Molecular and Biochemical Characterization of 2-Hydroxyisoflavanone Dehydratase. Involvement of Carboxylesterase-Like Proteins in Leguminous Isoflavone Biosynthesis\(^1\)\(^\text{[w]}\)

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Isoflavonoids are ecologically active secondary metabolites of the Leguminosae and known for health-promoting phytoestrogenic functions. Isoflavones are synthesized by 1,2-elimination of water from 2-hydroxyisoflavonones, the first intermediate with the isoflavonoid skeleton, but details of this dehydration have been unclear. We screened the extracts of repeatedly fractionated *Escherichia coli* expressing a *Glycyrrhiza echinata* cDNA library for the activity to convert a radiolabeled precursor into formononetin (7-hydroxy-4'-methoxyisoflavone), and a clone of 2-hydroxyisoflavone dehydratase (HID) was isolated. Another HID cDNA was cloned from soybean (*Glycine max*), based on the sequence information in its expressed sequence tag library. Kinetic studies revealed that *G. echinata* HID is specific to 2,7-dihydroxy-4'-methoxyisoflavonone, while soybean HID has broader specificity to both 4'-hydroxylated and 4'-methoxylated 2-hydroxyisoflavonones, reflecting the structures of isoflavonoids contained in each plant species. Strikingly, HID proteins were members of a large carboxylesterase family, of which plant proteins form a monophyletic group and some are assigned defensive functions with no intrinsic catalytic activities identified. Site-directed mutagenesis with soybean HID protein suggested that the characteristic oxyanion hole and catalytic triad are essential for the dehydratase as well as the faint esterase activities. The findings, to our knowledge, represent a new example of recruitment of enzymes of primary metabolism during the molecular evolution of plant secondary metabolism.

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2-hydroxyisoflavanone, via spontaneous dehydration (Akashi et al., 1999; Jung et al., 2000; Shimada et al., 2000). Also, the enzyme expressed in insect cells was reported to yield only isoflavone due to the presence of nonspecific dehydratases (Steele et al., 1999). On the other hand, a biochemical study of the 2-hydroxyisoflavanone dehydratase (HID) of *Pueraria lobata* has been reported (Hakamatsu et al., 1998). The substrate, 2,7,4'-trihydroxyisoflavanone, was prepared using microsomes of elicited *P. lobata* cells, and the production of daidzein was measured during the enzyme purification steps. The *P. lobata* HID protein was purified to apparent homogeneity, but, unfortunately, no information on the amino acid sequence was provided. More recently, we observed that a cell-free extract of *G. echinata* converted 2,7-dihydroxy-4'-methoxyisoflavanone into formononetin, but did not convert 2,7,4'-trihydroxyisoflavanone into daidzein (Akashi et al., 2000). This result indicated that *G. echinata* HID has a clear substrate specificity and distinguishes differently substituted 2-hydroxyisoflavonones.

The combination of in vitro reactions with microsomes of yeast expressing IFS (Akashi et al., 1999) as well as purified recombinant HI4'OMT protein (Akashi et al., 2003) allowed us to prepare a sufficient quantity of 2-hydroxyisoflavanone and its radiolabeled tracer. In this study, we examined HID activities in *G. echinata* and soybean for the presence of species-specific enzymes. To isolate the cDNA encoding HID, a cDNA library of elicited *G. echinata* cell cultures expressed in *Escherichia coli* was screened, assaying the activity in fractionated bacterial extracts. The *G. echinata* HID sequence was further used for the isolation of soybean HID cDNA. Surprisingly, the amino acid sequences of both HIDs possess the motifs characteristic of carboxylesterases, to which dehydratase activities have never been attributed. Analogous proteins, including human carboxylesterases, are widely distributed in living organisms, and plant carboxylesterase-like proteins form a monophyletic group. Furthermore, some plant proteins of this family are known for defensive functions with no intrinsic catalytic activities identified. Possibly, some of the dehydration in natural product biosynthesis is mediated by this group of enzymes. The implication of this finding to the molecular evolution of plant secondary metabolism is discussed.

**RESULTS**

**HID Activities in *G. echinata* and Soybean**

*G. echinata* cells constitutively accumulate formononetin and rapidly produce medicarpin on elicitor treatment (Fig. 1; Nakamura et al., 1999). Both products are 4'-methoxyisoflavonoids. Soybean mainly produces glycoconjugates of 4'-hydroxyisoflavones, such as daidzein, genistein, and glycitein (7,4'-dihydroxy-6-methoxyisoflavone), but minor quantities of formononetin and its derivatives also accumulate in soybean seeds (Dewick, 1993).

The crude enzyme preparation of elicited *G. echinata* cells efficiently converted 2,7-dihydroxy-4'-methoxyisoflavanone to formononetin (Table I). Only very low activity for the production of daidzein from 2,7,4'-trihydroxyisoflavanone and genistein from 2,5,7,4'-tetrahydroxyisoflavanone (0.7% and 3% of the formononetin production, respectively) with the same extract was detected. In contrast, the enzyme preparation of soybean seedlings was highly active to 2,5,7,4'-tetrahydroxyisoflavanone and yielded genistein, while it also showed activity toward 2,7,4'-trihydroxyisoflavanone, producing daidzein and formononetin (34% and 18% of genistein production, respectively; Table I). The production of isoflavones by spontaneous dehydration of 2-hydroxyisoflavonanes was negligible during 5-min incubation at the substrate concentration of 100 μM in the neutral buffer, pH 7.5. Thus, the enzyme-dependent dehydration of 2-hydroxyisoflavonanes to form isoflavones was clearly demonstrated, and the substrate specificities of HIDs of *G. echinata* and soybean were shown to be different.

![Figure 1. Isoflavonoid biosynthesis in leguminous plants. 4'-Hydroxylated isoflavones are biosynthesized from (2S)-flavanones by the reaction catalyzed by IFS and subsequent intramolecular 1,2-dehydration of 2-hydroxyisoflavonanes. 4'-Methoxylated isoflavonanes are produced from the IFS reaction products in 2 steps: 4'-O-methylation catalyzed by HI4'OMT and subsequent dehydration. In this study, HIDs of distinct substrate specificity toward 4'-hydroxylated and 4'-methoxylated 2-hydroxyisoflavonanes were characterized.](https://www.plantphysiol.org/)

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Cloning of *G. echinata* HID cDNA by Screening of the Fractionated Expression Library

A specific and highly sensitive assay of dehydratase activity yielding formononetin was achieved using [methyl-\(^{14}\)C]-2,7-dihydroxy-4'-methoxyisoflavonone that was prepared from 2,7,4'-trihydroxyisoflavanone, the product of the in vitro IFS reaction (Akashi et al., 1999), and [methyl-\(^{14}\)C]-S-adenosyl-L-Met with the recombinant HI4 OMT protein (Akashi et al., 2003). The first screening of the cDNA expression library of *G. echinata* (Akashi et al., 2003) was carried out with five cDNA pools (about 30,000 transformants/pool). The crude extracts of the pools were reacted with [methyl-\(^{14}\)C]-2,7-dihydroxy-4'-methoxyisoflavonone, and reaction mixtures were analyzed by thin-layer chromatography-autoradiography. Two pools were found to produce [\(^{14}\)C]formononetin, and one of the arbitrarily selected positive pools was subdivided into 10 pools of smaller size (about 3,000 clones/pool).

Expression of enzyme proteins and the assay were performed again, and the one resultant positive pool was subjected to the next round of subdivision. One positive pool among the 10 pools was repeatedly identified in the successive third (about 300 clones/pool) and fourth (30 clones/pool) screenings. A single *E. coli* clone showing 2,7-dihydroxy-4’-methoxyisoflavonone dehydratase activity was then isolated from the fourth pool.

The cDNA, designated *HIDM* (HID methoxy type; accession no. AB154414), had 1,178-bp nucleotides and encoded 328 amino acids (Fig. 2A). A conserved domain search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) suggested that *HIDM* is structurally related to the \(\alpha/\beta\)-hydrolase fold family protein (Ollis et al., 1992) and has a conserved sequence motif recorded for carboxylesterase (about 40–180 amino acids from the N terminus). A conserved sequence (His-85-Gly-86-Gly-87) constituting an oxyanion hole within the carboxylesterase motif (Satoh and Hosokawa, 1995), and a triad of amino acid residues (Thr-173, Asp-272, and His-304) that acts as the catalyst for ester hydrolysis outside the carboxylesterase motif, were also present, although a more general Ser residue in the catalytic triad of lipases and esterases (Nardini and Dijkstra, 1999; Manco et al., 2000) is substituted to Thr.

Cloning of a Soybean HID cDNA

Searches of expressed sequence tag (EST) databases of soybean, *Medicago truncatula*, and *L. japonicus*, leguminous plants known to produce isoflavones, revealed the presence of cDNAs homologous to *G. echinata* HIDM (>50% amino acid identity) in these plants. All these sequences were, however, only annotated as putative proteins. Phylogenetic analysis (Fig. 2B) indicated that soybean BM177194, *L. japonicus* AV425769, and *M. truncatula* TC43540 proteins form the same branch with *G. echinata* *HIDM* (>80% identity at the amino acid level). The soybean TC98460 protein had >60% identity with the 4 proteins and formed the adjacent branch with *M. truncatula* BG456496. The TC98460 sequence had the predicted initiation and stop codons. The cDNA of the coding region was cloned from soybean seedlings by reverse transcription (RT)-PCR and named *HIDH* (HID hydroxy type; accession no. AB154415). It showed 61% identity with *HIDM* at the amino acid level, and the characteristic carboxylesterase motifs were also conserved. On the other hand, the sequence of BM177194 contained only 5’-end 437 bps, and neither the fragment nor the full cDNA were amplified by RT-PCR and 3’-RACE, respectively, from the same soybean seedlings. Thus, the gene corresponding to BM177194 may not be expressed in the seedlings used in this study.

Biochemical Characterization of HIDs of *G. echinata* and Soybean

HIDM and HIDH proteins were expressed in *E. coli*, and purified recombinant proteins with six His residues at the N terminus were assayed for substrate specificities using HPLC for the product analysis. The chemical structures of the isoflavones were confirmed by comparison of the retention time values with those...
of authentic samples and electron ionization mass spectrometry (data not shown). Specific activities at the substrate concentration of 100 μM are listed in Table I. A high activity of formononetin production from 2,7-dihydroxy-4'-methoxyisoflavanone by *G. echinata* HID (HIDM protein) was observed. HIDM protein yielded only very small amounts of daidzein and genistein from respective substrates: The specific activity toward both 2,7,4'-trihydroxyisoflavonanone and 2,5,7,4'-tetrahydroxyisoflavonanone was 1/170th of that toward 2,7-dihydroxy-4'-methoxyisoflavonanone. Thus, the substrate preference of HIDM protein coincided with that of the *G. echinata* cell-free extract. On the other hand, recombinant HID of soybean (HIDH protein) had the highest activity toward 2,5,7,4'-tetrahydroxyisoflavonanone (relative activity, 100%) and also showed significant activity toward 2,7,4'-trihydroxyisoflavonanone (40%) and 2,7-dihydroxy-4'-methoxyisoflavonanone (16%). Thus, again, the substrate preference of HIDH protein coincided with that of soybean extract.

Recombinant *G. echinata* HIDM and soybean HIDH proteins hydrolyzed p-nitrophenyl butyrate in the standard assay of carboxylesterases (Heymann and Mentlein, 1981), but the magnitude of the activity was only 0.2% (HIDM) or 0.7% (HIDH) of the respective activity to the most preferred substrate (Table I). On the other hand, commercially available porcine liver carboxylesterase did not act on 2-hydroxyisoflavonanones at all (Table I).

To further characterize the enzyme properties, steady-state kinetic parameters for the recombinant HIDs were determined (Table II). HIDM and HIDH proteins showed comparable *Kₘ* values to the three substrates, with the HIDM *Kₘ*s displaying about one-half the levels of the HIDH *Kₘ*s. Interestingly, the relative ratios of *Kₘ* values for the three substrates displayed by HIDM and HIDH proteins were roughly the same (about 1:4:6 for both *Kₘ*s toward 2,7-dihydroxy-4'-methoxyisoflavonanone, 2,7,4'-trihydroxyisoflavonanone, and 2,5,7,4'-tetrahydroxyisoflavonanone, respectively), indicating that the affinities of the two enzymes to these substrates are conserved. However, these proteins exhibited quite different turnover numbers (*k₅₇₆* values) for differently substituted substrates. Thus, *G. echinata* HIDM protein had a high *k₅₇₆* for 2,7-dihydroxy-4'-methoxyisoflavonanone, but only 1% to

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th><em>G. echinata</em> HIDM</th>
<th>Soybean HIDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,7-Dihydroxy-4'-methoxyisoflavonanone</td>
<td>Formononetin</td>
<td>58 9.8 170</td>
<td>29 1.6 46</td>
</tr>
<tr>
<td>2,7,4'-Trihydroxyisoflavonanone</td>
<td>Daidzein</td>
<td>210 0.12 0.56</td>
<td>114 5.3 47</td>
</tr>
<tr>
<td>2,5,7,4'-Tetrahydroxyisoflavonanone</td>
<td>Genistein</td>
<td>304 0.19 0.62</td>
<td>170 18.1 106</td>
</tr>
</tbody>
</table>

Table II. Kinetic properties of recombinant HIDH and HIDM
The values are the averages from two independent experiments (maximum deviation was about 10%).
2% $k_{cat}$ levels for 4'-hydroxylated substrates. In contrast, soybean HIDH protein displayed the highest $k_{cat}$ for 2,5,7,4'-tetrahydroxyisoflavanone and about 30% and 10% $k_{cat}$ levels for 2,7,4'-trihydroxyisoflavanone and 2,7-dihydroxy-4'-methoxyisoflavanone, respectively. These differences in $k_{cat}$ values mainly caused different specificity constants ($k_{cat}/K_m$ ratios) of the two enzymes to the three substrates. Consequently, HIDM protein possessed a narrower substrate specificity, 2,7-dihydroxy-4'-methoxyisoflavanone being the most favored substrate, while HIDH protein exhibited broader substrate specificity toward both 4'-hydroxy- and 4'-methoxy-2-hydroxyisoflavanones.

The spontaneous dehydration of 2,7,4'-trihydroxyisoflavanone yielding daidzein in the neutral buffer, pH 7.5 at 30°C was shown to be a first-order reaction. The velocity is dependent on the substrate concentration, and the calculated rate constant was 3.1 $\times$ 10^{-5} s^{-1}, a much smaller value than the $k_{cat}$ (5.3 s^{-1}) displayed by the HIDH protein toward the same substrate. The half-life period of 2,7,4'-trihydroxyisoflavanone under this condition was 6.3 h.

**Site-Directed Mutagenesis of Soybean HID**

During ester hydrolysis by carboxylesterases, the amino acid residues of an oxyanion hole stabilize the negatively charged intermediate, and the nucleophilic hydroxyl of the Ser residue in the catalytic triad acts as the catalyst. To examine whether these residues also play catalytic roles in the HID reaction, mutations were introduced into the catalytic triad (Thr-164, Asp-263, and His-295) and oxyanion hole (Gly-78 and Gly-79) of soybean HIDH protein, and the purified recombinant proteins with six His residues were assayed for the activity. The mutants (D263N and H295A), in which a residue of the triad was replaced by Ser (a more general amino acid of the triad in hydrolases) and Ala (without hydroxyl), both HID and carboxylesterase activity were reduced to 3% to 4% and 1% of the wild type, respectively. Mutation in the oxyanion hole (G78A and G79A) also reduced both activities to about 10% of the wild type. Because the relative ratios of the reduction of HID and carboxylesterase activity with mutant proteins were almost identical (Table III), it was strongly suggested that these amino acid residues function in a similar manner during the catalytic process of both dehydration and ester hydrolysis.

**Phylogenetic Analysis of HIDs and the Carboxylesterase Gene Family**

The protein-protein BLAST search revealed that HID proteins had 30% to 40% identity with Nicotiana tabacum hsr203j (accession no. CAA54393; Pontier et al., 1994), Pisum sativum E86 (accession no. BAA85654; Ichinose et al., 2001), and some Arabidopsis (Arabidopsis thaliana) putative proteins. The genes of all these proteins are categorized into the plant carboxylesterase gene family, which was recently reported to consist of 7 clades by phylogenetic analysis of 20 Arabidopsis genes (representing 6 clades) and other plant carboxylesterase-like genes (Marshall et al., 2003). We extended the analysis of HID-like carboxylesterase proteins into those from evolutionarily more divergent organisms, including microorganisms and human (Homo sapiens). The phylogenetic tree comprising all the genes examined (102 sequences) is complicated (see Supplemental Figure 1), and a simplified scheme is shown in Figure 3. The whole carboxylesterase family contained monophyletic plant clades and five other clades (tentatively named clades A–E) to which genes of microbial and human origins belong. Except for clade C, consisting of Aspergillus nidulans genes, non-plant clades contain genes from evolutionarily separated species: For example, clade D includes genes of eubacteria (Mesorhizobium loti and Pseudomonas syringae), fungi (A. nidulans and Schizosaccharomyces pombe), and human. The phylogenetic relationships among plant carboxylesterases are essentially in agreement with the reported classification (Marshall et al., 2003), and eight clades are identified. The genes of Arabidopsis and rice (Oryza sativa), two species for which genome information is available, as well as those in ESTs of leguminous plants (M. truncatula and soybean), are scattered into diverse clades. HIDs are located in a small group of proteins from leguminous plants in clade III, which also contains Arabidopsis, rice, and potato (Solanum tuberosum) genes.

**DISCUSSION**

In this study, two cDNAs encoding HIDM of G. echinata and HIDH of soybean were cloned. HIDM and HIDH display different substrate specificities to 2-hydroxyisoflavonones. The substrate preferences re-
both the recombinant protein and the crude extract displayed a very high preference for 2,7-dihydroxy-4'-methoxyisoflavonane. Therefore, the activity in the crude extract should be attributed to the HIDM protein. Also, a preliminary RT-PCR analysis with *G. echinata* cells revealed a constitutive as well as an increased accumulation of *HIDM* mRNA upon yeast extract treatment in a coordinated manner with *IFS* and *HH4* OMT mRNAs (Supplemental Fig. 2), coinciding with the constitutive and elicitor-induced production of the isoflavonoids (Nakamura et al., 1999). The HIDM-type enzyme should also be involved in the biosynthesis of biochanin A (5,7-dihydroxy-4'-methoxyisoflavone) in legumes such as chickpea (Barz and Welle, 1992), because, in a preliminary experiment, HIDM did actually dehydrate 2,5,7-trihydroxy-4'-methoxyisoflavonane into biochanin A (data not shown). On the other hand, both the soybean extract and recombinant *HIDH* protein showed high substrate preferences for 2,5,7,4'-tetrahydroxyisoflavonane and also displayed significant activity toward 2,7,4'-trihydroxyisoflavonane and 2,7-dihydroxy-4'-methoxyisoflavonane. Thus, the HID activity of the crude extract of soybean could be attributed to HIDH protein. However, soybean EST libraries exhibited another HID candidate giving rise to the fragment clone BM177194, although in this study, its full sequence could not be obtained. Because this putative protein seems to be more similar to HIDM than to HIDH, its substrate specificity and expression in soybean will be quite interesting. Recent comprehensive studies on the expression of two soybean *IFS* genes have indicated distinct physiological functions of paralogous genes in the tissue-specific and pathogen- or rhizobia-responsive production of isoflavonones (Dhaubhadel et al., 2003; Subramanian et al., 2004). The biochemical characteristics and physiological functions of similar enzymes encoded by multiple genes in secondary metabolism are challenging subjects to be examined in the future.

The characteristics of the purified *P. lobata* HID (Hakamatsu et al., 1998) agree with the properties of soybean *HIDH*, except for the unusual *Km* value of *P. lobata* HID for 2,7,4'-trihydroxyisoflavonane (about 64-fold higher than that of soybean *HIDH*). Thus, the molecular mass (38 kD) of *P. lobata* protein is similar to the calculated average *M*, (35,137) of soybean HIDH protein, and the specific activity of *P. lobata* HID toward 2,7,4'-trihydroxyisoflavonane (56.8 nkatal mg$^{-1}$) is roughly identical to the activity of recombinant soybean *HIDH* protein (V_max value, 143 nkatal mg$^{-1}$). Characteristically, inhibition experiments indicated that His residues in *P. lobata* HID are important for the activity, and this is consistent with our finding that His is the central residue for the HID catalysis. Therefore, *HID* of *P. lobata* is likely to be a protein of the carboxylesterase-like family.

1,2-Dehydratases are grouped into the hydro-lyases (EC 4.2.1), which contain several types of proteins, but no hydrolase-like proteins are found among them. HID reaction is the first example of 1,2-dehydration by...
proteins classified into the carboxylesterases of the hydrolase family. Both mammalian carboxylesterases and plant carboxylesterase-like proteins have the $\alpha/\beta$-hydrolase fold common to Ser hydrolases that carry an oxyanion hole and the catalytic triad. In fact, site-directed mutagenesis of HIDH protein indicated that amino acid residues comprising the oxyanion hole and the catalytic triad are important for both HID and ester hydrolysis activities (Table III). Considering the established catalytic processes of carboxylesterase (Blow et al., 1969; Kraut, 1977) and microbial scytalone dehydratase, which is known to use a catalytic His residue for the dehydration of the substrate resembling 2-hydroxyisoflavanone (Lundqvist et al., 1994; Zheng and Brucic, 1998), a possible scheme for the HID reaction can be drawn, as in Figure 4. In this scheme, the nucleophilic oxygen of Thr in the catalytic triad acts as a base to abstract hydrogen from C-3 of the substrate, and the C-4 carbonyl is enolized to yield a negatively charged intermediate stabilized by the oxyanion hole. Subsequent abstraction of the hydroxyl from C-2 of the intermediate by the positively charged His residue yields an isoflavone. The initial hydrogen abstraction at C-3 is not necessarily accomplished by the Thr: The His can substitute for it. In the HID reaction shown in Figure 4, the stereochemistry of the 1,2-elimination of water is syn-oriented. This is in agreement with the predicted structure of the IFS reaction product, i.e. (2R, 3S)-2-hydroxyisoflavanone (Sawada et al., 2002), and the syn-elimination of water from this enantiomer. Further studies are, however, indispensable to establish the mechanism of the reaction.

The phylogenetic tree of the carboxylesterase family (Fig. 3; Supplemental Fig. 1) implies an interesting evolutionary process of this group of proteins. Several genes in the same clades are distributed to broad organisms of eubacteria, fungi, and human, and thus thought to be very old: primitive in evolution, in other words. However, whereas human carboxylesterases are known to catalyze the hydrolysis of a variety of ester-containing xenobiotics, fatty acids, and steroids (Satoh and Hosokawa, 1995), no clear intrinsic biochemical functions of carboxylesterases in microbes and plants have been identified. A very striking feature is that plant carboxylesterases are monophyletic, clearly distinguished from those of other higher and lower organisms. This scheme suggests that some specific function(s) that arose in an ancestral protein of the plant carboxylesterase family was significant, and the proteins were conserved and diverged during plant evolution. Regarding their physiological roles, it is noteworthy that P. sativum E86 (Ichinose et al., 2001), N. tabacum hsr203j (Pontier et al., 1994; Kiba et al., 2003; Takahashi et al., 2004), and grapevine (Vitis vinifera) BIG8.1 (accession no. AAN77692; Bézier et al., 2002) are induced in response to pathogenesis, and the L. japonicus hsr203j homolog (gene identity MDP051d06_f) is induced during the early stage of infection by symbiotic Mesorhizobium loti (Kouchi et al., 2004). Also, homologous sequences are also found in the EST database of Pinus radiata male cones (Walden et al., 1999) and a root hair-enriched M. truncatula cDNA library (Covitz et al., 1998). HIDs are the only proteins of the plant carboxylesterase family whose catalytic function is now clearly demonstrated, and, surprisingly, it is not ester hydrolysis. HIDs must have emerged within the clade III carboxylesterases in leguminous plants to dehydrate 2-hydroxyisoflavanones yielding isoflavones, the ecophysiologically significant substances such as biosynthetic intermediates of antimicrobial phytoalexins and chemical signals to nitrogen-fixing bacterial symbionts. The roles of other carboxylesterases in metabolic processes related to plant defense responses and/or organ-specific production of phytochemicals must be fascinating subjects for future studies.
(Shimada et al., 2003) producing 5-deoxyflavonoids, this work completes identification of enzymes of the legume-specific 5-deoxy- and either 4’-methoxy- or 4’-hydroxy-isoflavone pathways branching from general flavonoid metabolism. Among these, IFS should have evolved from the ancestral P450, which is also the ancestor of flavone synthase II, a P450 of the general flavonoid pathway, through critical events like the introduction of limited numbers of key amino acid residues for the unique aryl migration (Sawada et al., 2002; Y. Sawada and S. Ayabe, unpublished data). Also, type II chalcone isomerase must have emerged in the Leguminosae genome from the general type I chalcone isomerase through local gene duplication and subsequent mutations, resulting in the expansion of substrate specificity to 6’-deoxychalcone (Shimada et al., 2003). Extensive gene clusters of chalcone isomerases and other legume-specific (e.g. chalcone polyketide reductase and isoflavone reductase), as well as general enzymes (e.g. dihydroflavonol-4-reductase), in a model legume L. japonicus (Shimada et al., 2003; N. Shimada, T. Aoki, S. Sato, Y. Nakamura, S. Tabata, and S. Ayabe, unpublished data) further support the idea that gene duplication and mutation to gain new catalytic functions caused the diversity of secondary metabolism (Pichersky and Gang, 2000). During these processes, enzymes of primary metabolism also may have been recruited to secondary metabolism, as evidenced, for example, by the fact that chalcone polyketide reductase of the legume 5-deoxyflavonoid pathway and analogous codeinone reductase of morphinan alkaloid biosynthesis in Papaveraceae (Unterlinner et al., 1999) are members of the superfamily of aldo-keto reductases participating mainly in sugar metabolism. The findings in this study add another example of the recruitment: from hydrolyses to hydro-lyases of secondary metabolism. In this respect, it is interesting to note that Ser carboxypeptidase-like proteins catalyze the transacylation in plant natural product biosynthesis (Mikowskii and Strack, 2004; Stout and Chapple, 2004). The proteins also belong to the class of α/β hydrolyses, and the residues composing the catalytic triad are assumed to be important for the acyl-transfer reaction. Thus, the vast array of plant proteins classified as α/β hydrolyses can be interesting objectives to experimentally examine the recruitment mechanism during the molecular evolution of secondary metabolism. As the initial step to such a direction of research, we are planning to identify the biochemical basis for HID proteins to distinguish hydrolysis and dehydration through protein-engineering approaches.

**Preparation of Plant Cell-Free Extracts**

Glycrrhiza echinata cells (Ak-1 line; Akashi et al., 1999) were elicited with 0.2% (w/v) yeast extract (Invitrogen, Carlsbad, CA) for 24 h (Nakamura et al., 1999). Soybean (Glycine max L. Merr. cv Mikawashima; Tohoku, Tochigi, Japan) seeds were sown on filter paper in an Erlenmeyer flask, and seedlings were grown under 12-h-light/12-h-dark cycles at room temperature for 1 week. Filtered homogenates of these plant materials (10 g) in 10 mL of 100 mM potassium phosphate buffer, pH 7.5, containing 10% Suc and 14 mM 2-mercaptopethanol were centrifuged at 10,000g for 10 min. The ammonium sulfate fraction (30%–80% saturation) of the 10,000g supernatant, which had been treated with 2.5 g Dowex 1-X2 (20 min), was suspended in the same buffer for the initial extraction and, after desalting by passing through a Sephadex G-25 column, used as the enzyme (approximately 600 μg protein mL⁻¹).

**Enzyme Assays**

The buffer used was 100 mM potassium phosphate, pH 7.5, supplemented with 10% Suc and 14 mM 2-mercaptopethanol. For the HID assay, 5 nmol of each of the 2-hydroxyisoflavonanone sample in 2 μL 2-methoxyethanol was incubated with the enzyme preparation in a total volume of 50 μL at 30°C for 5 min. The ethyl acetate extract of the reaction mixture was analyzed by HPLC. HPLC conditions for daidzein or daidzein and formononetin were the same as described (Akashi et al., 2003). Genistein was analyzed using 50% methanol in water as the HPLC solvent. Specific activities were determined for cell-free extracts of G. echinata and soybean (approximately 10–100 μg protein) and also for purified recombinant HIDM and HIDH proteins (approximately 20–800 ng) from the peak areas of the isoflavones calibrated with those of standard samples. To determine the kinetic parameters, 2,7-dihydroxy-4’-methoxyisoflavonanone (5, 20, 50, 100, and 200 μM), 2,7,4’-trihydroxyisoflavonanone (5, 20, 50, 100, 200, and 400 μM), or 2,5,7,4’-tetrhydroxyisoflavonanone (10, 20, 50, 100, and 200 μM) was incubated with the recombinant protein (HIDM [50 ng] or HIDM [21 ng for 2,7-dihydroxy-4’-methoxyisoflavonanone dehydratase activity and 820 ng for 2,7,4’-trihydroxyisoflavonanone and 2,5,7,4’-tetrhydroxyisoflavonanone dehydratase activities]) in a total volume of 50 μL at 30°C for 5 min. Kₘ and kₐ values were calculated using a Lineweaver-Burk plot. In all the assays, enzymatically produced isoflavones were determined after the correction of preexisting and spontaneously formed isoflavones in the samples with the controls using the same concentrations of substrates in the buffer. To measure the rate of spontaneous dehydration, 2,7,4’-trihydroxyisoflavonanone (0.1, 1, 10, 50, and 100 μM) was incubated in the buffer in a total volume of 50 μL at 30°C for 0.5, 1, 2, 4, 8, 12, and 20 h. Daidzein production was determined by HPLC after the correction with the daidzein concentration at time 0. The velocity of dehydration was calibrated from the first 30-min incubations when the daidzein formation was linear with all the substrate concentrations tested. The initial velocities were proportional to the substrate concentrations, and the rate constant of dehydration was calculated from these values. The carboxylesterase activity of recombinant HID proteins was determined by the rate of p-nitrophenol production from p-nitrophenyl butyrate (Heymann and Mentlein, 1983). Porcine liver carboxylesterase (Sigma) was used as a positive control.

**Functional Expression Fractionation Screening of G. echinata HIDM**

The cDNA expression library (Akashi et al., 2003) constructed from yeast extract-treated G. echinata cells was the starting material. Five independent pools of cDNA fractions, each containing about 30,000 Escherichia coli clones, were prepared from the mother plate. Expression of protein and preparation of the crude extract of E. coli were performed as previously described (Akashi et al., 2003). 2,7,4’-Trihydroxyisoflavonanone (0.4 nmol) dissolved in 2 mL 2-methoxyethanol was preincubated with recombinant G. echinata HI4 ‘OMT’ (50 ng; Akashi et al., 2003) in the presence of 0.4 nmol [methy1-¹⁴C]-5-s-adenosyl-

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**MATERIALS AND METHODS**

**Chemical Materials**

Daidzein and genistein were obtained from Extrasynthese (Genay, France), and p-nitrophenyl butyrate from Sigma (St. Louis). Formononetin was from our laboratory stock. 2,7,4’-Trihydroxyisoflavonanone (Ayabe et al., 2002), 2,7-dihydroxy-4’-methoxyisoflavonanone (Akashi et al., 2003), and 2,5,7,4’-tetrhydroxyisoflavonanone (Akashi et al., 1999) were prepared as described.

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**Carboxylesterase-Like Dehydratase in Isoflavone Biosynthesis**
amino acids having E-values smaller than 1 from Arabidopsis thaliana and G. echinata HIDM were searched for cDNAs homologous to HIDM. Two specific primers containing Ndel or BamHI sites (underlined) were designed from the HIDM-like sequence of soybean (accession no. TC984660; TC98-F, 5’-GTCATATGGCCAGAGGA-TAGTGA-3’ and TC98-R, 5’-AGGATCTCTGAACCGCAGAACGA-3’). Total RNA was isolated from soybean seedlings using RNasey Plant Mini kit (Qiagen, Hilden, Germany). cDNAs were synthesized by Ready-To-Go T-Primed First Strand kit (Amersham Biosciences). cDNA (HIDM) obtained by the RT-PCR with the primers and soybean cDNAs as the template was cloned into pT7Blue T-vector (Novagen, Madison, WI).

Expression and Purification of Recombinant Proteins

Two primers containing Ndel or BamHI sites (underlined) were designed from G. echinata HIDM: HID-F, 5’-GTCATATGGCCAGAGGA-TAGTGA-3’; HID-R, 5’-ACCTAGGTAATTACCCCGCTA-3’. The coding region of HIDM was amplified by PCR with KOD polymerase (Toyobo, Tokyo) using HIDM and of the soybean HIDH as templates. The Ndel-BamHI fragments of the PCR product from G. echinata HIDM and of the soybean HIDH were subcloned into corresponding sites of pET28a (Novagen) to produce pET28a-HIDM and pET28a-HIDH. The vectors were transformed into E. coli BL21(DE3), and recombinant proteins were expressed and purified as described (Akashi et al., 2003).

Site-Directed Mutagenesis

Mutagenic primers (mutated sites in each primer are underlined) were designed from soybean HIDM: T164F primer, 5’-AGAATATGGCCAGAGGATAGTGA-3’; T164A primer, 5’-AGAATATGGCCAGAGGATAGTGA-3’. The coding region of HIDM was amplified by PCR with KOD polymerase (Toyobo, Tokyo) using HIDM and of the soybean HIDH as templates. The Ndel-BamHI fragments of the PCR product from G. echinata HIDM and of the soybean HIDH were subcloned into corresponding sites of pET28a (Novagen) to produce pET28a-HIDM and pET28a-HIDH. The vectors were transformed into E. coli BL21(DE3), and recombinant proteins were expressed and purified as described (Akashi et al., 2003).

Phylogenetic Analysis

The DNA sequences used for phylogenetic analysis were selected using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). Those were: (1) DNA encoding proteins similar to HIDM (having E-values smaller than 1 x 10^-3 at amino acid level) in the National Center for Biotechnology Information genome database (http://www.ncbi.nih.gov/Genomes) of Medicago truncatula (5 sequences), Lotus japonicus (5 sequences), Pseudomonas syringae pv. tomato (4 sequences), Arabidopsis thaliana (3 sequences), and G. echinata (2 sequences). The trees were constructed by the ClustalW program (Thompson et al., 1994) of the DNA Data Bank of Japan (Shizuka, Japan), and a neighbor-joining tree was produced from the results of 1,000 bootstrap replicates. The tree was displayed by TreeView (Page, 1996) or NJPlot software (Perrière and Gouy, 1996).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AB154414 and AB154415.

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