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Catalytic specificity of pea *O*-methyltransferases suggests gene duplication for (+)-pisatin biosynthesis

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Abstract

S-adenosyl-L-methionine: 2-hydroxyisoflavanone 4′-*O*-methyltransferase (HI4′OMT) methylates 2,7, 4′-trihydroxyisoflavanone to produce formononetin, an essential intermediate in the synthesis of isoflavonoids with methoxy or methylenedioxy groups at carbon 4′ (isoflavone numbering). HI4′OMT is highly similar (83% amino acid identity) to (+)-6a-hydroxymaackiain 3-*O*-methyltransferase (HMM), which catalyzes the last step of (+)-pisatin biosynthesis in pea. Pea contains two linked copies of *HMM* with 96% amino acid identity. In this report, the catalytic activities of the licorice HI4′OMT protein and of extracts of *Escherichia coli* containing the pea HMM1 or HMM2 protein are compared on 2,7,4′-trihydroxyisoflavanone and enantiomers of 6a-hydroxymaackiain. All these enzymes produced radiolabelled 2,7-dihydroxy-4′-methoxyisoflavanone or (+)-pisatin from 2,7,4′-trihydroxyisoflavanone or (+)-6a-hydroxymaakiain when incubated with [*methyl*-^{14}C]-*S*-adenosyl-L-methionine. No product was detected when (−)-6a-hydroxymaackiain was used as the substrate. HI4′OMT and HMM1 showed efficiencies (relative V_{max}/K_m) for the methylation of 2,7,4′-trihydroxyisoflavanone 20 and 4 times higher than for the methylation of (+)-6a-hydroxymaackiain, respectively. In contrast, HMM2 had a higher V_{max} and lower K_m on (+)-6a-hydroxymaackiain, and had a 67-fold higher efficiency for the methylation of (+)-6a-hydroxymaackiain than that for 2,7,4′-trihydroxyisoflavanone. Among the 15 sites at which HMM1 and HMM2 have different amino acid residues, 11 of the residues in HMM1 are the same as found in HI4′OMTs from three plant species. Modeling of the HMM proteins identified three or four putative active site residues responsible for their different substrate preferences. It is proposed that HMM1 is the pea HI4′OMT and that HMM2 evolved by the duplication of a gene encoding a general biosynthetic enzyme (HI4′OMT).

Keywords: *Glycyrrhiza echinata*; *Pisum sativum*; Leguminosae; Homology modeling; 2-Hydroxyisoflavanone; (+)-Pisatin; Pterocarpan

1. Introduction

Isoflavonoids are biosynthesized mainly by leguminous plants and play important roles in the adaptation and habituation of the producing plants to specific biological niches (Aoki et al., 2000; Dixon, 1999). The first compounds with an isoflavonoid skeleton in their biosynthesis are 2,5,7,4′-tetrahydroxyisoflavanone or 2,7,4′-trihydroxyisoflavanone **2** (Fig. 1). These compounds, collectively referred to as 2-hydroxyisoflavanone **2**, are produced from a general intermediate of flavonoid metabolism, (2*S*)-flavanone **1**, by the action of a legume-specific cytochrome P450 of the CYP93C subfamily (Akashi et al., 1999; Dixon, 1999). A subsequent intramolecular dehydration yields isoflavones such as daidzein **5** (Akashi et al., 2005), which lead to additional isoflavonoids such as the antimicrobial pterocarpans (Dixon, 1999; Aoki et al., 2000).

Abbreviations: D7OMT, daidzein 7-*O*-methyltransferase; HI4′OMT, 2,7,4′-trihydroxyisoflavanone 4′-*O*-methyltransferase; HMM, (+)-6a-hydroxymaackiain 3-*O*-methyltransferase; SAM, *S*-adenosyl-L-methionine.

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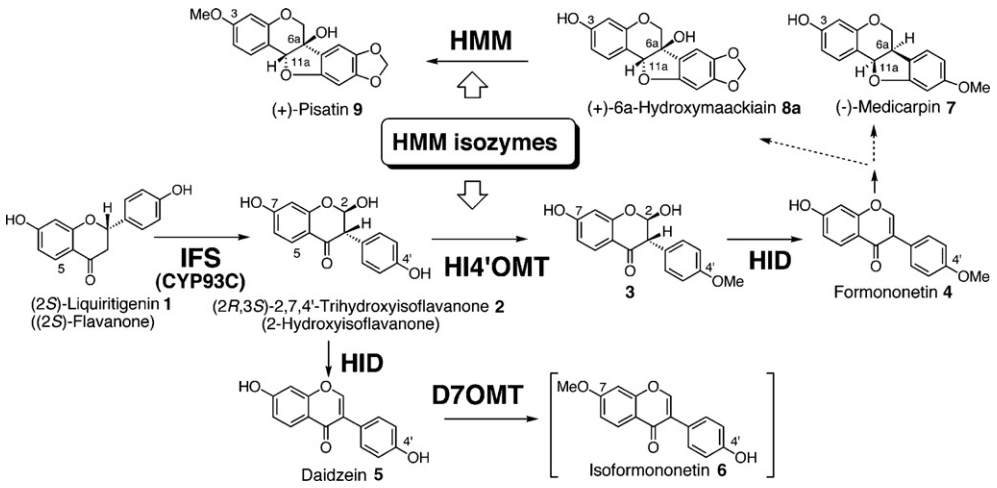

Fig. 1. Biosynthesis of isoflavones and the pterocarpan phytoalexins (+)-pisatin **9** in pea and (−)-medicarpin **7** in alfalfa and licorice. As shown, the pea HMM isozymes are proposed to function at two different steps in the biosynthesis of pisatin. In this figure, only the 5-deoxyisoflavonoid pathway is shown, but 5-hydroxyisoflavonoids are also produced by the IFS catalysis. The dotted arrows indicate that there are multiple steps between formononetin **4** and the indicated product. HMM, (+)-6a-hydroxymaackiain 3-*O*-methyltransferase; IFS, 2-hydroxyisoflavanone synthase; HI4′OMT, 2,7,4′-trihydroxyisoflavanone 4′-*O*-methyltransferase; HID, 2-hydroxyisoflavanone dehydratase; D7OMT, daidzein 7-*O*-methyltransferase.

Formononetin **4** (7-hydroxy-4′-methoxyisoflavone) is the precursor of the isoflavonoid derivatives with methoxy or methylenedioxy groups at carbon 4′ (isoflavone numbering) (Dixon, 1999). Formononetin **4** was proposed originally to be synthesized by the 4′-*O*-methylation of daidzein **5** (7,4′-dihydroxyisoflavone), an isoflavone found in soybean. However, the only known isoflavone *O*-methyltransferase in legumes that acts on daidzein **5** is the 7-*O*-methyltransferase (D7OMT) that yields isoformononetin **6** (7-methoxy-4′-hydroxyisoflavone) (Akashi et al., 2003). It is now known that formononetin **4** is produced by the action of 2,7,4′-trihydroxyisoflavanone 4′-*O*-methyltransferase (HI4′OMT), first characterized in licorice (*Glycyrrhiza echinata* L.) (Akashi et al., 2003). HI4′OMT acts on 2-hydroxyisoflavanone **2** to produce 2,7-dihydroxy-4′-methoxyisoflavanone **3**, which is dehydrated to yield formononetin **4** (Akashi et al., 2005). D7OMT and HI4′OMT have only about 50% identity and no substrate in common. On the other hand, HI4′OMT has 83% identity with (+)-6a-hydroxymaackiain 3-*O*-methyltransferase (HMM) of pea (*Pisum sativum* L.) (Wu et al., 1997). HMM catalyzes the last step in the biosynthesis of (+)-pisatin **9**, a pterocarpanoid phytoalexin, which is unusual in having a (+)-chirality (Fig. 1). Most pterocarpan phytoalexins, such as (−)-medicarpin **7** found in alfalfa and licorice, have a (−)-chirality (Dewick, 1988).

In pea, two closely linked *HMM* genes are found, and the *HMM* cDNAs encode the isozymes, HMM1 and HMM2, which share 96% amino acid sequence identity (Wu et al., 1997). Of the substrates tested, both HMMs showed highest activity on (+)-6a-hydroxymaackiain **8a** and essentially no activity on (−)-6a-hydroxymaackiain **8b** (structure not shown). Interestingly, while the licorice HI4′OMT preferred 2-hydroxyisoflavanone **2** as a substrate, it also had activity on (±)-medicarpin suggesting substrate overlap with the HMMs (Akashi et al., 2003). In this report, the catalytic activities of a purified recombinant licorice HI4′OMT protein and of extracts of *E. coli* containing the pea HMM1 or HMM2 protein are compared on 2,7,4′-trihydroxyisoflavanone **2**, as well as on enantiomers of 6a-hydroxymaackiain **8a** and **8b**. The results indicate that HI4′OMT and HMM1 have very similar activities but that HMM1 and HMM2 differ greatly in their substrate preferences. These results combined with homology modeling data have lead us to a proposal for the molecular evolution of the specific *O*-methyltransferase involved in the biosynthesis of (+)-pisatin **9**.

2. Results and discussion

Both HI4′OMT and the HMMs produced the expected radiolabelled products when [*methyl*-^{14}C]-*S*-adenosyl-L-methionine (SAM) along with 2,7,4′-trihydroxyisoflavanone **2** or (+)-6a-hydroxymaakiain **8a** were used as the substrates. No product was detected when (−)-6a-hydroxymaackiain **8b** was used as the substrate.

The steady-state kinetic data for HI4′OMT and the HMM enzymes are shown in Table 1. The Michaelis constants (K_m's) for the reactions of HI4′OMT and HMM1 with the two methyl acceptors were 3 μM for 2,7,4′-trihydroxyisoflavanone **2** and slightly higher (6–8 μM) for (+)-6a-hydroxymaackiain **8a**, indicating that these proteins have somewhat greater affinity for 2,7,4′-trihydroxyisoflavanone **2** than for (+)-6a-hydroxymaackiain **8a**. In contrast, HMM2 showed a higher K_m (23 μM) for 2,7,4′-trihydroxyisoflavanone **2** and a lower K_m (0.5 μM) for (+)-6a-hydroxymaackiain **8a** indicating an approximately 50-fold higher affinity for the pisatin **9** precursor than for 2,7,4′-trihydroxyisoflavanone **2**. The V_{max} and V_{max}/K_m values cannot be compared because the HMMs were not purified proteins. However, one can compare the efficiencies of

Table 1
Kinetic properties of recombinant licorice HI4′OMT, pea HMM1 and HMM2

	Substrate	K_m [μM]	V_{max} [pkatal mg^{-1}]	V_{max}/K_m [pkatal mg^{-1} μM^{-1}]	V_{max}/K_m [%][c]
HI4′OMT[a]	2,7,4′-Trihydroxyisoflavanone **2**	3	20,000	6600	100
	(+)-6a-Hydroxymaackiain **8a**	8	2700	340	5
HMM1[b]	2,7,4′-Trihydroxyisoflavanone **2**	3	1800	600	100
	(+)-6a-Hydroxymaackiain **8a**	6	910	150	25
HMM2[b]	2,7,4′-Trihydroxyisoflavanone **2**	23	340	15	100
	(+)-6a-Hydroxymaackiain **8a**	0.5	520	1000	6700

The values are the averages from two independent experiments (maximum deviation was about 10%).
[a] Purified recombinant licorice HI4′OMT.
[b] 10,000*g* supernatant of an extract of *Escherichia coli* expressing pea HMM1 or HMM2.
[c] Relative ratio of V_{max}/K_m within the individual enzyme reaction to the two methyl acceptors (2,7,4′-trihydroxyisoflavanone = 100%).

the enzyme reactions in terms of relative V_{max}/K_m with each substrate (see Table 1). Whereas the V_{max} ratios of HI4′OMT and HMM1 with (+)-6a-hydroxymaackiain **8a** were <50% of their ratios with 2,7,4′-trihydroxyisoflavanone **2**, HMM2 had a 1.5-fold higher V_{max} on (+)-6a-hydroxymaackiain **8a** than on 2,7,4′-trihydroxyisoflavanone **2**. As a result, HI4′OMT and HMM1 showed efficiencies (relative V_{max}/K_m) for the methylation of 2,7,4′-trihydroxyisoflavanone **2** 20 and 4 times higher than the efficiencies for (+)-6a-hydroxymaackiain **8a**, respectively. In contrast, HMM2 showed a 67-fold higher efficiency for the methylation of (+)-6a-hydroxymaackiain **8a** than that for 2,7,4′-trihydroxyisoflavanone **2**. Thus, we suggest that in pea, HMM1 is the equivalent of the HI4′OMT

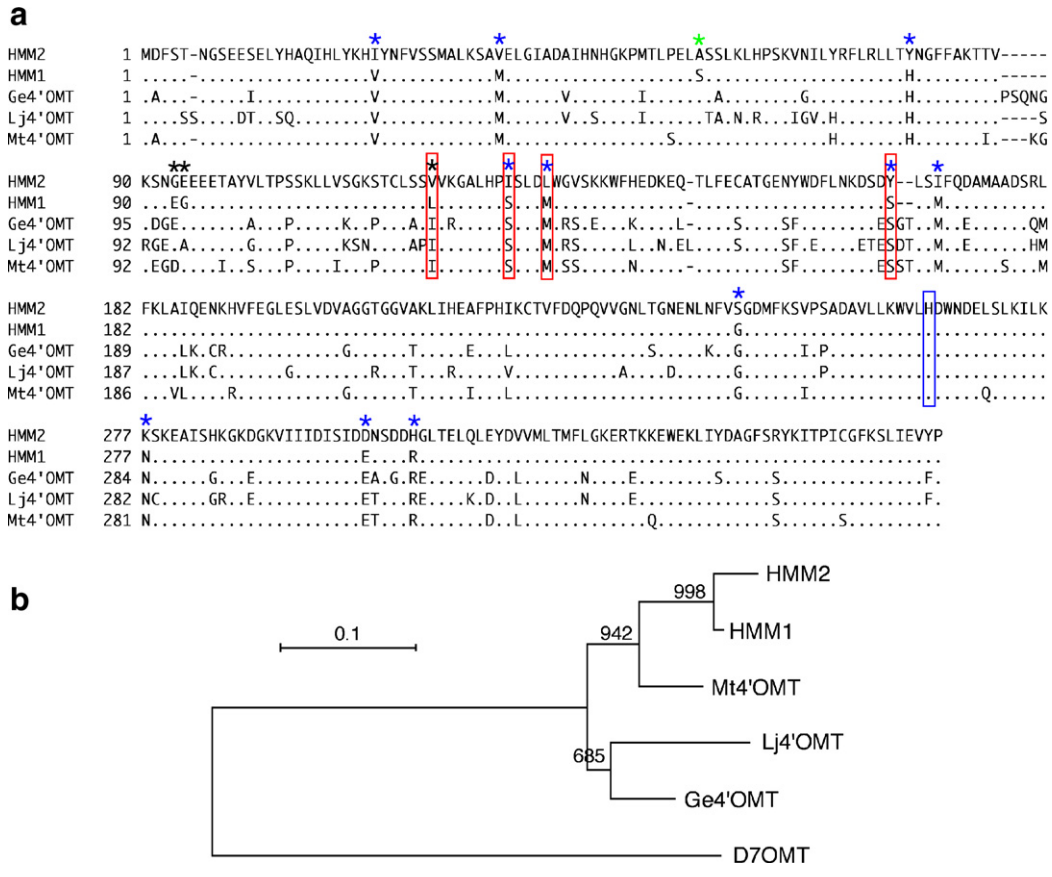

Fig. 2. Alignments (a) and a phylogenetic tree (b) of the HMMs of pea and related OMT proteins. (a) The aligned amino acid sequences of HMM2, HMM1, the HI4′OMTs of licorice (Ge4′OMT) and *Lotus japonicus* (Lj4′OMT), and a putative HI4′OMT of *Medicago truncatula* (Mt4′OMT). Dots indicate the amino acid residues identical to those of HMM2. Gaps (-) are inserted to optimize alignment. The conserved catalytic His 264 is denoted by the blue box, and the residues that differ between HMM1 and HMM2 in the putative active site are indicated with red boxes. Amino acid residues different between HMM1 and HMM2 are marked by stars: blue, unique amino acid residues in HMM2; green, residues common to HMM2 and the HI4′OMTs. (b) The tree was constructed from the amino acid sequences of the proteins in (a) with the alfalfa D7OMT as the outgroup. The numbers above the branches are the bootstrap values for 1000 replicates. The scale bar shows the amino acid substitution ratio.

found in other legumes and is involved in formononetin **4** biosynthesis and that HMM2 likely has a different substrate *in planta* and is involved primarily in the biosynthesis of (+)-pisatin **9**.

Fig. 2a shows the alignment of the amino acid sequences of the HMMs and HI4′OMTs of licorice, *Lotus japonicus*, and *Medicago truncatula*. In total, there are 15 sites at which HMM1 and HMM2 have different amino acid residues (indicated with stars over the sequences in Fig. 2a). At 11 (blue stars) of these 15 sites, the amino acid residues in HMM1 and the other HI4′OMTs are identical. The amino acid residues at these 11 sites are not conserved in HMM2. Therefore, these amino acid residues could be important in determining the differential substrate preferences of the HMM1/HI4′OMTs and HMM2. The structure of the alfalfa D7OMT protein, based on X-ray crystallography, has been reported by Zubieta et al. (2001). Also, the homology models of chavicol *O*-methyltransferase and eugenol *O*-methyltransferase of sweet basil based on the alfalfa D7OMT structure have identified the amino acid residues that are critical for the substrate preferences of these OMTs (Gang et al., 2002). The amino acid identity of the chavicol/eugenol *O*-methyltransferases and D7OMT is ~43%, whereas the identity of HMM/HI4′OMT and D7OMT is ~49%. Therefore, the D7OMT-based homology models of the HMM proteins are expected to be more useful for identifying which of the unconserved amino acid residues are critical in determining the substrate preferences of the HMM isozymes.

The hypothesized binding of the substrates to the common active sites of HI4′OMT/HMMs was verified first *in silico*. When plausible stereostructures of (+)-6a-hydroxymaackiain **8a** and the four optical isomers of 2,7,4′-trihydroxyisoflavanone **2** are compared, the overall structures of the (2*R*, 3*S*)- and (2*S*, 3*R*)-forms with their quasi-equatorial 2-hydroxy and 3-phenyl conformations can be superimposed onto (+)-6a-hydroxymaackiain **8a** (Fig. 3a). We favor the (2*R*, 3*S*)-2,7,4′-trihydroxyisoflavanone **2** as the likely substrate as we expect it to be the initial product from the action of the CYP93C involved in the synthesis of 2-hydroxyisoflavanone **2** from the (2*S*)-flavanone **1** (Fig. 1) (Sawada et al., 2002; Sawada and Ayabe, 2005). The homology modeled structures of the HMM1 and HMM2 proteins also had the same overall conformation (data not shown). Among the 15 sites where the residues of HMM1 and HMM2 differ, four sites (residues 120, 128, 132 and 167) were located at the substrate-binding region (indicated with red boxes in the alignment (Fig. 2a)). While L120/V120 of HMM1/HMM2 both differ from the corresponding residue (I) of HI4′OMT, S128, M132 and S167 are conserved in HMM1 and HI4′OMT. A closer inspection of the active site (Fig. 3b) shows that when the hydroxyls of the substrates are positioned next to the conserved catalytic H264 (blue) involved in their methylation, the different hydrophobic residues, M132 (red) of HMM1 and the L132 (green) of HMM2, are near the 7-hydroxyl of 2-hydroxyisoflavanone **2** and the methylenedioxy of (+)-6a-hydroxymaackiain **8a**. Also, the differences at positions 128 (S vs. I) and 167 (S

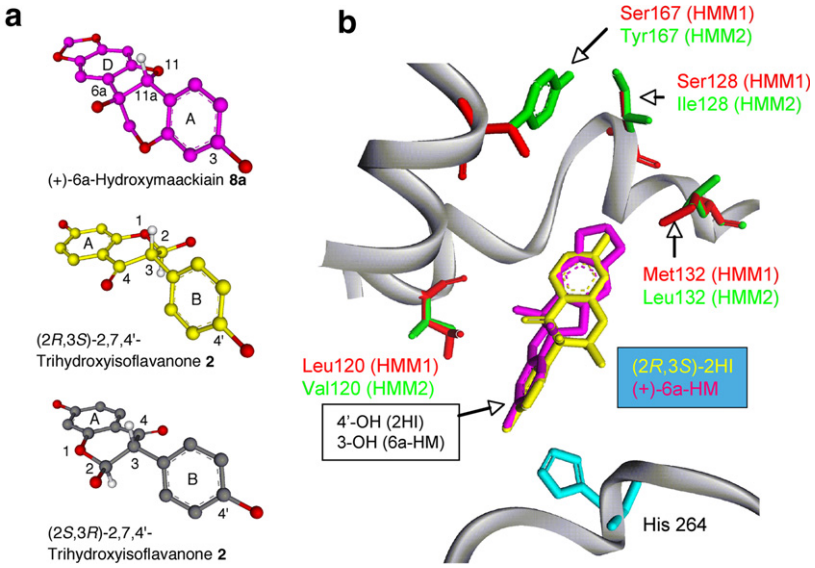

Fig. 3. Stereoviews of the substrates of HI4′OMT and HMM (a) and the active site of a homology-modeled HMM with the substrates superimposed and docked (b). (a) Substrates are shown as balls and sticks. Carbons of (+)-6a-hydroxymaackiain **8a**, (2*R*,3*S*)- and (2*S*,3*R*)-2,7,4′-trihydroxyisoflavanone **2** are displayed as pink, yellow and gray balls, respectively. Oxygens are shown as red balls, and hydrogens at C-11a of (+)-6a-hydroxymaackiain **8a** and C-2 and C-3 of 2,7,4′-trihydroxyisoflavanones **2** are displayed as white balls. (b) Conserved catalytic H264 (blue), the amino acid residues at positions 120, 128, 132 and 167 of HMM1 (red) and HMM2 (green), (+)-6a-hydroxymaackiain **8a** [(+)-6a-HM, pink] and (2*R*,3*S*)-2,7,4′-trihydroxyisoflavanone **2** [(2*R*,3*S*)-2HI, yellow] are shown as sticks. The distance between H264 and each of the methyl-accepting hydroxyls of the substrates is about 3 Å. Residues at position 132 (M and L) are located within 4 Å, and residues at positions 128 (S and I) and 167 (S and Y) are within 8 Å, of the 7-hydroxyl of 2-hydroxyisoflavanone **2** and the methylenedioxy of (+)-6a-hydroxymaackiain **8a**, respectively.

vs. Y) involve amino acids which differ in size, hydrophobicity and in the presence of a hydroxyl, which if present, might participate in hydrogen bonding with the substrate (position 128). We expect the residues at these three sites, and possibly at position 120 as well, to be involved in determining the differences in the catalytic activities of HMM1 and HMM2, particularly the high affinity (low K_m) of HMM2 for the (+)-pisatin **9** precursor.

HI4′OMT is expected to be a highly conserved gene found in all legumes that produce isoflavonoids with methoxy and methylenedioxy groups at the carbon equivalent to the 4′-position of an isoflavone. Pea is one such legume and the enzymatic data presented here indicate that what was originally identified as HMM1 is the pea HI4′OMT. In contrast, HMM2, which has a very high efficiency for the methylation of (+)-6a-hydroxymaackiain **8a**, would appear to be an enzyme primarily involved in the last step of (+)-pisatin **9** biosynthesis. These results are consistent with an interesting mechanism for the molecular evolution of enzymes of secondary metabolism. It has been demonstrated that two (or more) *HMM* genes, presumably *HMM1* and *HMM2*, are present on an 18.7-kb *Bam*HI fragment of the pea genome (Wu et al., 1997). Several examples of clusters of paralogous genes encoding enzymes of secondary metabolism with different specificities are found in plants (Shimada et al., 2003; Shimada et al., 2005), and gene duplication followed by the accumulation of mutations is an accepted mechanism to account for the extreme diversity of plant secondary products (Ober, 2005).

The phylogenetic tree indicates that the pea HMMs form a monophyletic clade with strong bootstrap support in a clade of legume HI4′OMTs (Fig. 2b). The branch length of HMM2 (0.033) is about five times longer than that of HMM1 (0.007), suggesting a higher accumulation of amino acid substitutions in the HMM2 sequence than in the HMM1 sequence. Also, a reconstruction of the ancestral sequence of the HMMs revealed that 13 amino acid residues (V24I, M37V, H80Y, E93G, L120V, S128I, M132L, C167Y, M170I, G244S, N277K, E300D, R305H), including all the active site residues highlighted in Fig. 2a and Fig. 3b, have been replaced to generate the HMM2 sequence in contrast to only three amino acids (A58S, E94G, C167S) for the HMM1 sequence.

3. Concluding remarks

A consideration of the amino acid substitutions leading to HMM2 and the molecular modeling studies has allowed the identification of four active site residues predicted to be critical for the differences in HMM1 and HMM2 catalysis. While most legumes produce pterocarpans with (−)-chirality such as (−)-medicarpin **7** (Fig. 1), the garden pea is somewhat unusual in that it synthesizes a (+)-pterocarpan, (+)-pisatin **9** (Dewick, 1988). Thus, it would be expected that there would be specific enzymes involved in the synthesis of this pterocarpan. It is hypothesized that in pea, the duplication of a gene encoding a general biosynthetic enzyme (HI4′OMT) resulted in the emergence of a second enzyme that acts later in the same pathway for the production of a unique phytoalexin, (+)-pisatin **9**.

4. Experimental

4.1. Chemicals

The (+)- and (−)-6a-hydroxymaackiain **8a** and **8b** were obtained as previously described (VanEtten et al., 1983). The 2,7,4′-trihydroxyisoflavanone **2** was prepared *in vitro* using yeast microsomes expressing CYP93C2 (Ayabe et al., 2002).

4.2. Enzymes and enzyme assay

To prepare the HMM isozymes, HMM1 and HMM2 cDNAs in pBluescript SK(−) were transformed into *E. coli* DH5α. Extracts containing HMM1 and HMM2 were prepared as described previously (Wu et al., 1997) except the induction by β-D-thiogalactopyranoside was for 5 h and the extraction buffer was 100 mM potassium phosphate buffer (pH 7.5) containing 10% (w/v) sucrose and 14 mM 2-mercaptoethanol. Purified recombinant licorice HI4′OMT with six histidine residues at the N-terminus was prepared as previously described (Akashi et al., 2003).

HI4′OMT activity was assayed in a reaction mixture containing 2,7,4′-trihydroxyisoflavanone **2** (Akashi et al., 2003) dissolved in 2 μl 2-methoxyethanol, 150 μM SAM (2.4 nmol [^{14}C]SAM (2.26 GBq/mmol, Amersham Biosciences, Buckinghamshire, UK) and 12.6 nmol unlabeled SAM) and either the crude extract of *E. coli* with HMM1 (350 ng protein), or HMM2 (1.75 μg protein) or the purified recombinant HI4′OMT (17.5 ng protein), in a total volume of 100 μl. To determine the kinetic parameters, 1, 3, 9, 30 and 100 μM of 2,7,4′-trihydroxyisoflavanone **2** were used. The reaction was carried out at 30 °C for 5 min and terminated by the addition of EtOAc (60 μl). The EtOAc extract of the mixture (30 μl) was subjected to silica-gel TLC [LK6DF (Whatman, Maidstone, UK)] using $CHCl_3$:acetone:25% aq. ammonia solution (70:29:1, v/v). The product, [^{14}C]-2,7-dihydroxy-4′-methoxyisoflavanone **3**, was collected from the developed TLC and its radioactivity measured by liquid scintillation counting. K_m and V_{max} values were calculated using a Lineweaver–Burk plot. For the assay of HMM activity, (+)-6a-hydroxymaackiain **8a** (0.3, 0.75, 1.5, 3, 9 or 30 μM) was incubated with *E. coli* extracts containing HMM1 (875 ng protein), or HMM2 (1.17 μg protein) or the purified recombinant HI4′OMT (350 ng) in the presence of 150 μM SAM (2.4 nmol [^{14}C]SAM and 12.6 nmol unlabeled SAM) in a total volume of 100 μl at 30 °C for 5 min (Preisig et al., 1989; Wu et al., 1997). For resolving the 6a-hydroxymaackiain *O*-methyltransferase product, LK6DF TLC plates were developed with toluene:EtOAc (3:2, v/v).

4.3. Homology modeling

The structural formulae of 2,7,4′-trihydroxyisoflavanone **2** and (+)-6a-hydroxymaackiain **8a** were drawn using ChemDraw Pro (CambridgeSoft Corporation, Cambridge, MA, USA). The stereochemical analysis was performed using DS ViewerPro 5.0 (Accelrys Inc., San Diego, CA, USA). The three-dimensional homology models of HMM1 and HMM2 were constructed using MODBASE program (http://salilab.org/modbase). Of the 10 HMM1 and HMM2 models constructed, only those, which aligned with the reported stereostructure of D7OMT (Zubieta et al., 2001), were selected for further analyses. The superimposition of the HMM1 and HMM2 models was carried out using i-Mol software (http://www.pirx.com/iMol/). The substrate–protein complex was generated by the manual docking of the individual structures using DS ViewerPro 5.0.

4.4. Phylogenetic and evolutionary analyses

Amino acid sequences of the OMTs were aligned using Clustal W (Thompson et al., 1994). The phylogenetic tree was constructed with the neighbor-joining method (Saitou and Nei, 1987) and displayed by NJPlot software (Perrière and Gouy, 1996). For the reconstruction of ancestral sequences, DNA sequences of the OMTs were aligned according to the protein sequence alignment using CodonAlign software (http://www.sinauer.com/hall/), and maximum likelihood analyses under codon models were performed using the codeml program in the PAML package (Yang, 1997).

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