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Stress responses in alfalfa (*Medicago sativa* L). XXII. cDNA cloning and characterization of an elicitor-inducible isoflavone 7-*O*-methyltransferase

Xian-Zhi He, J. T. Reddy and Richard A. Dixon*
*Plant Biology Division, Samuel Roberts Noble Foundation, P.O. Box 2180, Ardmore, OK 73402, USA (*author for correspondence)*

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Abstract

Medicarpin, the major phytoalexin in alfalfa, is synthesized via the isoflavonoid branch of phenylpropanoid metabolism. The methyl group at the 9 position of medicarpin is generally accepted to arise via the methylation of the 4′ position (B-ring) of daidzein. Surprisingly, the isoflavone-*O*-methyltransferase (IOMT), which is induced along with other enzymes involved in medicarpin biosynthesis, methylates the A-ring 7-hydroxyl group of daidzein *in vitro*, a reaction that probably does not occur in *vivo.* Utilizing internal amino acid sequence information from purified alfalfa IOMT, we have isolated three full-length IOMT cDNA clones. A search of the protein databases revealed sequence similarities to *O*-methyltransferases from various sources. The highest match (50.5% identity) was found between IOMT8 and 6a-hydroxymaackiain 3-*O*-methyltransferase from *Pisum sativum.* The molecular weight of alfalfa IOMT expressed in *Escherichia coli* was similar to that of purified IOMT from alfalfa cell cultures (41 kDa by SDS-PAGE). The recombinant enzyme catalyzed the *O*-methylation of A-ring hydroxyl group(s) of isoflavones, and could also methylate the pterocarpan (+) 6a-hydroxymaackiain. Alfalfa contains multiple IOMT genes, and closely related sequences are present in the genomes of chickpea and cowpea, species that also produce B-ring methylated isoflavonoids *in vivo.* Northern blot analysis indicated that IOMT transcripts are rapidly induced following elicitation, prior to the increase in IOMT activity and medicarpin accumulation. The possible role of the isoflavone 7-OMT in the synthesis of formononetin *in vivo* is discussed.

Introduction

Phytoalexins are low-molecular-weight antimicrobial compounds that accumulate in plants as a result of infection or stress. The major phytoalexin in alfalfa (*Medicago sativa* L.) is the pterocarpan medicarpin which has been shown to accumulate in fungal pathogen infected plants, in $CuCl_2$-treated alfalfa seedlings, or following exposure of cell suspension cultures to elicitor derived from yeast cell walls [6, 28]. Previous studies have implicated medicarpin as an important factor in defense against a number of fungal pathogens [1, 2, 26, 42, 43].

Medicarpin is synthesized from L-phenylalanine via the isoflavonoid branch of phenylpropanoid metabolism. 4-Coumaroyl-CoA and malonyl-CoA are condensed, with reduction, to form a trihydroxychalcone, which is then isomerized to the corresponding dihydroxyflavanone. An aryl ring migration, catalyzed by isoflavone synthase, forms the basic isoflavone carbon skeleton. Medicarpin production then involves a series of reactions including methylation, hydroxylation, reduction and dehydration [48] (Figure 1). This proposed biosynthetic pathway has been elucidated primarily via radiolabeled precursor feeding studies in alfalfa seedlings elicited by exposure to $CuCl_2$ [10, 11, 40] and by direct isolation of the intermediates from infected white clover [52]. Much of this pathway has now been confirmed by the characterization of the specific enzymes involved and the cloning of their genes

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers AF000975 (IOMT6), U97125 (IOMT8) and AF000976 (IOMT9).

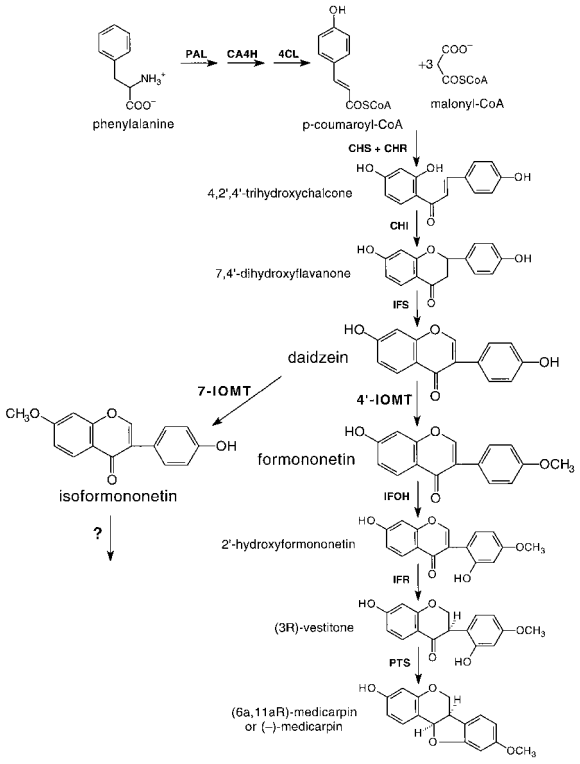


Figure 1. Biosynthetic pathway leading to (−)medicarpin in alfalfa. The names of the enzymes catalyzing each step are abbreviated as follows: PAL, L-phenylalanine ammonia-lyase; CA4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; CHS, chalcone synthase; CHR, chalcone reductase; CHI, chalcone isomerase; IFS, isoflavone synthase; IOMT, isoflavone-*O*-methyltransferase; IFOH, isoflavone 2′-hydroxylase; IFR, isoflavone reductase; PTS, pterocarpan synthase (this is composed of a separate reductase and dehydratase).

[12]. However, the nature of the enzymatic step leading to the methyl group at the 9 position of medicarpin (4′ position, isoflavone numbering) is still a matter of debate.

Transmethylation reactions of flavonoid/isoflavonoid biosynthesis catalyzed by position-specific *O*-methyltransferases involve the transfer of an intact methyl group from *S*-adenosyl-L-methionine (SAM) to the phenolic hydroxyl groups. The methylation of polyphenolic compounds, in addition to glucosylation, reduces the chemical reactivity of the phenolic hydroxyl groups and plays a crucial role in directing intermediates toward specific biosynthetic pathways. *O*-methylation of flavonoids/isoflavonoids also increases their lipophilicity [39] and may in some cases increase their antimicrobial activity [16].

The isoflavone *O*-methyltransferase (IOMT) as depicted in Figure 1 methylates 7,4′-dihydroxyisoflavone (daidzein), the first isoflavonoid intermediate in the pathway, at the 4′ position to yield 7-hydroxy-4′-methoxyisoflavone (formononetin). However, in chickpea (*Cicer arietinum*) cell cultures, which produce medicarpin upon elicitation, an isoflavone 7-*O*-methyltransferase, which methylated the 7-position of 5,7,4′-trihydroxyisoflavone (genistein, the 5-hydroxy derivative of daidzein) to produce 5,4′-dihydroxy-7-methoxyisoflavone (prunetin), was identified [20]; this enzyme activity had been erroneously described as a 4′-*O*-methyltransferase in an earlier report [50]. Likewise, treatment of alfalfa cell suspension cells with yeast elicitor results in a massive induction of IOMT activity [6] which, like the chickpea enzyme, was shown to be an isoflavone 7-*O*-methyltransferase (7-IOMT) [14]. The purified alfalfa enzyme catalyzed the methylation of the A-ring of daidzein to produce 4′-hydroxy-7-methoxyisoflavone (isoformononetin) [14, 23], a rare naturally occurring compound [29]. Isoformononetin is unlikely to be involved in the formation of medicarpin because it is a very poor substrate for 2′-hydroxylation [14], the step following methylation in medicarpin formation. A very low level of isoflavone 4′-*O*-methyltransferase activity resulting in the formation of formononetin was detected in extracts from elicited alfalfa suspension cells [14], but this contrasts with the strongly increased extractable activities of all other known enzymes in the pathway leading to medicarpin upon elicitation [6]. It has not been possible to purify this activity [23].

The above situation is further complicated by the observation that radiolabeled daidzein is not incorporated into medicarpin in $CuCl_2$-treated seedlings of alfalfa, whereas formononetin is a good precursor [10]. Although this suggests that the methoxyl group at the 9 position of medicarpin originates from formononetin, the direct precursor for 4′-*O*-methylation is unclear. It has been suggested that methylation of the 4′ position of the isoflavone is an integral part of the aryl migration reaction [11], but this now seems unlikely in view of the demonstration that this reaction is catalyzed by a methyl group donor-independent cytochrome P450 enzyme [22, 35, 37]. In *Pueraria lobata*, the enzyme catalyzing the aryl migration first converts 7,4′-dihydroxyflavanone into 2,7,4′-trihydroxyisoflavanone; this intermediate then undergoes dehydration at the 2 position to yield daidzein [22], catalyzed by a water-soluble dehydratase that has been purified to homogeneity from

P. lobata [21]. Similarly, a microsomal preparation from soybean could convert 7,4′-dihydroxyflavanone directly into daidzein with the corresponding 2-hydroxyisoflavanone as an intermediate [37], and, in alfalfa, microsomal preparations can catalyze the conversion of 5,5,4′-trihydroxyflavanone (naringenin) to genistein with no requirement for methylation [35]. Finally, a mutant of subterranean clover (*Trifolium subterraneum*), which had lost the ability to produce formononetin, accumulated large amounts of daidzein, suggesting that daidzein is the immediate precursor of formononetin [51].

There have been some previous reports on the cloning of cDNAs encoding flavonoid/isoflavonoid *O*-methyltransferases. These include a chalcone 2′-OMT from alfalfa roots involved in the biosynthesis of an inducer of Rhizobial nodulation genes [41], a flavonol OMT from *Chrysosplenium americanum* with 3′/5′-OMT activity for partially methylated flavonols [17], and putative flavonoid OMT cDNA clones isolated from powdery mildew infected barley [18] and from maize roots where they may encode enzymes involved in suberin biosynthesis [24]. Most relevant to the present studies, a cDNA clone encoding an OMT from pea that converts (+) 6a-hydroxymaackiain to the phytoalexin pisatin has been isolated (H.D. VanEtten, personal communication). This reaction is equivalent, at the pterocarpan level, to methylation of an isoflavone at the 7 position.

We here report the cloning of cDNAs encoding isoflavone 7-*O*-methyltransferase (7-IOMT) from alfalfa, as a prelude to functional analysis of its involvement in medicarpin biosynthesis by reverse genetic approaches. We have previously reported the purification and characterization of this enzyme [23] and preliminary peptide sequence data. We describe the production of catalytically active 7-IOMT protein in *E. coli*, the substrate specificity of the recombinant enzyme, and the elicitor-induced expression of 7-IOMT transcripts in alfalfa cell cultures.

Materials and methods

Plant materials and chemicals

Callus and suspension cultures of alfalfa (*Medicago sativa* cv. Apollo) were initiated, maintained, and treated with elicitor as previously described [32]. Cells from elicited and control flasks were harvested by filtration, frozen in liquid N_2, and stored at −80 °C until used for enzyme assay, determination of phenolic compounds or isolation of RNA. Plants were grown and maintained in the greenhouse under standard conditions.

Flavonoids were purchased from Indofine chemicals (Somerville, NJ) except for isoformononetin (Apin Chemicals, Oxon, UK). S-[^{3}H-methyl]adenosyl-L-methionine (2.2–3.1 TBq/mmol) was obtained from Amersham (Arlington Heights, IL). Medicarpin was from our laboratory collection, and (+) maackiain and (+) 6a-hydroxymaackiain were gifts from Dr Hans VanEtten (University of Arizona). All other chemicals were obtained from Sigma (St. Louis, MO).

PCR amplification of the 7-IOMT cDNA fragment

Degenerate oligonucleotide primers for PCR amplification were designed based on the amino acid sequences of three tryptic peptides obtained from the purified enzyme [23]. Since the relative positions of the three tryptic fragments were unknown, degenerate oligonucleotide primers in both the sense and antisense directions for each amino acid sequence were designed based on regions of minimal degeneracy in the genetic code. For the P2 peptide (His-Ile-Tyr-Ala-Phe-Ile-Asp-Ser-Met), primers 5′-CA(C,T)ATITA(C,T)GCITT(C,T)ATICA-3′ (primer P2a) and 5′-TCIAT(G,A)AAIGC(G,A)TAIAT(G,A)TG-3′ (primer P2b) were made. For the P3 peptide (Tyr-Leu-Ala-His-Asn-Gly-Phe-Phe-Glu-Ile-Ile-Thr), primers 5′-GCICA(C,T)AA(C,T)GGITT(C,T)TT(C,T)GA-3′ (primer P3a) and 5′-TC(G,A)AA(G,A)AAICC(G,A)TT(G,A)TGIGC-3′ (primer P3b) were made. For the P4 peptide (Leu-Phe-Ile-Glu-Ala-Gly-Phe-Gln-His-Tyr-Lys), primers 5′-GA(A,G)GCIGGITT(C,T)CA(A,G)CA(C,T)TA(C,T)AA-3′ (primer P4a) and 5′-ATG(C,T)TG(G,A)AAICCIGC(T,C)TCIAT(G,A)AA-3′ (primer P4b) were made. The oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer. The PCR reaction mixture contained 1 μM of each primer, 2.5 units of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, IN), 200 μM each dNTP, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$ 0.1% Triton X 100 and template DNA in a total volume of 100 μl. Template DNA was prepared by boiling a portion of an alfalfa cDNA library [33] and adding 10 μl to the reaction mix. The DNA was denatured at 94 °C for 4 min and amplified through 30 cycles of 1 min of denaturation at 94 °C, 1.5 min annealing at 42 °C and 2 min extension at 72 °C, and a final extension of 7 min at 72 °C. Of the

6 combinations of primers, only the P3a-P4b combination generated a single band PCR product, of 800 bp. Gel-purified PCR products were ligated to pGEM-T Vector (Promega, Madison, WI) and sequenced using a T7 Sequencing kit (Pharmacia, Alameda, CA) according to the manufacturer's instructions. The sequence was analyzed using PCGene software (IntelliGenetics, Mountain View, CA).

Isolation and sequence analysis of IOMT cDNA clones

A λZAPII (Stratagene, La Jolla, CA) cDNA library constructed from elicited alfalfa cell suspension culture RNA [33] was screened with the above 800 bp PCR fragment that had been ^{32}P-labeled by random priming. Phage DNA was transferred to Hybond-N membranes (Amersham) and fixed as recommended by the manufacturer. Prehybridization was performed at 65 °C for 2 h in 5× Denhardt's solution, 5× SSC and 0.5% SDS. Hybridization with a heat-denatured probe was allowed to proceed in the same solution at 65 °C overnight. The membrane was washed under high stringency conditions (0.2× SSC, 0.1% SDS) at 42 °C for 20 min and at 65 °C 3 times for 30 min (each time). Positive plaques were identified by autoradiography and further purified by two additional rounds of screening. The helper phage R408 was used to rescue pBluescript plasmids according to Stratagene's *in vivo* excision protocol. Double stranded full-length IOMT clones were sequenced in both directions using synthetic oligonucleotide primers as necessary to extend the sequence.

Expression of alfalfa IOMT in E. coli *and enzyme activity assay*

The pIOMT8 cDNA was reamplified by PCR using the carboxy-terminal M13 reverse primer and an amino terminal primer which introduces an *Nde*I site at the start of translation (5′-GGCCATATGGCTTCATCAATTAATG-3′). The amplification product was digested with *Nde*I and *Bam*HI, gel-purified and ligated to *Nde*I/*Bam*HI-digested expression vector pET15b (Novagen, Madison, WI). The recombinant plasmids were used to transform *E. coli* DH5α competent cells. A single colony bearing the pET15b-IOMT8 plasmid was isolated. To express IOMT activity, the pET15b-IOMT8 plasmid was transformed into *E. coli* BL21(DE3) (Novagen) competent cells. A single colony was used to inoculate 10 ml LB liquid medium containing 100 μg/ml carbenicillin. When the cell culture reached approximately 0.6 OD_{600} units, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. After an additional 3 h of culture, protein extracts were prepared from the bacterial cells according to the manufacturer's protocol (Novagen) and analyzed for IOMT activity as described [23]. *E. coli* BL21(DE3) harboring pET15b without an insert was used as a negative control. Potential substrates were tested at a final concentration of 1.7 μM. Radiolabeled products were analyzed by silica gel TLC as described [23], using the following solvent systems. System 1: chloroform/methanol/triethylamine, 8:1:1; System 2: chloroform/acetone/25% ammonia, 9:10:5; System 3: chloroform/methanol, 50:2; System 4: benzene/methanol, 9:1.

HPLC analysis of medicarpin and enzyme reaction products

Medicarpin and its conjugated form, medicarpin-3-*O*-glucoside-6″-*O*-malonate (MGM), were extracted from frozen cells using cold acetone and quantified by HPLC as described previously [15].

HPLC analysis of enzyme reaction products was carried out as described [14] using a gradient from 20–55% solvent B in solvent A (over 40 min) followed by 95% solvent B (2 min). Solvent A was H_2O adjusted to pH 10 with triethylamine, and solvent B was tetrahydrofuran. The eluant was monitored at 287 nm. Identification by HPLC was based on the coincidence of retention time of the authentic compounds and radioactivity collected in fractions.

DNA and RNA gel blot analysis

Genomic DNA was isolated [8] from shoot tips of greenhouse-grown young plants of alfalfa (cv. Apollo), chickpea (*Cicer arietinum*), soybean (*Glycine max*), bean (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*), tobacco (*Nicotiana tabacum*) and potato (*Solanum tuberosum*). Genomic DNA was digested with *Eco*RI, *Hind*III, and *Bam*HI, subjected to electrophoresis through a 0.8% agarose gel, and transferred to a Hybond-N membrane by capillary blotting. The membranes were hybridized with the ^{32}P-labeled 800 bp IOMT probe, and washed either under low stringency conditions (0.5× SSC, 0.2% SDS) at 42 °C for 20 min followed by 3 times for 30 min each at 65 °C, or under high-stringency conditions as described above.

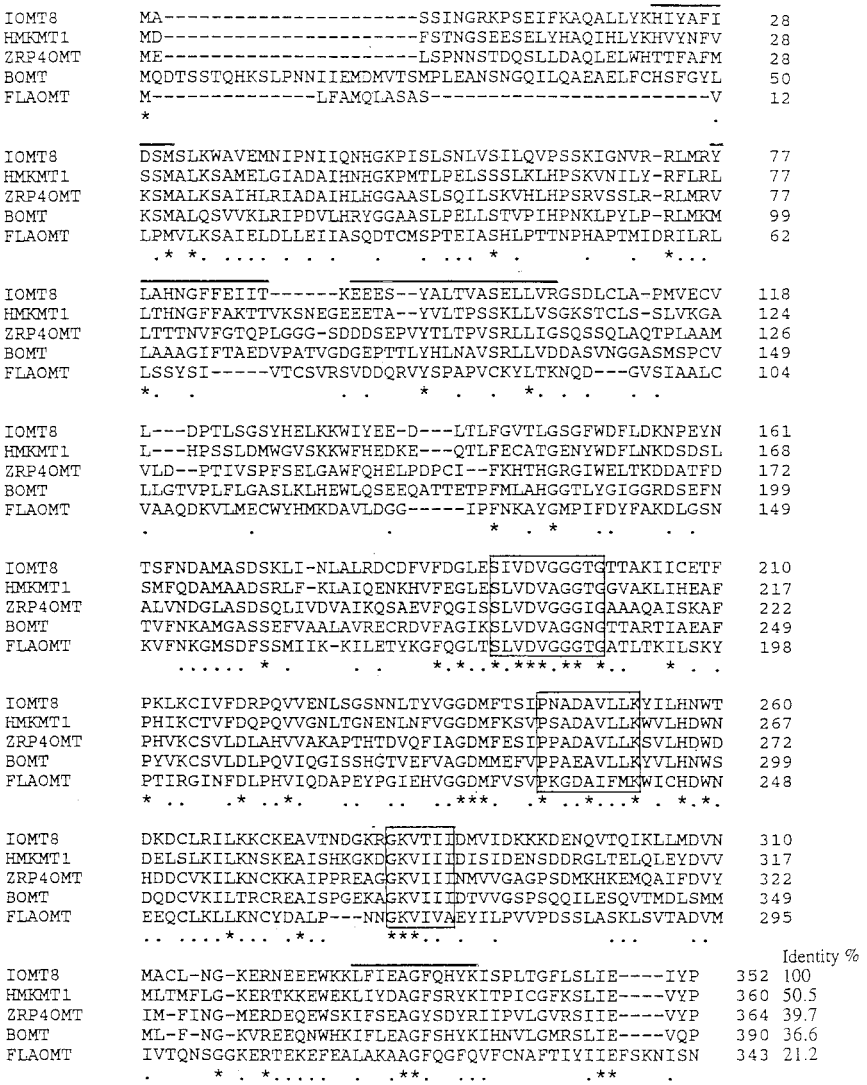


Figure 2. Alignment of the deduced amino acid sequence of IOMT8 (accession number U97125) to various plant phenolic OMTs. HMKMT1, 6a-hydroxymaackiain 3-OMT from pea (H.D. VanEtten, personal communication, accession number U69554); ZRP4OMT, maize putative OMT expressed in roots [24] (accession number L14063); BOMT, barley putative OMT responding to fungal infection [18] (accession number X77467); FLAOMT, flavonol 3′/5′-OMT from *Chrysosplenium americanum* [17] (accession number U16794). Identical amino acids are marked by *. Conserved amino acids are marked by (.). The regions representing amino acids conserved in enzymes that require S-adenosyl-L-methionine as a cosubstrate are shown in the boxes. The sequences overlined represent previously reported [23] IOMT tryptic peptide fragments.

Total RNA was isolated [4] from an elicited alfalfa cell suspension culture at different time points after elicitation. RNA (10 μg/lane) was denatured and separated by electrophoresis through a 1% agarose/formaldehyde gel [47] and transferred to a Hybond-N membrane by capillary blotting. Membranes were hybridized with the 800 bp IOMT probe, and washed under high-stringency conditions as described above for Southern analysis. RNA loading and transfer were checked by reprobing with a soybean 18S ribosomal RNA probe [13].

Results

Isolation and sequence analysis of IOMT cDNA clones

To obtain a probe for screening an alfalfa cell culture cDNA library for IOMT clones, degenerate oligonucleotide primers were designed, in both sense and antisense orientations, based on tryptic peptide sequences from purified alfalfa IOMT [23]. These were used for PCR amplification of alfalfa cDNA library DNA. An 800 bp fragment was generated with the combination of P3a and P4b primers, and was subcloned and sequenced. The tryptic peptides P1, P3 and P4 were contained within the sequence.

The cDNA library [33] (200 000 plaques) was screened using the ^{32}P-labeled 800 bp IOMT PCR fragment as a probe at high stringency, and more than 100 positive clones were detected. Ten positive clones were purified through 3 rounds of plaque purification. Following autoexcision into pBluescript and preliminary 5′ and 3′ end sequencing, three potentially full-length cDNA clones, designated as IOMT 6, 8, and 9, were identified. The nucleotide sequences of these 3 clones were determined. There was considerable nucleotide sequence identity (99.05 ± 0.65%) between these clones, not only in the coding region but also in the 5′- and 3′-untranslated regions. The first ATG at the 5′ end for the three clones (nucleotides 25–30) was surrounded with sequence (AAAAAATGGCT) consistent with the consensus sequence (AAACAATGGCT) for translation start sites proposed for plant genes [30]. The major difference between the clones was in the length of the 3′-untranslated regions, that were 199, 151, and 148 nucleotides for IOMT 6, 8, and 9 respectively. A potential polyadenylation site was located in the 3′-untranslated regions within the expected distance from the poly(A) tail [31]. All three cDNAs used TAA as the stop codon.

Analysis of the deduced IOMT amino acid sequence

The deduced amino acid sequence of the IOMT8 cDNA is shown in Figure 2. The polypeptide, translated from the longest ORF from nucleotide 25 to 1080, contained 352 amino acid residues with a calculated M_r of 39 603, close to the value of 41 000 estimated by SDS-PAGE for the purified IOMT protein [23]. The four tryptic peptides sequenced from the purified protein [23] were identified in the derived open reading frame. Comparison of the deduced amino

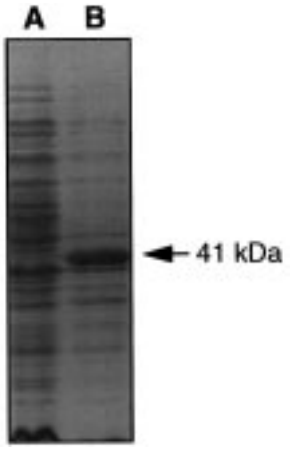


Figure 3. SDS-polyacrylamide gel electrophoresis of alfalfa IOMT8 expressed in *E. coli.* Lane A contains proteins from non-induced cells, and lane B contains proteins from 0.5 mM IPTG induced cells. Proteins were stained with Coomassie blue R-250. The expressed IOMT is indicated by the arrow.

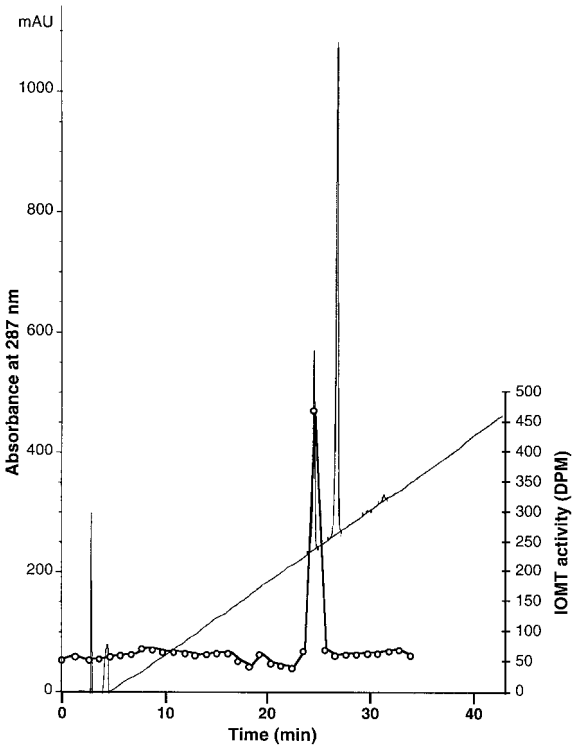
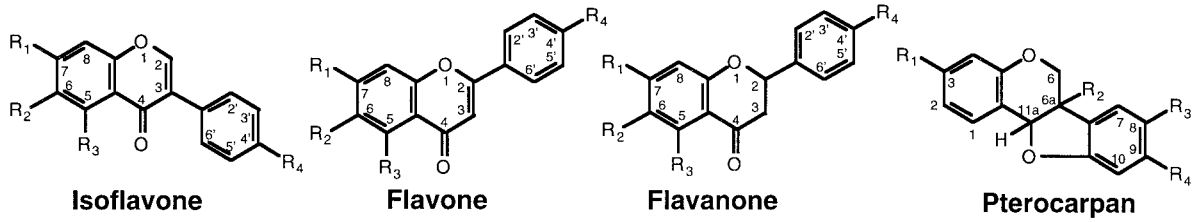


Figure 4. HPLC profile of UV absorbance of authentic isoformononetin (retention time 24.4 min) and formononetin (retention time 26.6 min) at 287 nm (—), and of radioactivity in the reaction product formed by incubating daidzein and [^{3}H-methyl] SAM with IOMT expressed in *E. coli* (o—o).

Table 1. Substrate specificity of alfalfa isoflavone-*O*-methyltransferase expressed in *E. coli.*

Substrates[a]	R1	R2	R3	R4	Activity[b] %
Isoflavone:					
Daidzein	OH	H	H	OH	100
Genistein	OH	OH	OH	OH	12.9
6,7,4′-Trihydroxyisoflavone	OH	OH	H	OH	136
Prunetin	OMe	H	OH	OH	0
Formononetin	OH	H	H	OMe	0
Biochanin A	OH	H	OH	OMe	0
Isoformononetin	OMe	H	H	OH	0
Pterocarpan:					
(+)6a-Hydroxymaackiain	OH	OH	OCH_2O	OCH_2O	49.2
(+)Maackiain	OH	H	OCH_2O	OCH_2O	1.7
(−)Medicarpin	OH	H	H	OMe	0
Flavone:					
Apigenin	OH	H	OH	OH	0
Flavanone:					
Naringenin	OH	H	OH	OH	0

a. Basic structures of substrates:



b. Activities were determined by counting the radioactive spots from TLC plates developed in solvent systems as described in Materials and methods and are given relative to the 7-*O*-methylation of daidzein (100%). The 7-*O*-methyltransferase preparation contained 0.18 pkat of activity per mg protein using daidzein as substrate.

acid sequences from all three alfalfa IOMT clones showed extensive sequence identity (98.9%) with very few amino acid changes, and these were all in regions that are not conserved between different plant OMTs (see Figure 2). IOMT 9 differed from IOMT 6 and 8 by Glu-38 to Gly, Glu-46 to His and to Asp-292 to Asn. In addition, IOMT 8 differed from IOMT 6 and 9 by Glu-293 to Lys.

Comparison of IOMT8 with other plant OMT sequences showed sequence identity ranging from 21.2% to 50.5% (Figure 2), with the highest degree of identity to 6a-hydroxymaackiain 3-*O*-methyltransferase from pea (H.D. VanEtten, personal communication; GenBank accession number U69554). The amino acid sequence alignment highlights a number of conserved regions between all the OMTs. The proposed SAM binding sites for OMTs were identified within the polypeptide [34], as shown in Figure 2.

Substrate and product specificity of IOMT expressed in E. coli

To confirm that the cDNA clone did indeed encode an isoflavone-*O*-methyltransferase, IOMT8 was cloned into the pET15b vector for expression of enzyme activity in *E. coli.* As shown in Figure 3, a strongly expressed protein band of M_r 41 000 was observed after induction by IPTG. Although a low level of IOMT activity was observed in uninduced cultures, IPTG induction led to an 8.8-fold increase in IOMT activity against daidzein as substrate. No IOMT activity was measurable in *E. coli* cells harboring empty pET15b vector. As shown in Table 1, the expressed enzyme could methylate daidzein, genistein and 6,7,4′-

trihydroxyisoflavone as previously reported for the purified enzyme [23]. In addition, it also methylated (+)6a-hydroxymaackiain (HMK) with 49% activity compared to that with daidzein as substrate, but had very low activity against (+)maackiain. As reported for the purified enzyme previously [23], isoformononetin was the only reaction product of daidzein *O*- methylation, as identified by TLC (Rf 0.88 in solvent system 1), and HPLC (Figure 4), in comparison with the authentic product. Prunetin was the major reaction product from genistein, as determined in comparison with the authentic product by TLC (Rf 0.88 in solvent system 1 and 0.35 in solvent system 2). The reaction product with HMK is most likely pisatin, in comparison with the reported Rf value on TLC plates [28] (Rf 0.81 in solvent system 3, 0.63 in solvent system 4). IOMT expressed in *E. coli* could not further methylate formononetin, isoformononetin, prunetin or medicarpin, and flavones and flavanones were not methylated.

Presence and genomic organization of IOMT in alfalfa and other species

Southern blot analysis of alfalfa genomic DNA showed three to four hybridizing bands with high molecular weights in *Eco*RI or *Bam*HI digests, and six major bands following digestion with *Hin*dIII (Figure 5A). The IOMT cDNA clones do not contain *Eco*RI or *Bam*HI sites, but do contain a single *Hin*dIII site. The Southern blot data therefore suggest that IOMT genes are present as a small family in the alfalfa genome. The IOMT probe also hybridized to genomic DNA fragments from chickpea, soybean, bean and cowpea under low-stringency conditions, but not to genomic DNA from tobacco or potato (Figure 5B). However, under high-stringency condition, signals were only seen with alfalfa, chickpea and cowpea (data not shown). Tobacco and potato do not produce isoflavonoids, and the isoflavonoids of bean and soybean are not methylated. Chickpea produces medicarpin [27], and cowpea produces methylated 2-arylbenzofuran phytoalexins which may be derived from a 7-*O*-methylated isoflavonoid [46]. Thus, the presence of IOMT-related gene sequences correlates with the production of both 4′- and 7-*O*-methylated isoflavonoids by the plants in this small survey.

Developmental expression of IOMT

No IOMT transcripts were detected in the total RNA fraction from flowers, leaves, stems and roots of 2-month-old alfalfa plants. Correct sized transcripts (approximately 1.5 kb) were, however, detected in root tissue of one-week-old alfalfa seedlings (data not shown), consistent with constitutive accumulation of methylated isoflavonoids in young roots [49].

Elicitor-induced changes in IOMT transcripts and enzymatic activity in relation to the phytoalexin response

Northern blot analysis showed the presence of IOMT transcripts of about 1.5 kb in the total RNA fraction from elicitor-treated alfalfa suspension cells harvested at different times after elicitation. The transcript level rapidly increased after elicitor treatment; it was first detected after 1 h, and reached its first peak at 2 h and its maximum level at 12 h after elicitation. Little transcript was detected in untreated cells throughout the time course (Figure 6A). These changes in IOMT transcripts precede the accumulation of IOMT extractable activity and medicarpin and MGM in the elicited cultures (Figure 6B).

Discussion

We have isolated three full-length cDNA clones encoding an isoflavone-*O*-methyltransferase from alfalfa. Identity of the clones was confirmed by location of the internal peptide sequences obtained from the purified IOMT protein within the deduced amino acid sequence of the clones and by expression of IOMT enzyme activity in *E. coli.* The expressed enzyme had the same substrate specificity as the purified enzyme from elicited cell cultures [14, 23], and primarily methylates the 7-hydroxyl-group of isoflavonoids.

Sequence alignments of IOMT8 with other plant OMTs showed highest sequence identity to 6a-hydroxymaackiain (HMK) 3-OMT and, as predicted, the expressed IOMT had relatively high activity with (+)6a-hydroxymaackiain, but low activity with (+)maackiain. The formation of pisatin by methylation of the 3 position of the pterocarpan HMK is the analogous reaction to the formation of isoformononetin from daidzein at the isoflavone level. In alfalfa, pterocarpans with the (–) configuration are produced whereas pea produces pterocarpans with the (+) configuration [9]. It is therefore interesting that the alfalfa IOMT has activity against a (+) pterocarpan.

IOMT transcript levels increased as early as 1 h after elicitation, reaching maximum levels at 12 h after

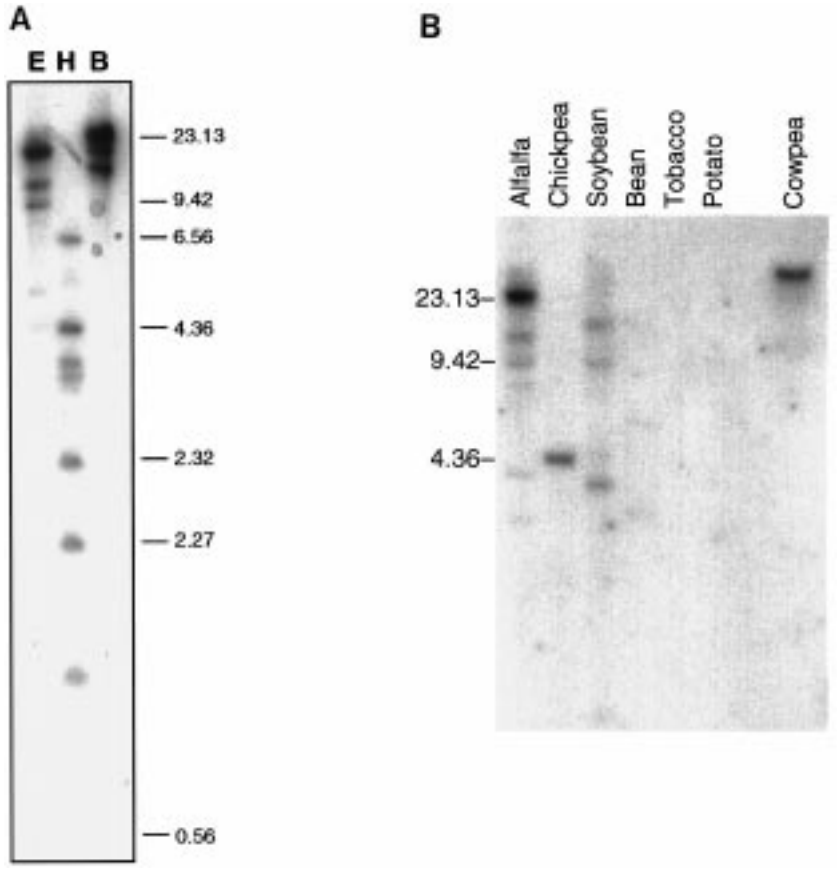


Figure 5. Southern-blot analysis of genomic DNA from various plants for the presence of IOMT-related sequences. A. Genomic DNA from alfalfa was digested with *Eco*RI (E), *Hin*dIII (H), or *Bam*HI (B) and the blot was hybridized with a ^{32}P-labeled 800 bp IOMT probe at high stringency. B. Genomic DNA from alfalfa, chickpea, soybean, bean, tobacco, potato and cowpea was digested with *Eco*RI and hybridized with the IOMT probe at low stringency. Hybridizing bands in the soybean and bean lanes were not detected under high stringency conditions (data not shown). The size of the DNA size standards is indicated in kb on the left of the picture.

elicitation. Maximum levels of free medicarpin were reached at 24 h after elicitation. The elicitation pattern of IOMT transcripts is therefore similar to those of other isoflavonoid pathway enzymes such as isoflavone reductase and vestitone reductase, particularly with respect to the rapid response to elicitation within 1–2 h [19, 44]. IOMT is therefore coordinately regulated with the other enzymes involved in medicarpin biosynthesis.

Multiple IOMT genes are present in the alfalfa genome, as confirmed by Southern blot and cDNA sequence analysis. This is not unexpected considering the heterotetraploid nature of the plant. Multiple phenolic OMT genes have also been reported in poplar [3], maize [5] and *Chrysosplenium americanum* [17]. Gene sequences closely related to IOMT are present in chickpea and cowpea. Chickpea, like alfalfa, accumulates medicarpin as a phytoalexin [27]. Furthermore, following inoculation of cowpea seedlings with *Colletotrichum lindemuthianum*, an isoflavone-derived 2-arylbenzofuran phytoalexin with two methoxyl groups is produced [46]. Although the methyltransferase(s) for 2-arylbenzofuran methylation in cowpea is unknown, it may be closely related to the alfalfa isoflavone *O*-methyltransferase.

The alfalfa isoflavone *O*-methyltransferase purified from cell cultures [23] or expressed in *E. coli* possesses 7-IOMT activity, in contrast to the 4′-OMT activity believed to exist *in vivo*. The correlative evidence above links the presence and expression of 7-IOMT to the formation of 4′-*O*-methylated isoflavonoids. Very good incorporation of 4,2′,4′-trihydroxychalcone (Figure 1) into formononetin and medicarpin in alfalfa [11], and of 5,7,4′-trihydroxyflavanone into biochanin A in

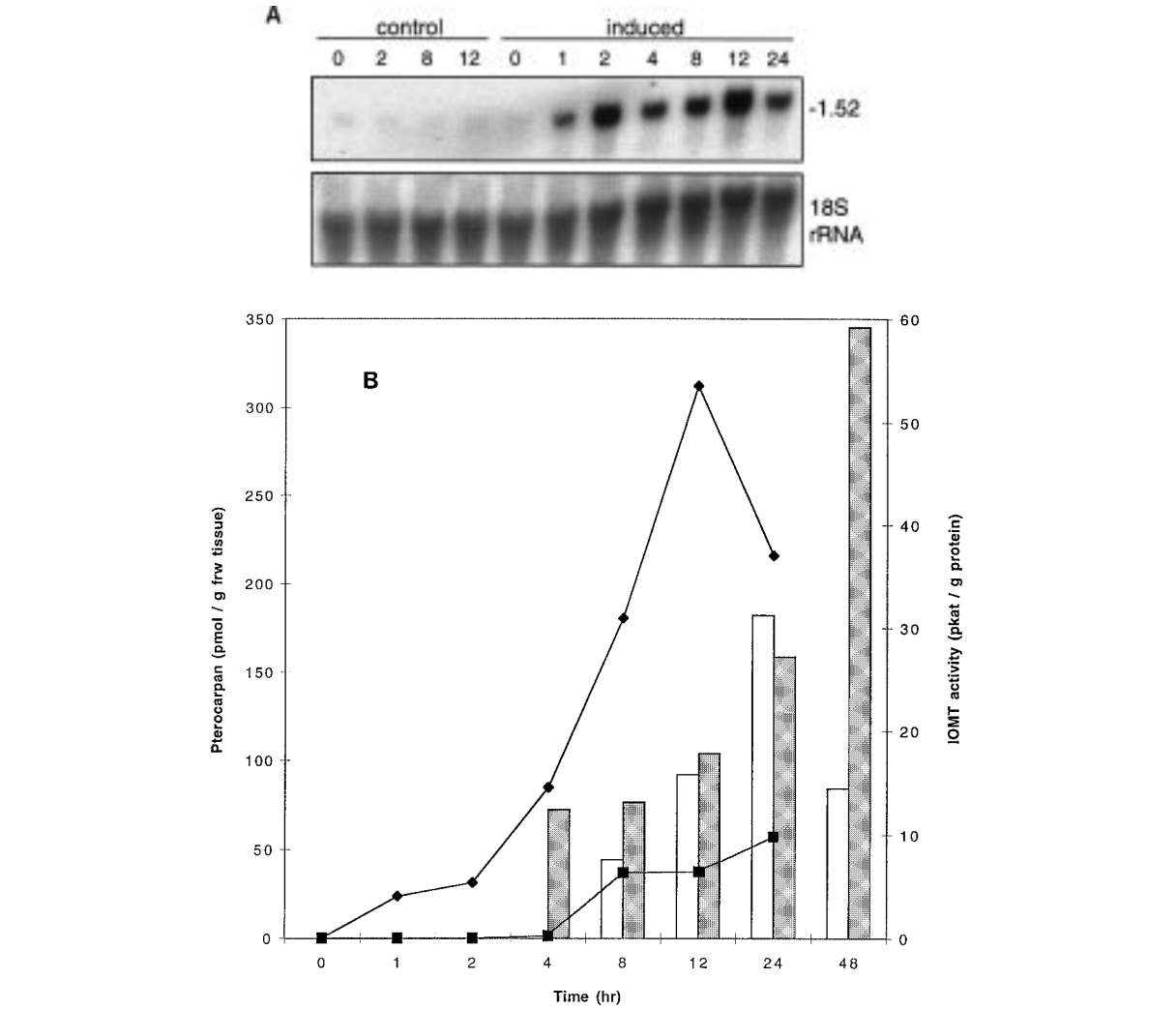


Figure 6. Elicitor induction of IOMT transcripts (**A**) and medicarpin and IOMT activity (**B**) in cell suspension cultures. Northern-blot analysis was performed on total RNA from alfalfa cells with (induced) or without (control) elicitor treatment for the time (in h) shown at the top of the lanes. The blot was probed with the ^{32}P-labeled 800 bp IOMT PCR fragment. A soybean 18S rRNA [13] probe was used to reprobe the blot to check the RNA loading in each lane (lower panel). ♦—♦, IOMT activity in elicited cells; ■—■, IOMT activity in unelicited cells; open bar = medicarpin level and solid bar = medicarpin conjugate (malonyl glucoside) level in elicited cells. Medicarpin or medicarpin conjugate were not detected in unelicited controls.

chickpea [38], have been shown in feeding experiments. A subterranean clover mutant which had lost the ability to produce formononetin accumulated substantial amounts of daidzein [51]. Furthermore, 7-*O*-glucosyltransferases, with substrate specificity toward formononetin rather than daidzein in alfalfa, or toward formononetin and biochanin A in chickpea, have been isolated and characterized [9, 45]. These studies all suggest that 4′-*O*-methylation of isoflavonoids occurs at a stage after 7,4′-dihydroxyflavanone formation but earlier than glucosylation, and therefore implicate daidzein as the immediate substrate for IOMT and precursor of formononetin.

All the enzymes so far characterized in the isoflavone branch of the phenylpropanoid pathway respond to elicitation with highly increased extractable activities [12]. It would be expected that the methyltransferase activity would respond similarly at both enzyme activity and transcript levels, as observed for the 7-IOMT activity. We therefore propose that the elicitor-induced 7-IOMT is the enzyme that catalyzes the 4′-*O*-methylation of daidzein *in vivo.*

This reaction may occur in a metabolic channel in which a microenvironment is created that favors 4′-*O*-methylation over 7-*O*-methylation. This hypothesis would explain why radiolabeled daidzein is poorly incorporated into formononetin and medicarpin in alfalfa [11], and why only very low concentrations of extractable free daidzein are detected in alfalfa and subterranean clover [49, 51]. Furthermore, blocking the methylation step by inhibition of *S*-adenosyl-L-homocysteine hydrolase with tubericidin caused the accumulation of 7,4′-dihydroxyflavanone (Figure 1) with concomitant reduction of isoflavonoid phytoalexin biosynthesis in alfalfa cell cultures [7], suggesting the possibility of a linked functional metabolic unit from flavanone to methylated isoflavone. The enzymes that flank IOMT, isoflavone synthase and isoflavone 2′-hydroxylase, are both membrane-associated cytochrome P450s [27, 36, 37] that could provide anchoring of loosely associated enzymes to the surface of the endoplasmic reticulum. It is possible that the 4′-methoxyl group is introduced during the aryl migration even though it is not required for the reaction; perhaps aryl migration favors ionization of 4′-hydroxyl over that of the 7-hydroxyl group.

An alternative explanation is that no studies to date have reproduced the conditions necessary for obtaining optimal isoflavone 4′-OMT activity *in vitro*, and that the 7-IOMT we have cloned is not involved in medicarpin biosynthesis. A single report describes the production of alfalone, an isoflavone methylated at both 4′ and 7-positions, in alfalfa cell cultures exposed to a fungal naphthoquinone [36]. Surprisingly, these cultures were not reported to produce medicarpin. The availability of 7-IOMT clones now makes it possible to address the question of the *in vivo* function of this enzyme using reverse genetic approaches.

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