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cDNA cloning of a BAHD acyltransferase from soybean (*Glycine max*): Isoflavone 7-*O*-glucoside-6″-*O*-malonyltransferase

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Abstract

A cDNA from soybean (*Glycine max* (L.) Merr.), *GmIF7MaT*, encoding malonyl-CoA:isoflavone 7-*O*-glucoside-6″-*O*-malonyltransferase, was cloned and characterized. Soybeans produce large amounts of isoflavones, which primarily accumulate in the form of their 7-*O*-(6″-*O*-malonyl-β-D-glucosides). The cDNA was obtained by a homology-based strategy for the cDNA cloning of some flavonoid glucoside-specific malonyltransferases of the BAHD family. The expressed gene product, GmIF7MaT, efficiently catalyzed specific malonyl transfer reactions from malonyl-CoA to isoflavone 7-*O*-β-D-glucosides yielding the corresponding isoflavone 7-*O*-(6″-*O*-malonyl-β-D-glucosides) (IF7MaT activity). The k_{cat} values of GmIF7MaT were much greater than those of other flavonoid glucoside-specific malonyltransferases with their preferred substrates, while the K_m values were at comparable levels. *GmIF7MaT* was expressed in the roots of *G. max* seedlings more abundantly than in hypocotyl and cotyledon. Native IF7MaT activity was also observed in the roots, suggesting that GmIF7MaT is involved in the biosynthesis from isoflavone 7-*O*-β-D-glucosides to the corresponding isoflavone 7-*O*-(6″-*O*-malonyl-β-D-glucosides) in *G. max*. This protein is a member of flavonoid glucoside-specific acyltransferases in the BAHD family.

Keywords: Soybean; *Glycine max*; Leguminosae; Malonyltransferase; Acyltransferase; Malonyl-CoA; BAHD; Isoflavone; Isoflavonoid; Glucoside; Malonylglucoside

1. Introduction

Isoflavones are a class of plant flavonoids with a 3-phenylchromone structure. They are distributed almost exclusively in legumes and serve as chemoattractants for specific symbionts, genetic inducers of the nodulation genes of symbiotic rhizobial bacteria, and precursors of antimicrobial phytoalexins and/or phytoanticipins (Hungria and Stacey, 1997; Dixon and Ferreira, 2002). Since many studies have shown that dietary intake of these isoflavones from soybeans is closely related to decreasing the risk of various diseases, there is growing interest in the biomedical aspects of isoflavones, in particular, the major isoflavones of soybean (*Glycine max* (L.) Merr.) seeds, **1a** and **1b** (see Fig. 1 for their structures). The pharmacological properties of soybean isoflavones include a chemoprotectant activity, the prevention of cardiovascular diseases and osteoporosis, the improvement of blood–cholesterol levels, and phytoestrogen activity causing a relief of postmenopausal symptoms (Jung et al., 2000; Dixon and Ferreira, 2002).

The isoflavones **1a** and **1b** are derived from the corresponding (2*S*)-flavanones, *i.e.*, liquiritigenin and naringenin, respectively, which undergo 2-hydroxylation catalyzed by 2-hydroxyflavanone synthase, a microsomal cytochrome P450 enzyme (Akashi et al., 1999; Steele et al., 1999; Jung et al., 2000). In *G. max* and some other legumes, the resulting product, 2,7,4′-tetrahydroxyisoflavanone and 2,5,7,4′-trihydroxyisoflavanone, then undergo the enzymatic dehydration to produce **1a** and **1b**, respectively (Akashi et al., 2005). The amount of isoflavones **1** in *G. max* seeds is actually negligible. The most abundant forms are isoflavone conjugates **2** and **3** (see Fig. 1 for their structures) (Wang and Murphy, 1996). These conjugates

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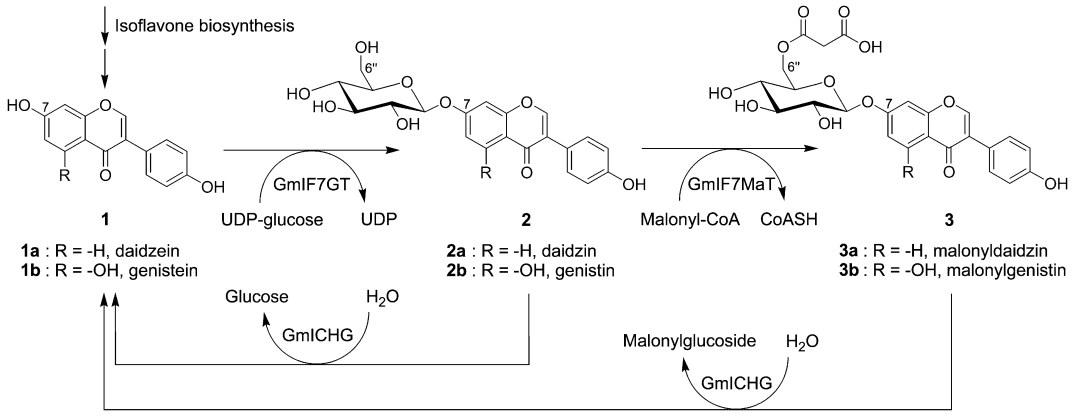

Fig. 1. Proposed biosynthetic pathway of isoflavone conjugates in *G. max*. The isoflavone conjugates are synthesized from the corresponding isoflavone aglycones by the combined actions of GmIF7GT and GmIF7MaT. GmICHG catalyzes the hydrolysis of isoflavone conjugates to produce isoflavone aglycones. GmIF7GT, UDP-glucose:isoflavone 7-*O*-glucosyltransferase; GmIF7MaT, malonyl-CoA:isoflavone 7-*O*-glucoside-6″-*O*-malonyltransferase; GmICHG, isoflavone conjugate-hydrolyzing β-glucosidase.

are more water-soluble than the aglycones and accumulate in vacuoles as latent forms (Barz and Welle, 1992). We recently reported the cloning of a cDNA, *GmICHG*, encoding an isoflavone conjugate-hydrolyzing β-glucosidase (ICHG) of *G. max*, along with the molecular properties and cellular localization of the enzyme (Suzuki et al., 2006). This enzyme is localized in the cell wall and intercellular space in the roots of *G. max* seedlings and hydrolyzes not only isoflavone conjugates **2** but also **3** directly to produce isoflavone aglycones **1** (Fig. 1), suggesting that GmICHG plays an important role in the conversion of isoflavone conjugates in vacuolar pools to bioactive aglycones released from the roots. On the other hand, there are also some reports regarding the biosynthesis to isoflavone conjugates from isoflavone aglycones (Köster and Barz, 1981; Koester et al., 1984; Nagashima et al., 2004). The enzymatic activities of UDP-glucose:isoflavone 7-*O*-glucosyltransferase (IF7GT) (Köster and Barz, 1981) and malonyl-CoA:isoflavone 7-*O*-glucoside-6″-*O*-malonyltransferase (IF7MaT) (Koester et al., 1984) in chick pea (*Cicer arietinum* L.) were characterized over two decades ago. Therefore, it is obvious that isoflavone conjugates **3** are synthesized from the corresponding aglycones **1** by the action of IF7GT followed by that of IF7MaT (Fig. 1). The cDNA encoding an isoflavone-specific glucosyltransferase was isolated from *Glycyrrhiza echinata* cell-suspension cultures (Nagashima et al., 2004). However, the cDNA encoding IF7MaT remains to be identified.

We have studied plant acyltransferases responsible for the malonylation of flavonoid glucosides, such as anthocyanins (Suzuki et al., 2001, 2002, 2004b) and quercetin 3-*O*-β-D-glucoside (Suzuki et al., 2004a), and established the identified malonyltransferases are members of the BAHD superfamily (St-Pierre and De Luca, 2000). It is expected, from these findings, that IF7MaT should be a new member of this family. We here describe the cDNA cloning, heterologous expression, and transcription analysis of a BAHD member of *G. max*, *GmIF7MaT*. The cDNA was obtained from the roots of a *G. max* seedling by a homology-based strategy that has been employed in the cDNA cloning of some flavonoid glucoside-specific malonyltransferases in the BAHD family members (Suzuki et al., 2002, 2004a). Recombinant GmIF7MaT was highly specific for isoflavone 7-*O*-glucosides **2** and catalyzed its 6″-*O*-malonylation, suggesting that this gene encodes IF7MaT, which is involved in the biosynthesis of isoflavone conjugates in *G. max*.

2. Results and discussion

2.1. cDNA cloning of GmIF7MaT by a homology-based strategy

Many members of the BAHD superfamily have been characterized, showing that these enzymes catalyze acyl-CoA dependent *O*- or *N*-acylations of a wide variety of plant secondary metabolites (D'Auria, 2006). Benzylalcohol *O*-acetyltransferase (BEAT) of California wildflower (*Clarkia breweri*) (Dudareva et al., 1998) and anthranilate *N*-hydroxycinnamoyl/benzoyltransferase (HCBT) of *Dianthus caryophyllus* (Yang et al., 1997) are, for instance, involved in the biosynthesis of the floral scent and phytoalexins, respectively. The BAHD members share two highly conserved sequences, HXXXD (motif 1; see Fig. 2a) and DFGWG (motif 3; see Fig. 2a). The crystal structure of vinorine synthase (VS), which is the first solved protein structure of the BAHD superfamily, revealed that motif 1 is located near the putative active center (Ma et al., 2005), supporting the suggestion that the histidine residue in motif 1 of BAHD enzymes is involved in the primary catalytic mechanism (Suzuki et al., 2003; Bayer et al., 2004). Motif 3 is located far away from the active center, which suggests that this motif plays a role in maintaining the conformational integrity rather than in the primary catalysis (Ma et al., 2005). Recent advances in plant

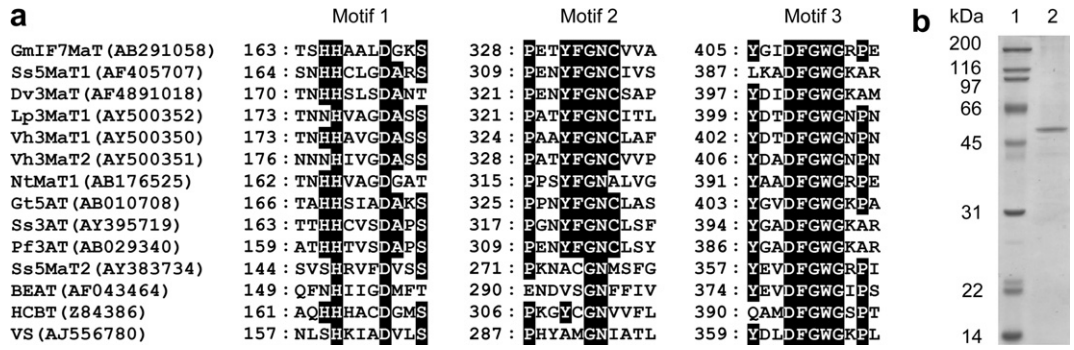

Fig. 2. Characterization of GmIF7MaT. (a) Alignment of the amino acid sequences covering motifs 1–3 of GmIF7MaT and the BAHD family members. The enzymes used for the alignment are as follows: isoflavone glucoside malonyltransferase, GmIF7MaT (this study); anthocyanin malonyltransferases, Ss5MaT1 (Suzuki et al., 2001), Dv3MaT (Suzuki et al., 2002), and Ss5MaT2 (Suzuki et al., 2004b); flavonol glucoside malonyltransferases, Lp3MaT1 and Vh3MaT1 (Suzuki et al., 2004a); phenolic-glucoside malonyltransferases, Vh3MaT2 (our unpublished data) and NtMaT1 (Taguchi et al., 2005); anthocyanin aromatic acyltransferases, Gt5AT (Fujiwara et al., 1998), Ss3AT (our unpublished data), and Pf3AT (Yonekura-Sakakibara et al., 2000); BAHD members that do not belong to the phenolic-glucoside acyltransferase subfamily, BEAT (Dudareva et al., 1998), HCBT (Yang et al., 1997), and VS (Bayer et al., 2004; Ma et al., 2005). The DDBJ/GenBank Accession Numbers of these enzymes are shown in parentheses. (b) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of molecular size markers (lane 1) and GmIF7MaT purified from *E. coli* JM109 harboring pQE-*GmIF7MaT*. Proteins were stained with Coomassie Brilliant Blue R-250.

genome sequence analyses imply that numerous BAHD members are widely distributed in the plant kingdom. The *Arabidopsis* genome contains about 70 genes of the family (Dudareva and Pichersky, 2000). It is extremely difficult, however, to predict the biochemical functions of these BAHD homologues on the basis of their primary structures due to the catalytic versatility and overall sequence diversity of the BAHD family members.

We have studied plant acyltransferases involved in the malonylation of anthocyanins (Nakayama et al., 2003). The malonyltransferase first identified is malonyl-CoA:anthocyanin 5-*O*-β-D-glucoside-6‴-*O*-malonyltransferase from scarlet sage (*Salvia splendens*) (Suzuki et al., 2001), which is a member of the BAHD family. This enzyme shares the YFGNC sequence (motif 2; see Fig. 2a), in addition to motifs 1 and 3, with anthocyanin aromatic acyltransferases (Fujiwara et al., 1998; Yonekura-Sakakibara et al., 2000). Motif 2 appeared to be conserved exclusively among anthocyanin acyltransferases of BAHD family members, leading to the establishment of a homology-based strategy for the specific isolation of anthocyanin acyltransferase cDNAs: the amplification of partial cDNAs encoding a region between motifs 2 and 3 of BAHD proteins by PCR followed by the isolation of full-length cDNAs using the amplified cDNAs as probes (Suzuki et al., 2002). By this strategy, we have successfully cloned cDNAs encoding not only anthocyanin malonyltransferases (Suzuki et al., 2002) but also flavonol glucoside-specific malonyltransferases (Suzuki et al., 2004a). We, therefore, adopted this strategy to identify *GmIF7MaT* cDNA in this study. A partial cDNA of 252 bp was amplified by a reverse transcription-PCR (RT-PCR) using two degenerate primers based on motifs 2 and 3 and a total RNA isolated from *G. max* roots as a template. Using the partial cDNA amplified as a probe, the full-length cDNA was obtained by a plaque hybridization of a cDNA library prepared from *G. max* roots (about 300,000 clones) to give 31 positive clones. The nucleotide sequence of the cDNA, termed *GmIF7MaT*, was determined (DDBJ/GenBank Accession Number AB291058). Some partial cDNAs (*e.g.*, DDBJ/GenBank Accession Numbers CX708072, CF808906, and BM188331) obtained from *G. max* in the Soybean EST Project showed identity to the *GmIF7MaT* sequence. The *GmIF7MaT* encoded a protein of 467 amino acids, which is a BAHD homologue containing all of motifs 1, 2, and 3 (Fig. 2a). The calculated molecular weight of GmIF7MaT was 51,663. GmIF7MaT showed sequence similarity to flavonoid glucoside-specific acyltransferases (identity, 32–36%); these sequence identities were generally greater than the values to other BAHD members, such as BEAT (24%), HCBT (24%), and VS (25%).

2.2. *GmIF7MaT encodes a BAHD member having IF7MaT activity*

To elucidate the catalytic activity of the gene product of *GmIF7MaT*, hexa-histidine-tagged GmIF7MaT was prepared from the crude extracts of cells of *Escherichia coli* JM109 harboring pQE-*GmIF7MaT* that overexpress *GmIF7MaT* under the control of the *T5* promoter. The recombinant protein was purified to near-homogeneity by Ni^{2+}-ion affinity chromatography, anion exchange column chromatography, and hydrophobic interaction column chromatography (Fig. 2b). The apparent molecular mass of the purified GmIF7MaT was 52 kDa, as estimated by means of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Fig. 2b). The molecular mass of GmIF7MaT was estimated to be 46 kDa by gel filtration analysis, indicating that GmIF7MaT is monomeric, as are the other BAHD members characterized (Nakayama et al., 2003; D'Auria, 2006).

The purified protein was incubated at 30 °C for 20 min with malonyl-CoA and **2a** or **2b**. The reaction products were analyzed by HPLC. As expected, GmIF7MaT catalyzed the malonyl-CoA-dependent malonyl transfer to **2a** and **2b** to produce **3a** and **3b**, respectively (Fig. 1). The malonyl transfer reactions proceeded at constant rates over 30 min. The specific activities were 540 and 410 nanokat/mg protein for **2a** and **2b**, respectively. The kinetic parameters are summarized in Table 1. The k_{cat} values were approximately 3–18 times larger than those of other flavonoid glucoside-specific malonyltransferases for their preferred substrates (Nakayama et al., 2003; Suzuki et al., 2004a), while the K_m values were at comparable levels. Neither *p*-nitrophenyl β-D-glucoside, quercetin 3-*O*-β-D-glucoside, nor the following anthocyanins served as substrates (relative activity, <0.1% of specific activity for genistin): cyanidin 3-*O*-β-D-glucoside, cyanidin 3-*O*-(6″-*O*-malonyl-β-D-glucoside), pelargonidin 3,5-*O*-β-D-diglucoside, pelargonidin 3-*O*-(6″-*O*-malonyl-β-D-glucoside)-5-*O*-β-D-glucoside, and cyanidin 3-*O*-(6″-*O*-*p*-coumaryl-β-D-glucoside)-5-*O*-β-D-glucoside. For acyl donors, this enzyme showed the highest activity toward malonyl-CoA. No acyl transfer activity to genistin from acetyl-CoA, *p*-coumaroyl-CoA, and caffeoyl-CoA was detected (relative activity, <0.1%). This acyl-donor profile was similar to those of other flavonoid glucoside-specific malonyltransferases (Nakayama et al., 2003; Suzuki et al., 2004a). Methylmalonyl-CoA and succinyl-CoA were also extremely poor substrates (relative activity, 0.7%), in contrast to the observations for other flavonoid glucoside-specific malonyltransferases, which show effective acyl transfer activities from succinyl-CoA as well as malonyl-CoA to their acyl acceptor (8-57% of specific activity for malonyl-CoA) (Nakayama et al., 2003; Suzuki et al., 2004a). Thus, GmIF7MaT had efficient IF7MaT activity with high acyl-donor and acyl-acceptor specificities.

2.3. Suggested involvement of GmIF7MaT in the biosynthesis of malonylated isoflavone glucosides in G. max

GmIF7MaT was expressed mainly in the roots more than in the hypocotyl and cotyledon of *G. max* seedlings that were grown under light (Fig. 3a). The accumulations of the *GmIF7MaT* transcripts were estimated by quantitative RT-PCR using primers specific for *GmIF7MaT* cDNA (Fig. 3a, inset). The transcription profile was similar to the accumulation profile of isoflavones, including malonylated isoflavone glucosides, **3a** and **3b**, in *G. max* (Graham, 1991). Dark-grown seedlings displayed a similar expression pattern of the gene (Fig. 3a). In the *G. max* plants that were grown for 3.5 months until soybean seeds were fully formed in the pods, *GmIF7MaT* was expressed broadly in roots, stems, leaves, and pods including seeds (Fig. 3a).

We determined the native IF7MaT activity in crude extracts prepared from various organs of *G. max* using

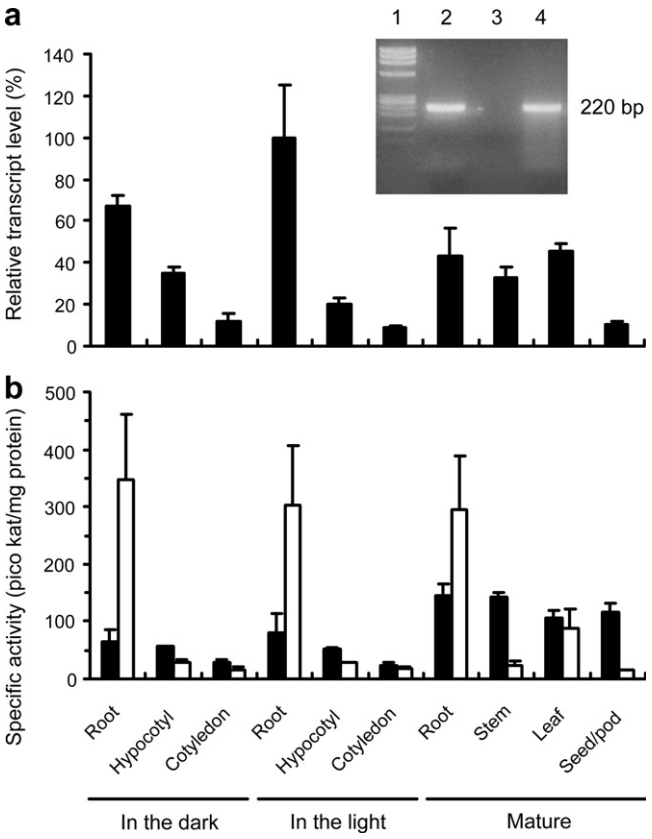

Fig. 3. Relative accumulation of *GmIF7MaT* transcripts (a) and IF7MaT and ICHG activities (b) in various organs of *G. max* plant: seedlings grown for 5–7 days in the dark (in the dark) and in available light (in the light). The *G. max* plants were also grown for 3.5 months until soybean seeds were fully formed in the pods (mature). (a) The transcription levels of*GmIF7MaT* transcripts in 100 ng of total RNA were estimated by quantitative RT-PCR. The relative average values of four independent determinations are presented with error bars with ±standard deviations. Agarose gel electrophoresis (inset) showed the specific amplification of a DNA fragment of *GmIF7MaT* by quantitative RT-PCR: lane 1, HaeIII-digest of ϕx174 DNA; lane 2, RT-PCR using *GmIF7MaT* cDNA as a template; lane 3, RT-PCR using *GmAT133* cDNA (DDBJ/GenBank Accession No. AB291059) encoding a GmIF7MaT homologue as a template; lane 4, RT-PCR using total RNA isolated from roots of *G. max* seedlings as a template. (b) The specific activities of the IF7MaT (black bars) and ICHG (white bars) in the crude extracts prepared from various organs of *G. max* were determined. The IF7MaT activity was determined using malonyl-CoA and **2b** as substrates. The ICHG activity was determined using **3b** as a substrate; other reaction conditions were the same as those described for the IF7MaT assay. The average values of three independent determinations are presented with error bars with ±standard deviations.

Table 1
Kinetic parameters of the IF7MaT activity of recombinant GmIF7MaT (30 °C, pH 7.0)[a]

	k_{cat} (s^{-1})	K_m for malonyl-CoA (μM)	K_m for acyl donor (μM)
2a	52 ± 3	5.0 ± 0.6	3.8 ± 0.4
2b	36 ± 1	1.6 ± 0.2	6.6 ± 0.8

[a] The reactions were carried out at 30 °C in a mixture consisting of 20 mM potassium phosphate, pH 7.0, 5–15 μM of malonyl-CoA, 2–30 μM of **2a** or **2b**, and 1.5 ng (0.29 nM) enzyme. The kinetic parameters were determined as described under Experimental procedures.

malonyl-CoA and genistin as substrates. The IF7MaT activity was higher in roots than in other organs (Fig. 3b). We also determined the GmICHG activity in crude extracts because the GmICHG activity should be responsible for the underestimation of IF7MaT activity due to the degradation of the reaction substrate (*i.e.*, **2b**) and the reaction product (*i.e.*, **3b**) in the assay of IF7MaT activity at pH 7 (Suzuki et al., 2006). In fact, a significant amount of **1b** was produced during the reactions to determine the native IF7MaT activity (data not shown). The GmICHG activity, as well as the IF7MaT activity, was the highest in the roots (Fig. 3b), clearly indicating that the enzyme responsible for IF7MaT activity was distributed mainly in roots. Thus, the transcription profile of *GmIF7MaT* was similar to the distribution profile of IF7MaT activity as well as the accumulation profile of isoflavone conjugates **3**. These results suggest that the BAHD protein encoded by *GmIF7MaT* is responsible for the native IF7MaT activity found in *G. max* and also imply that GmIF7MaT is involved in the biosynthesis from isoflavone 7-*O*-glucosides **2** to the corresponding malonylated forms **3** in *G. max*. Because no acetyl transfer activity of GmIF7MaT was detectable, as described above, an acetyltransferase(s) other than GmIF7MaT may be responsible for the production of isoflavone 7-*O*-(6″-*O*-acetyl-β-D-glucosides), which are minor components of soybean isoflavonoids (Mathias et al., 2006); otherwise, the acetylated form of conjugates may be produced by decarboxylation of the malonylated form.

2.4. Phenolic-glucoside acyltransferases in the BAHD family

We have shown that many anthocyanin malonyltransferases (Nakayama et al., 2003; Suzuki et al., 2004b), in addition to malonyl-CoA:flavonol 3-*O*-glucoside-6″-*O*-malonyltransferases (Suzuki et al., 2004a), are members of the BAHD family. In this study, we identified an isoflavone glucoside malonyltransferase, GmIF7MaT, as a new member of the family. A phylogenetic analysis revealed that these malonyltransferases, along with anthocyanin aromatic acyltransferases (Fujiwara et al., 1998; Yonekura-Sakakibara et al., 2000), could be phylogenetically categorized into a certain subfamily in the BAHD family (data not shown), with some exceptions (Suzuki et al., 2004b; Yoshihara et al., 2006). Interestingly, this subfamily includes a BAHD enzyme from tobacco cells (*Nicotiana tabacum* L.), NtMaT1, which is responsible for the malonylation and, hence, the detoxification of exogenous phenolic glucosides by the cells (Taguchi et al., 2005). We have also cloned a cDNA, *Vh3MaT2* (DDBJ/GenBank Accession Number AY500351), encoding a BAHD protein categorized into the subfamily, which malonylates some phenolic glucosides (our unpublished data). Thus, it is likely that this subfamily consists of BAHD members catalyzing the acylation of phenolic glucosides including flavonoid glucosides.

The members belonging to the subfamily, phenolic-glucoside acyltransferases, share the motif 2 sequence. The amino acid sequence, -GN-, in motif 2 is absolutely conserved among all members of the BAHD family (Fig. 2a). In the crystal structure of VS, this GN motif is located near the putative active center (Ma et al., 2005). The crystal structure of an anthocyanin malonyltransferase homologue complexed with malonyl-CoA, which has been clarified very recently (Unno et al., 2007), also showed that the side chain of the asparagine residue in the GN motif is hydrogen-bonded with the histidine residue in motif 1 that is proposed to act as a general base in the primary catalytic mechanism. These findings indicate that the -GN- sequence in motif 2 is involved in the catalytic mechanism rather than in the binding of an acyl-acceptor substrate. Although the functional significance of the specific conservation of motif 2 among phenolic-glucoside acyltransferases remains to be clarified, the motif 2 sequence, other than the -GN- portion, might be a "remnant motif", which might have remained during the course of diversification of BAHD proteins toward the phenolic-glucoside acyltransferase function.

3. Experimental

3.1. Plant materials and chemicals

Soybean seeds (*G. max* L. Merr. cv. Wase-Hakucho; Takii, Kyoto, Japan) were germinated at room temperature (20–23 °C) in the dark (or in the light) on an agar substratum consisting of 10 mM potassium phosphate, pH 7.0, 1% (w/v) sucrose, and 0.5% (w/v) agar. After cultivation for 5–7 days, grown seedlings were harvested. The *G. max* plants were also grown for 3.5 months until soybean seeds were fully formed in the pods. The plant materials were separated into the respective organs and stored at −80 °C until used. Isoflavones and their conjugates were purchased from Fujicco (Kobe, Japan). Quercetin 3-*O*-β-D-glucoside was kindly provided by Professor Tsukasa Iwashina (Tsukuba Botanical Garden, National Science Museum, Tsukuba, Japan). *p*-Coumaroyl-CoA and caffeoyl-CoA were kindly provided by Dr. Yoshikazu Tanaka (Suntory Research Center, Osaka, Japan). Anthocyanins were obtained as described previously (Suzuki et al., 2004a). Other chemicals commercially available were of analytical grade.

3.2. Preparation of crude extracts of G. max

All operations were performed at 4 °C. The plant material (1 g, fresh weight) was homogenized in 0.3 g polyvinylpolypyrrolidone and 4 ml of a buffer consisting of 100 mM potassium phosphate, pH 7.0, 30 mM 2-mercaptoethanol, 5 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride with a mortar and pestle. The extract was centrifuged and then cleared by the addition of polyethyleneimine to give a final concentration of 0.1% (w/v) followed by centrifugation. The supernatant was filtered through a filter disk (0.22 μm, Kanto chemicals).

3.3. cDNA cloning of GmIF7MaT

Two degenerate PCR primers were synthesized on the basis of two amino acid sequences, YFGNC (motif 2; see Fig. 2a) and DFGWG (motif 3; see Fig. 2a). Motif 2 is shared exclusively among flavonoid glucoside-specific acyltransferases in the BAHD family, and motif 3 is conserved generally in the BAHD family members. The forward primer was designed on the basis of the sequence around motif 2 as follows: 5′-(A/G)(A/C)(T/C)TA(T/C)TT-(T/C)GG(T/G/C)AA(T/C)TG-3′. The reverse primer was designed on the basis of the sequence around motif 3 as follows: 5′-CTT(T/C)CCCCA(T/C)CC(A/G)AAATC-3′. About 250-bp DNA fragments were amplified from a total RNA isolated from roots of the *G. max* seedling by a RT-PCR with the primers and the Qiagen One-Step RT-PCR Kit (Qiagen). The amplified fragments were cloned into pCR2.1-TOPO (Invitrogen) and sequenced. The possible fragments encoding the partial amino acid sequence of the BAHD enzyme were DIG-labeled using the PCR DIG Probe Synthesis Kit (Roche) and hybridized with about 300,000 plaques of a cDNA library of roots of the *G. max* seedling that had been constructed using the λZAPII-cDNA synthesis Kit (Stratagene) (Suzuki et al., 2006). DIG-labeled DNA was detected using the DIG-DNA detection kit (Roche). The thermal cycling conditions for RT-PCR and the hybridization conditions were as described previously (Suzuki et al., 2004a). The cDNA of positive clones obtained were subcloned into pBluescript SK- following the manufacturer's instructions and sequenced.

3.4. Heterologous expression in E. coli cells

The *GmIF7MaT* sequence was amplified by PCR using primers 5′-GAGCTC*ATG*GCAGAGACACCAACC-3′ (with the start codon of *GmIF7MaT* shown in italics and an SacI site shown by underlining) and GGTACC-*TTA*TGATTCCAAGGACTC-3′ (with the stop codon of *GmIF7MaT* shown in italics and a KpnI site shown by underlining). The amplified fragment was cloned into pCR4Blunt-TOPO (Invitrogen) and sequenced to confirm the absence of PCR-generated errors. From the plasmid, the *GmIF7MaT* sequence was excised with SacI plus KpnI and cloned between the SacI and KpnI sites of pQE-30 (Qiagen), resulting in pQE-*GmIF7MaT*, which contains the sequence encoding MRGSHHHHHHGSACEL-GmIF7MaT under the *T*5 promoter. This plasmid was used for the transformation of *E. coli* JM109.

Transformant cells, *E. coli* JM109 harboring pQE-*GmIF7MaT*, were cultured at 27 °C for 16 h in an LB medium supplemented with 50 μg/ml ampicillin and 1% lactose to allow constant expression of the *T*5 promoter. All operations were performed at 4 °C. The harvested *E. coli* cells were suspended in buffer A (50 mM sodium phosphate, pH 8.0, 0.5 M NaCl, 20% glycerol, 7 mM 2-mercaptoethanol, and 10 mM imidazole) containing 2 mg/ml lysozyme, incubated for 30 min, disrupted by sonication, and centrifuged. The cell lysate was cleared by the addition of polyethyleneimine to give a final concentration of 0.1% (w/v) followed by centrifugation. The cleared lysate was applied to a Ni^{2+}-nitrilotriacetic acid agarose spin column (Qiagen) equilibrated with buffer A. The column was washed successively with buffer A and buffer B (50 mM sodium phosphate, pH 8.0, and 7 mM 2-mercaptoethanol) containing 50 mM imidazole. Materials were eluted from the column with buffer B containing 200 mM imidazole. For further purification, the eluate was passed through a Q-Sepharose column (1 ml; GE Healthcare) equilibrated with buffer B containing 200 mM imidazole and subjected to a chromatography on Resource PHE column (1 ml; GE Healthcare). The chromatography conditions were as follows: the enzyme solution was 20%-saturated with ammonium sulfate and applied to a Resource PHE column equilibrated with buffer C (20 mM potassium phosphate, pH 7.0, and 7 mM 2-mercaptoethanol) containing ammonium sulfate at 20% saturation at a flow rate of 1 ml/min by the fast protein liquid chromatography system (Amersham). The column was washed with the same buffer for 10 min. Materials on the column were eluted with a 0–100% linear gradient of buffer C containing 50% (v/v) ethylene glycol over 10 min. Proteins were quantified using a kit (Bio-Rad) with bovine serum albumin as the standard. The molecular weight of native proteins was estimated by chromatography on a gel filtration column (HiLoad Superdex 200 16/60 prep grade, GE Healthcare) with isocratic elution in buffer C containing 0.15 M NaCl at a flow rate of 1 ml/min.

3.5. Enzyme assay

The standard reaction mixture (100 μl) consisted of 20 mM potassium phosphate, pH 7.0, 60 μM malonyl-CoA, 120 μM isoflavone 7-*O*-glucoside **2 b**, and 1.5 ng (0.29 nM) enzyme. After incubation at 30 °C for 20 min, the reaction was stopped by adding 200 μl of ice-cold 0.5% TFA in water. The reaction products were analyzed by reversed-phase HPLC using the Dynamax system (Rainin Instruments Co.) equipped with an UV–Vis detector model SPD-10A *VP* (Shimadzu). The HPLC conditions for the analysis of isoflavonoids were as follows: column, *J′*sphere ODS-M80 (4.6 × 150 mm, YMC); column temperature, 20 °C; detection, 260 nm; flow rate, 0.7 ml/min. After 50 μl of the reaction mixture had been injected into the column equilibrated with 13.5% CH_3CN–H_2O (13.5:86.5, v/v, with the H_2O containing 0.1% TFA), the column was initially developed isocratically for 3 min, followed by development by a linear gradient from CH_3CN–H_2O (13.5:86.5 → 10:90, v/v, with the H_2O containing 0.1% TFA) for 15 min. The HPLC conditions for analysis of anthocyanins (Suzuki et al., 2001, 2002), flavonols (Suzuki et al., 2004a), and other glucosylated phenolic compounds (Suzuki et al., 2004a) were as described previously. The kinetic parameters were determined by

non-linear least squares fitting of the initial reaction velocities on the equation for the double-displacement mechanism, $[E]/v = K_{mA}/k_{cat}[A] + (1 + K_{mB}/[B])/k_{cat}$, although it has been suggested that the acyltransfer reactions catalyzed by BAHD enzymes proceed via ternary complex formation (Suzuki et al., 2003; Ma et al., 2005), because the reciprocal plots showed apparently parallel lines, as observed in other flavonoid glucoside-specific malonyltransferases (Suzuki et al., 2003), and because double reciprocal plots of ternary complex mechanisms are known to yield parallel patterns under some circumstances (Segel, 1975).

3.6. Transcription analysis by quantitative RT-PCR

Total RNA was isolated from the individual organs of the *G. max* seedling (5–7 days old) using the RNeasy Plant Mini Kit (Qiagen). The *GmIF7MaT* transcripts in 100 ng of total RNA were estimated by quantitative RT-PCR on the LightCycler Quick System model 330 (Roche). Quantitative RT-PCR was carried out with QuantiTect SYBR Green RT-PCR Kit (Qiagen) and the following primers for 220 bp amplification: 5′-AGAAGTTAACAACCACCG-3′ and 5′-GGTAGTGAAAACAGCAAC-3′. The thermal cycling conditions were 50 °C for 20 min and then 95 °C for 15 min, followed by 35 cycles of 94 °C for 15 s, 55 °C for 20 s, and 72 °C for 15 s.

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