction and purification of OsFNS were performed as described by Kim et al. (2006b). For the FNS enzyme assay, purified OsFNS was incubated for 3 h at 37°C with 50 μM $FeSO_4$, 100 mg mL^{-1} of catalase, 160 μM oxoglutarate, 1 mM ascorbate, and 60 μM *S*- or *R*-naringenin in 500 μL of 10 mM Tris/HCl buffer (pH 8.0). The reaction product was analyzed via HPLC, as described by Kim et al. (2006a).

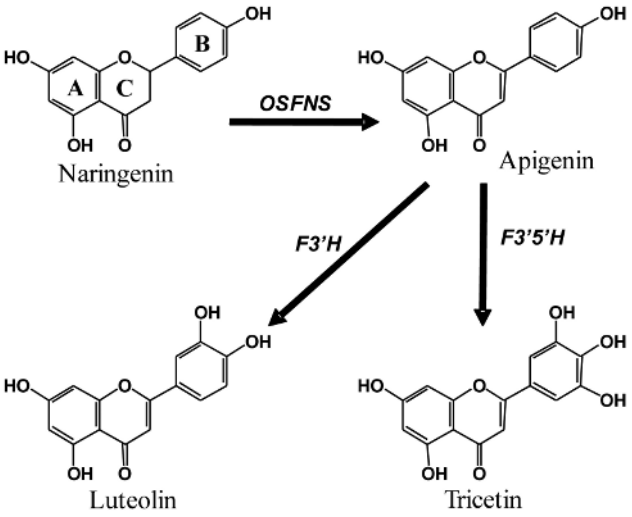

Figure 2. Biosynthetic pathway for flavones (apigenin) and flavonol (luteolin and tricetin) from flavanone (naringenin). F3′H, flavone 3′-hydroxylase; F3′,5′H, flavone 3′,5′-hydroxylase.

RESULTS AND DISCUSSION

Analysis of Flavonoids in Rice

Most of the total flavonoids extracted from our 90-d-old rice leaves were glycosylated, which resulted in their complexity. To simplify the evaluation of their composition, we removed the *O*-linked sugars by acid hydrolysis. These sugars are released by such a process whereas the C-linked sugars are resistant (Markham, 1982b). HPLC analysis of the acid hydrolysates revealed seven major peaks (Fig. 1), all of which contained the UV-spectra characteristic of flavones. The maximum absorption spectra were observed at approximately 330 to 350 nm (Markham, 1982a). Structures for the compounds corresponding to each peak (Table 1) were determined by LC-MS/MS, as defined by published data (Ferreres et al., 2003). The first six peaks were determined to be *C*-linked flavonoids, with Peaks 1 and 2 being 6-*C*-arabinosyl-8-*C*-glucosyl luteolin and 6-*C*-glucosyl-8-*C*-arabinosyl luteolin, respectively. The others included 6-*C*-arabinosyl-8-*C*-glucosyl apigenin (Peak 3), 6-*C*-glucosyl luteolin (Peak 4), 8-*C*-glucosyl luteolin (Peak 5), and 8-*C*-glucosyl 3′-*O*-methyl luteolin (chrysoeriol) (Peak 6). Peak 7 was tricin (3′, 5′-*O*-dimethyl tricetin). Tricin is probably *O*-glycosylated *in vivo* but sugars are released during hydrolysis. We note here that all the flavonoids found in these rice leaves are flavone derivatives (luteolin, apigenin, and tricetin).

Characterization of *OsFNS*

Flavone biosynthesis is initiated by flavone synthase (FNS), which converts naringenin into apigenin, the latter eventually being converted by flavonoid 3′,5′-hydroxylase (F3′, 5′H) to form luteolin and tricetin (Fig. 2). Neither FNS nor F3′, 5′H have yet been characterized in rice, the former being a dioxygenase and the latter, a cytochrome P450. Thus, we searched the rice genome and found four FNS homologues. Because FNS has high similarity with F3H, we cloned and expressed them in *E. coli* to determine whether they were FNS or F3H. Of these, three turned out to be F3H (results to be reported elsewhere). We named the clone encoding *FNS* as *OsFNS*.

OsFNS is 1027-bp long and encodes a 38.7-kDa protein. It contains a well-conserved Fe^{2+} binding site and an oxoglutarate binding site. We cloned its ORF and sub-cloned that into *E. coli* expression vector pGEX 5X-2. This recombinant OsFNS was expressed well and, after purification to

Table 1. Flavonoid compounds from rice leaves identified by LC-MS/MS.

Peak no.	Compound	Rt (min)	$[M - H]^-$	MS2 $[M-H]^-$ (Relative abundances (%) of ions from $[M-H]^-$)				
				-60	-90	-120	A+113	A+83
1	6-C-arab-8-C-glc-luteolin	7.0	579	519 (32)	489 (100)	459 (75)	399 (19)	369 (13)
2	6-C-glc-8-C-arab-luteolin	7.3	579	519 (25)	489 (100)	459 (17)	399 (6)	369 (2)
3	6-C-arab-8-C- glc-apigenin	7.7	563	503 (45)	473 (100)	443 (80)	383 (15)	353 (14)
4	6-C-glc-luteolin	7.9	447	-	357 (100)	327 (55)	-	-
5	8-C-glc-luteolin	8.2	447	-	357 (41)	327 (100)	-	-
6	8-C-glc-chrysoeriol	9.3	461	-	371 (12)	341 (100)	-	-
7	Tricin	15.4	329	MS2 314			MS3 285 , 290	

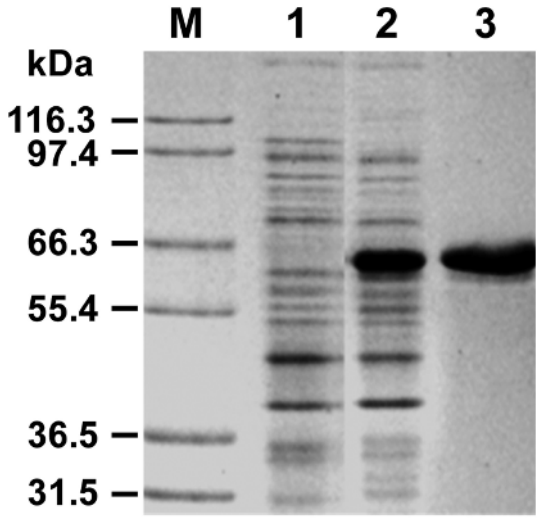

Figure 3. SDS–PAGE analysis of recombinant protein. M, molecular weight size marker; 1, *E. coli* lysate before induction; 2, *E. coli* lysate after induction; 3, purified recombinant OsFNS.

homogeneity through a glutathione *S*-transferase (GST) affinity column, it was found to a molecular weight of 64.7 kDa, which included the 26-kDa molecular weight of GST (Fig. 3).

We reacted the purified recombinant OsFNS with 2*S*-naringenin and other cofactors (Fe^{2+}, oxoglutarate, and ascorbate) to see whether it utilized naringenin. Because the enzymatic reaction product by chalcone synthase is a 2*S*-naringenin, it is generally known that FNSs utilize 2*S*-naringenin much more effectively than 2*R*-naringenin (Turnbull et al., 2004). Our separation of 2*S*-naringenin from the enantiomeric mixture of naringenin was done as described by Miyahisa et al. (2006). The reaction product showed a new peak with a retention time that differed from the substrate under HPLC. Its molecular mass was 272 Da, which was reduced 2 Da compared to that of naringenin and corresponded to that of apigenin. MS/MS analysis showed the same pattern of reaction fragments with apigenin (Fig. 4), thereby indicating that the reaction product is apigenin and that OsFNS encodes a flavone synthase. Two kinetic parameters for *S*-naringenin -- Km and Vmax -- were determined to be 20 μM and 8.3 μkcat mg^{-1}. Without the addition of oxoglutarate, OsFNS activity was completely lost; the omission of Fe^{2+} resulted in a 65% decline in that activity. This demonstrates that OsFNS is dependent on both Fe^{2+} and oxoglutarate.

The composition and biosynthetic pathway for flavonoids is still unknown in rice. More careful analysis of its phenolic compounds has now shown that rice grains contain tricin, which is effective against breast and colon cancers (Hudson et al., 2000; Cai et al., 2004). Our results clearly proved the presence of tricin in rice leaves. Several C-glycosides of flavonoids in that species also have a role in conferring resistance to the brown planthopper, *Niaparvata lugens* (Stevenson et al., 1996). Our study makes it obvious that one of the dominant flavonoid aglycones in rice leaves is tricin. In addition, the composition of major flavonoids in rice seems to shift from apigenin to tricin as those plants mature (unpublished observation). Our approach to identifying those flavonoids and cloning the genes involved in flavonoid biosynthesis will help elucidate the flavonoid biosynthesis pathway in rice, and will be useful in engineering flavonoid composition or content in that species.

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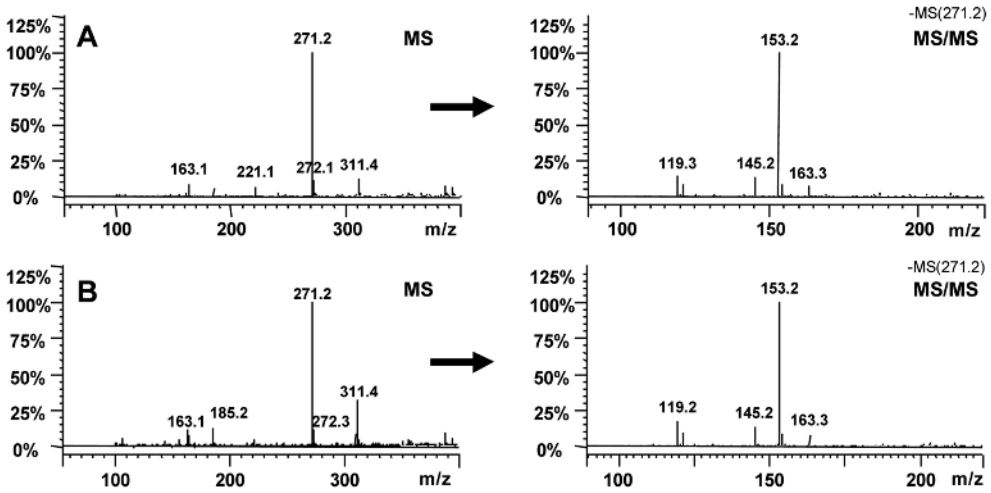

Figure 4. MS and MS/MS spectra of naringenin reaction product with OsFNS (**A**) and authentic apigenin (**B**).

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