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Preliminary Communication

New Scheme of the Biosynthesis of Formononetin Involving 2,7,4′-Trihydroxyisoflavanone but Not Daidzein as the Methyl Acceptor

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***Glycyrrhiza echinata* cell-free extract produced isoformononetin by the 7-*O*-transmethylation of daidzein from *S*-adenosyl-L-methionine (SAM). When the yeast microsome expressing 2-hydroxyisoflavanone synthase was mixed with the cell-free extract and incubated with liquiritigenin and SAM, formononetin emerged. Furthermore, the cell-free extract yielded formononetin on incubation with 2,7,4′-trihydroxyisoflavanone and SAM. We propose a novel pathway of formononetin biosynthesis involving 2,7,4′-trihydroxyisoflavanone as the methyl acceptor.**

Key words: *Glycyrrhiza echinata*; Fabaceae; cytochrome P450; flavonoid; *O*-methyltransferase

Formononetin is a common *O*-methylated isoflavone of leguminous plants that is an essential intermediate in the biosynthesis of isoflavonoid phytoalexins (*e.g.* medicarpin, vestitol, and maackiain) and rotenoids (Fig. 1).[1,2] Formononetin is schematically biosynthesized by the 4′-*O*-methylation of daidzein, a simple isoflavone found in soybean. However, earlier feeding experiments with red clover have shown that both isoliquiritigenin and formononetin but neither 2′,4′-dihydroxy-4-methoxychalcone nor daidzein are involved in the biosynthesis of medicarpin and maackiain, implying that 4′-*O*-methylation of daidzein is an unlike process in this plant.[3] Although an *O*-methyltransferase (OMT) toward isoflavones in chickpea cell cultures has been reported,[4] the reaction product was later shown to be the 7-*O*-methylated isoflavone.[5] In alfalfa, too, 7-*O*-methylation of isoflavones instead of 4′-*O*-methylation has been reported,[6] and furthermore, isoflavone 7-OMT has been purified[7] and its cDNA has been cloned.[8] Importantly, the 7-*O*-methylated products, isoformononetin (7-*O*-methyldaidzein) and 7-*O*-methylgenistein, are not major constituents *in vivo*, while 4′-*O*-methylation of the isoflavone skeleton should take place during phytoalexin production in these plants. Thus, reexamination of this methylation step has been strongly warranted.[9]

The isoflavone skeleton is biosynthesized by a microsomal cytochrome P450 (P450) 2-hydroxyisoflavanone synthase (IFS) and subsequent dehydration.[10–12] Recently, alfalfa 7-OMT was proposed to be associated with IFS and another P450, isoflavone 2′-hydroxylase, to form a metabolic "compartment" or "channel" where it actually methylates the 4′-hydroxy of daidzein,[1,8] but no experimental proof to this hypothesis has been presented. On the other hand, the ionic mechanism of isoflavone biosynthesis has been hypothesized to have a spirodienone-type intermediate, which is methylated at the 4′-carbonyl to give 2,7-dihydroxy-4′-methoxyisoflavanone and subsequently dehydrated to give formononetin (Fig. 1).[13]

Until very recently, however, IFS protein has not been identified in spite of very strong interest in its reaction mechanism, and the activity has only been assayed in plant microsomes; thus the detailed analysis of the OMT in connection with the formation of the isoflavone skeleton has been impossible. In 1999, a P450 cDNA (CYP93C2) cloned from *Glycyrrhiza echinata* L. (Fabaceae) was shown to encode IFS.[14] IFS cDNAs have also been cloned from soybean[15,16] and other legumes including *Lotus japonicus*.[17] Because cultured *G. echinata* cells produce a large quantity of formononetin,[18] we examined formononetin biosynthesis using a combined system of heterologously expressed IFS and *G. echinata* cell-free extract. We propose here a novel pathway of formononetin biosynthesis that involves 2,7,4′-trihydroxyisoflavanone but not daidzein as the methyl acceptor. The new scheme resolves the experimental conflicts so far encountered, and, although preliminary in nature, provides information about the mechanism of isoflavone skeleton formation and methylation.

First, transmethylation of daidzein in *G. echinata* cell-free extract was examined. Elicitor (yeast extract)-treated (12 h) *G. echinata* AK-1 cells[18] were homogenized with 0.1 M potassium phosphate buffer

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Abbreviations: IFS, 2-hydroxyisoflavanone synthase; OMT, *O*-methyltransferase; P450, cytochrome P450; SAM, *S*-adenosyl-L-methionine

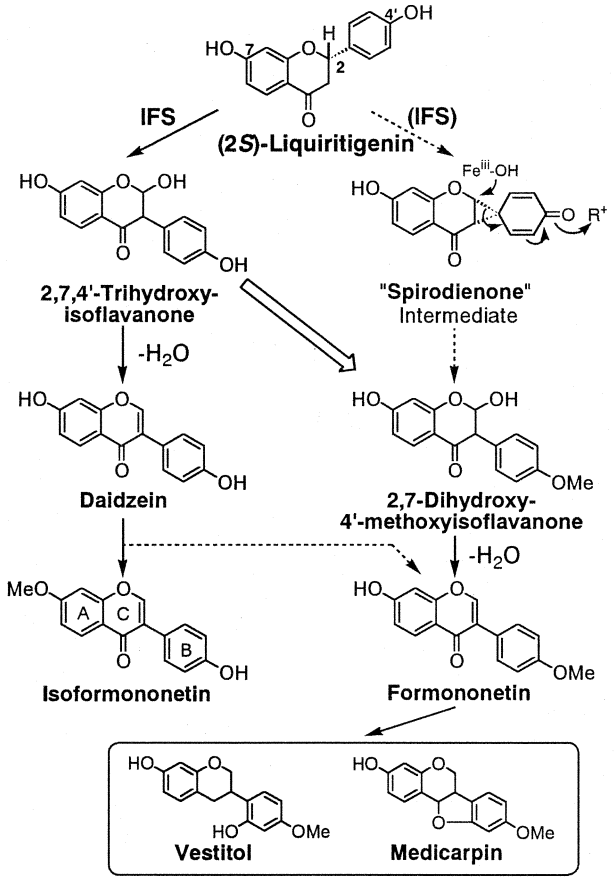

Fig. 1. Biosynthesis of Isoflavones and Isoflavonoid Phytoalexins in Leguminous Cells.

The open arrow indicates the new pathway described in this study, and the dashed arrows indicate the pathways which are unlikely to be present in the cells.

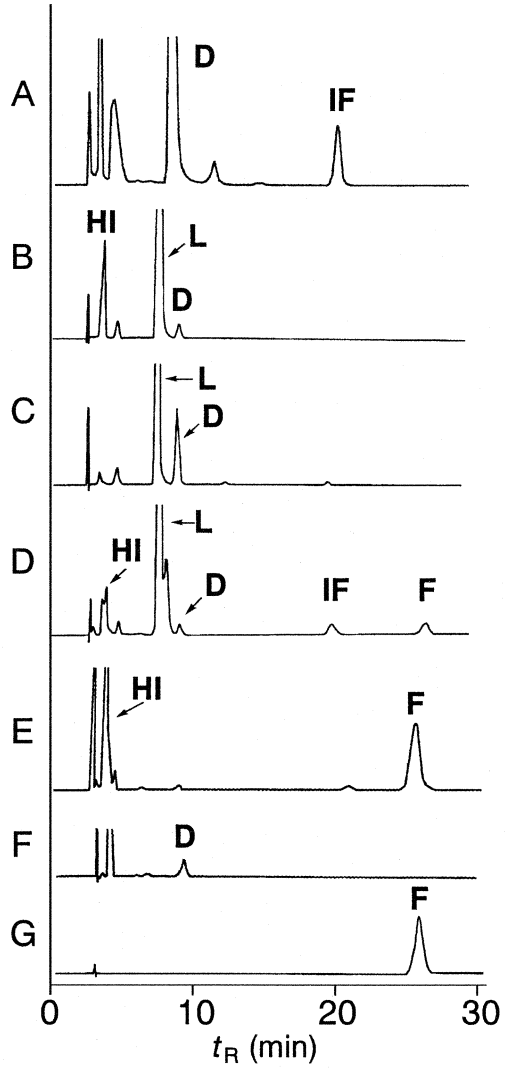

Fig. 2. HPLC Chromatograms of the Products from the Reactions with *G. echinata* Cell-free Extracts (15,000×*g* supernatant) or with the Mixture of Microsomes of the Recombinant Yeast Harboring CYP93C2 cDNA and *G. echinata* $(NH_4)_2SO_4$ Precipitates.

Column, Shim-pack CLC-ODS (6.0×150 mm, Shimadzu); solvent, 50% methanol in H_2O; flow rate, 1 ml/min at 40°C. HPLC was monitored at 248 nm (A, B, C, E, F, G) or 285 nm (D). The ordinate scales of the HPLC charts are equal. A, daidzein and SAM reacted with 15,000×*g* supernatant; B, liquiritigenin reacted with the recombinant yeast microsome; C, products of acid treatment of materials in B; D, liquiritigenin, SAM and NADPH reacted with the mixture of the yeast microsome and the $(NH_4)_2SO_4$ precipitates; E, 2,7,4′-trihydroxyisoflavanone and SAM reacted with 15,000×*g* supernatant; F, 2,7,4′-trihydroxyisoflavanone and SAM without 15,000×*g* supernatant; G, standard formononetin. Abbreviations: D, daidzein, F, formononetin, HI, 2,7,4′-trihydroxyisoflavanone; IF, isoformononetin; L, liquiritigenin.

(pH 7.5) containing 10% (w/v) sucrose and 14 mM 2-mercaptoethanol. The 15,000×*g* supernatant of the homogenate after treatment with Dowex 1-x2 (equal volume of the cells) was used as the cell-free extract (about 0.2 mg protein/ml). Daidzein (39 nmol) and SAM (230 nmol) were incubated with 0.7 ml of the extract at 30°C for 2 h, and the ethyl acetate layer of the mixture was analyzed by reversed phase HPLC. As shown in Fig. 2(A), a major product appeared at t_R 21.5 min, but it was not formononetin (t_R 26 min, see Fig. 2(G)). The transmethylation product was identified as isoformononetin by the electron impact mass spectrum [M^+ *m/z* 268 ($C_{16}H_{12}O_4$) and fragment ions of *m/z* 151 ($C_8H_7O_3$; corresponding to the methylated A-ring) and 118 (C_8H_6O; corresponding to a C-ring without methyl) resulting from retro-Diels-Alder fragmentation]. Therefore, the *in vitro* methyltransferase activity of *G. echinata* toward daidzein is identical to that of alfalfa and chickpea.[5,6] The daidzein 7-OMT activity was not detected in control cells, and on elicitation, it increased to the maximum at 24 h (Fig. 3(A)).

The effect of combined incubation of IFS protein and *G. echinata* extract was then examined. The microsome of the recombinant yeast expressing CYP93C2 reacted with liquiritigenin yielded 2,7,4′-trihydroxyisoflavanone together with a minor quantity of daidzein (Fig. 2(B)).[14] The product is easily dehydrated to give daidzein on acid treatment

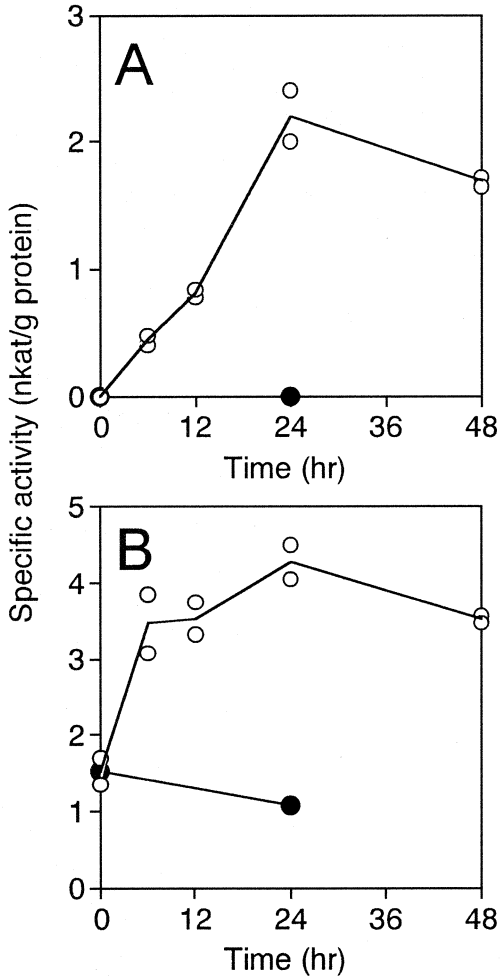

Fig. 3. Courses of Daidzein 7-OMT Activity (A) and the Formononetin Production from 2,7,4′-Trihydroxyisoflavanone and SAM (B) in Cultured *G. echinata* Cells Treated with Yeast Extract (0.1% of medium).

The data were collected from two independent measurements. Open and closed circles represent the activity in elicited and control cells, respectively.

(Fig. 2(C)). It is assumed that in plant cells a dehydratase substitutes for acid in isoflavone biosynthesis.[12] For the combined assay, 0.7 ml of the $(NH_4)_2SO_4$ fraction (30 to 80% saturation) of the *G. echinata* cell-free extract (about 0.05 mg protein/ml; after Sephadex G-25 column) was mixed with the same volume of the suspension of the recombinant yeast microsome (about 1 mg protein/ml),[14] and incubated with (*RS*)-liquiritigenin (39 nmol), SAM (230 nmol), and 1 mM NADPH. The products were analyzed with HPLC as above. As shown in Fig. 2(D), a peak of formononetin appeared in addition to the peaks of isoformononetin, daidzein and the IFS reaction product, 2,7,4′-trihydroxyisoflavanone. The structure of formononetin was confirmed by electron impact mass spectrum [M^+ m/z 268 and a fragment ion m/z 132 (C_9H_8O; corresponding to the methylated C-ring); identical to the spectrum of a standard sample] of the product recovered from HPLC. Without the addition of the plant cell-free extract, no methylated products were formed (data not shown); thus the methyltransferase(s) of *G. echinata* must be responsible for the production of both formononetin and isoformononetin. The formation of isoformononetin in Fig. 2(D) can be the result of 7-*O*-methylation of daidzein, which is the product of spontaneous dehydration (as in Fig. 2(B)) rather than a dehydratase reaction. Judging from the results of Figs. 2(A) and 2(D), formononetin formation must be independent of daidzein production.

Finally, the reaction of *G. echinata* cell-free extract with 2,7,4′-trihydroxyisoflavanone was examined. 2,7,4′-Trihydroxyisoflavanone was prepared from liquiritigenin using the recombinant yeast microsome expressing IFS.[14] *G. echinata* cell-free extract and SAM were then reacted with 2,7,4′-trihydroxyisoflavanone in the same manner as daidzein incubation. As shown in Fig. 2(E), a significant amount of formononetin was formed. Because formononetin was never detected in *G. echinata* cell-free extract (see Fig. 2(A)), the substrate should have been converted into formononetin. It is also noted that only a negligible amount of daidzein was produced while the substrate remained substantial (Fig. 2(E)). Without *G. echinata* cell-free extract, no reaction product was observed except for a minute amount of daidzein formed spontaneously (Fig. 2(F)).

The only reasonable explanation for these results is that a methyltransferase in *G. echinata* extract catalyzes 4′-*O*-methylation of 2,7,4′-trihydroxyisoflavanone. The product, 2,7-dihydroxy-4′-methoxyisoflavanone, would be readily dehydrated to give formononetin by a dehydratase also contained in the extract that does not use 2,7,4′-trihydroxyisoflavanone as the substrate (Fig. 1). The production of formononetin from 2,7,4′-trihydroxyisoflavanone and SAM in the *G. echinata* cells changed on elicitation with yeast extract (Fig. 3(B)). In contrast to the daidzein 7-OMT activity (Fig. 3(A)), the formononetin-forming activity in control cells (time 0) was rather high, and elicited cells showed about a three-fold increase of the activity in 24 h.

To further clarify the reaction courses, the purification or cDNA cloning and the characterization of the methyltransferase and dehydratase must be accomplished. Whether the enzyme showing daidzein 7-OMT *in vitro* is actually the 2-hydroxyisoflavanone 4′-OMT and a channeling mechanism is operating in this transmethylation reaction[1] is of particular interest. However, the *in vitro* methylation of 2,7,4′-trihydroxyisoflavanone demonstrated in this study makes the hypothetical "spirodienone" intermediate[13] in formononetin biosynthesis unnecessary.

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