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Characterization of flavone synthase I from rice

*Yoon Jung Lee, Jeong Ho Kim, Bong Gyu Kim, Yoongho Lim & Joong-Hoon Ahn**
Department of Bioscience and Biotechnology, Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Korea

Flavones are synthesized from flavanones through the action of flavone synthases (FNSs). There are two FNSs, FNS I and II. FNS I is a soluble dioxygenase present in members of the *Apiaceae* family and FNS II is a membrane bound cytochrome P450 enzyme that has been identified in numerous plant species. In this study, we cloned *OsFNS I-1* from rice by RT-PCR, expressed it in *E. coli*, and purified the recombinant protein. By NMR analysis, we found that OsFNS I-1 converted the flavanone (2*S*)-naringenin into the flavone, apigenin. Moreover, we found that the cofactors oxoglutarate, $FeSO_4$, ascorbate and catalase are required for this reaction. *OsFNS I-1* encodes a flavone synthase I. This is the first type I FNS I found outside of the *Apiaceae* family. [BMB reports 2008; 41(1): 68-71]

INTRODUCTION

Flavonoids, derivatives of 1,3-diphenylpropan-1-one, are phytochemicals with various biological functions in plants. Flavonoids mediate the interactions of plants with other microorganisms and have a role in protecting plants against ultraviolet light. Naringenin (5,7,4'-trihydroxyflavanone), is a basic flavonoid and serves as a starting material for isoflavone, flavone, flavonol, and anthocyanin synthesis (1, 2). The formation of a double bond between carbon 2 and 3 of naringenin results in the production of apigenin (5,7,4'-trihydroxyflavone). Compared with the flavanones, flavones including isoflavones exhibit diverse biological properties including antioxidative, antitumor, antiinflammatory, antibacterial, antiviral, and antiatherosclerotic activities (3-5). Thus, conversion of flavanone into flavone is of great interests due to the various biological activities of flavone. However, a great number of flavones are mainly isolated directly from plant sources because the chemical synthesis of flavones can be complicated.

Rice contains several flavonoids including flavonen (apigenin) and flavonols (luteolin, and quercetin) (6, 7), all of which contain double bond between carbon 2 and 3. The formation of flavones from flavanones can be catalyzed by two different enzymes, flavone synthase I (FNS I) and flavone synthase II (FNS II). In a wide range of plants, flavone formation is catalyzed by flavone synthases II (FNS II), a member of the cytochrome P450 protein family. FNS I has previously been identified only in the *Apiaceae* plant family and is classified as a Fe^{2+}- and 2-oxoglutarate-dependent dioxygenase (3). Among Fe^{2+}- and 2-oxoglutarate-dependent dioxygenases, FNS I, flavonol synthase (FLS), flavanone 3-hydroxylase (F3H) and anthocyanin synthase (ANS) are all involved in flavonoid biosynthesis. ANS and FLS share amino acid homology whereas FNSI and F3H are also closely related (8). Thus, it is difficult to predict functions of these proteins without biochemical characterization of each gene. So far, no rice gene in charge of converting flavanone into flavone has been biochemically characterized. Here, we reported characterization of the first flavone synthase I, *OsFNS I-1* from rice.

RESULTS AND DISCUSSION

We searched the Institute of Genome Research (TIGR) rice gene index with FNS I from parsley to find rice homologue and found one gene, *OsFNS I-1*. Its open reading frame (ORF) was amplified using reverse transcription and polymerase chain reaction (RT-PCR). *OsFNS I-1* consists of 1029-bps and BLAST analysis of OsFNS I-1 revealed high identity with Fe^{2+}- and 2-oxoglutarate-dependent oxygenases from *Medicago truncatula* (64%) and *Arabidopsis thaliana* (63%). These proteins contain Fe^{2+} binding sites and dependent dioxgenase binding sites (9) which were conserved in *OsFNS I-1* (Fig. 1).

Because FNS I and F3H have a high degree of amino acid homology, it is difficult to predict functional differences between the two proteins based on their amino acid sequences. In order to determine the substrate for OsFNS I-1, the full length *OsFNS I-1* cDNA was subcloned into the *E. coli* expression vector and expressed in *E. coli*. The recombinant OsFNS I-1 fusion protein was purified to near homogeneity. The molecular weight of the recombinant OsFNS I-1 was in agreement in the predicted molecular mass of OsFNS I-1 (38.7-kDa) and the glutathione *S*-transferase (26-kDa) (Fig. 2).

In order to determine a substrate for OsFNS I-1, (2*S*)- naringenin, was first tested. (2*S*)-Naringenin is served as a substrate for both FNS I and F3H. HPLC analysis of the *OsFNS I-1* reaction product after incubation with (2*S*)-naringenin revealed a new peak with a retention time of 15 min. This peak had the same retention time and UV-spectra with authentic apigenin

*Corresponding Author. Tel: 82-2-450-3764; Fax: 82-2-3437-6106; E-mail: jhahn@konkuk.ac.kr

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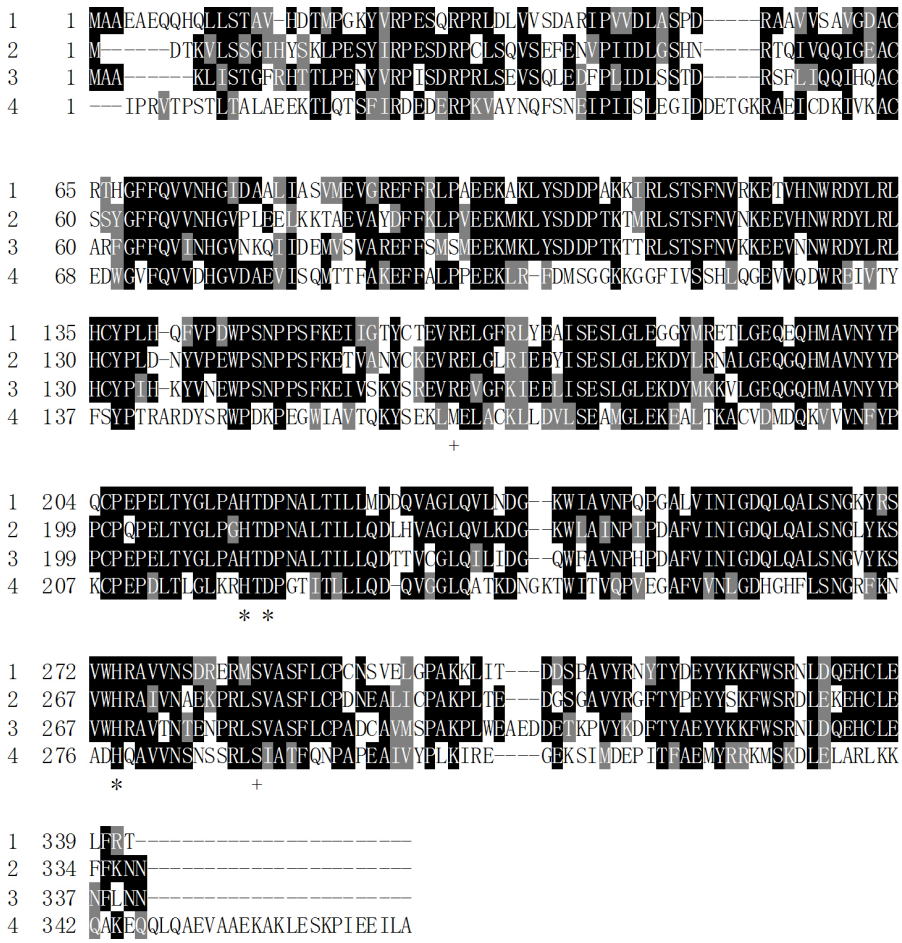

Fig. 1. Alignment of the flavone synthases from several plants. The sequences listed here correspond to 1, OsFNS I-1 (GenBank Number, 375364-43); 2, *Medicago truncatula* 2-oxoglutarate-Fe(II) oxygenase (GenBank Number, 92878635); 3. *Arabidopsis thaliana* oxidoreductase (GenBank Number, 152-38567); 4, Petunia 2-oxoglutarate 3-dioxygenase (GenBank Number. 1345-562). Note that Fe^{2+} binding sites are indicated as * and oxoglutarate binding sites are indicated as + under amino acids.

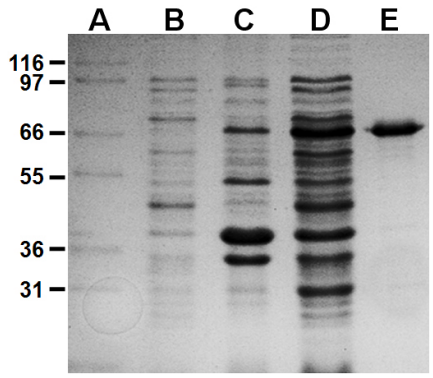

Fig. 2. Expression and purification of the recombinant OsFNS I-1. A, Molecular weight size markers; B, *E. coli* lysate before induction; C, *E coli* lysate after induction; D, Soluble protein after sonication, E, purified recombinant OsFNS I-1.

(Fig. 3). However, the reaction with (*2R*)-naringenin produces apigenin about 13% compared to (*2S*)-naringenin (data not shown). This difference is likely to be due to the structural differences between the two enantiomers. It is generally known that flavone synthases are chirally selective toward (*2S*)-naringenin (8) and there have been no reports of FNS I utilizing (*2R*)-naringenin. FNS I from parsley has been shown to use (*2S*)-naringenin as a substrate (10). It has not been tested whether this enzyme actually used (*2R*)-naringenin because (*2S*)-naringenin is synthesized naturally through the reactions of chalcone synthase and chalcone isomerase using 4-coumaroyl-CoA and malonyl-CoA. Taxifolin was also tested as a substrate of OsFNS I-1 but it was not metabolized (data not shown). By Lineweaver-Burk analysis, the K_m and of (*2S*)-naringenin was determined to be 29 μM and the V_{max} 43 nkat/mg.

In order to confirm the structure of the OsFNS I-1 reaction product with (*2S*)-naringenin, a fraction of the eluent (about 1 mg) corresponding to the retention time of the new peak was collected and its structure was determined using nuclear magnetic resonance (NMR) spectroscopy. The NMR data of authentic apigenin and those of the reaction product were compared. As a result, they were agreed to each other. The NMR data are as follows: ^{1}H NMR (DMSO-d_6) δ 6.20 (1H, *d*, *J* = 2.0, H-6),

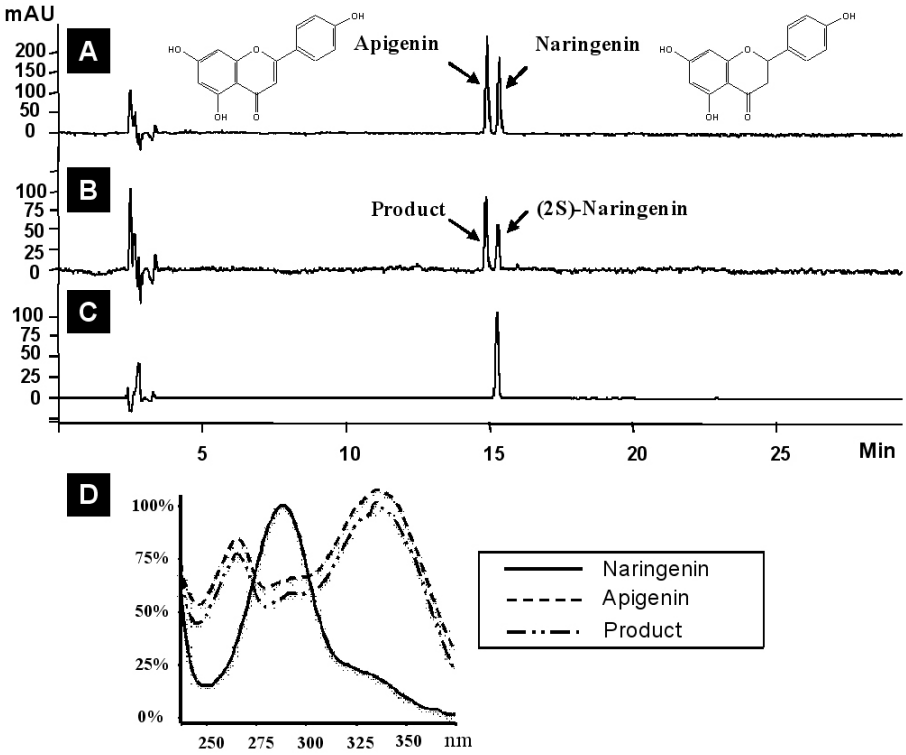

Fig. 3. HPLC analysis of naringenin reaction products with OsFNS I-1. A, authentic apigenin and naringenin; B, reaction product of (2*S*)-naringenin with OsFNS I-1; C, reaction product of (2*S*)-naringenin with glutathione *S*-transferase; D, UV-spectra of authentic compounds (naringenin, apigenin, and reaction product).

6.44 (1H, *d*, *J* = 2.0, H-8), 6.68 (1H, *s*, H-3), 6.90 (2H, *d*, *J* = 9.0, H-3'/H-5'), 7.92 (2H, *d*, *J* = 9.0, H-2'/H-6'). Therefore, the reaction product of naringenin was determined to be apigenin.

Next, we examined whether OsFNS I-1 was Fe^{2+} and oxoglutarate-dependent. Various combinations of ascorbate, $FeSO_4$, catalase, and oxoglutarate were added to reaction mixtures containing the purified OsFNS I-1 and (2*S*)-naringenin. When $FeSO_4$ was absent from the reaction mixture, no product was detected. In fact, the OsFNS I-1 activity increased with increasing concentrations of $FeSO_4$ up to 50 μM and decreased to half of its maximum activity as the concentration of $FeSO_4$ reached 200 μM. These results demonstrate that OsFNS I-1 is Fe^{2+} dependent and the optimum concentration of Fe^{2+} for OsFNS I-1 activity is 50 μM. We also found that ascorbate is also a key cofactor. Like Fe^{2+}, no reaction product was generated in the absence of ascorbate. As the concentration of ascorbate was increased from 500 μM to 2 mM, the OsFNS I-1 activity also increased. However, for concentrations of ascorbate higher than 1 mM, the production of apigenin was minimal. Consequently, an optimum concentration of ascorbate was determined to be 1 mM. OsFNS I-1 also was inactive in the absence of 2-oxoglutarate. Optimum concentration of 2-oxoglutarate for the activity of OsFNS I-1 was determined to be 160 μM. Based on the identity of the reaction product and the dependence on Fe^{2+} and oxoglutarate, we concluded that *OsFNS I-1* encodes a flavone synthase I. This is the first type I FNS found outside of the *Apiaceae* family.

Up to now, no Fe^{2+}- and 2-oxoglutarate-dependent dioxygenases have been characterized in rice. The identification of OsFNS I-1 will help our understanding of the flavonoid biosynthesis pathway in rice.

MATERIALS AND METHODS

Cloning and expression of *OsFNS I-1*

cDNA was synthesized with Omniscript reverse transcriptase (Quiagen, Hilden, Germany) using total RNA from two week-old rice seedling under the manufacture's instruction. In order to clone the *OsFNS I-1*, polymerase chain reaction (PCR) was carried out using the above cDNA as template. The primer sequences 5'-TGTCGTCCTCTTCTCCTCC-3' (forward) and 5'-TAGAGTGAGCTTGCGATGAG-3' (reverse) were designed based on the *OsFNS I-1* sequence (TIGR rice gene index accession number TC301317).

The full length *OsFNS I-1* cDNA was amplified and cloned into the *E. coli* expression vector pGEX 5X-3 which contains a glutathione *S*-transferase as a tag. The resulting fusion protein was induced with IPTG (isopropyl-β-D-thiogalactoside) in *E. coli* as described (11). The recombinant *OsFNS I-1* fusion protein was purified with a glutathione *S*-transferase affinity column (Amersham Biotech, USA) according to the manufacturer's instruction.

Enzyme assay and reaction product analysis

Purified OsFNS I-1 was incubated with 1 mM ascorbate, 50 μM $FeSO_4$, 100 mg/ml catalase, 160 μM oxoglutarate, and 60 μM of *S* or *R*-naringenin in 500 μl of 10 μM Tris/HCl buffer (pH 8.0) for 3 hr at 37°C. The reaction product was analyzed using HPLC as described by (11).

Enatiomers of naringenin were separated using high performance liquid chromatography (HPLC, Varian, USA) equipped with a chiral column (Chiralpack® AD-RH column, 150 × 4.6 mm, Daicel Inc., Exton, PA, USA) as described by (12). The structure of reaction product was determined using nuclear magnetic resonance (NMR) spectroscopy. The reaction product was dissolved in dimethylsulfoxide-d6 (DMSO-d_6) and transferred into a 5 mm NMR tube for ^{1}H NMR analysis.

Effects of various cofactors such as ascorbate, $FeSO_4$, catalase, and oxoglutarate were investigated with reaction mixtures containing 40 μg of the purified OsFNS I-1 and 60 μM of (2*S*)-naringenin.

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