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ISOFLAVONE *O*-METHYLTRANSFERASE ACTIVITIES IN ELICITOR-TREATED CELL SUSPENSION CULTURES OF *MEDICAGO SATIVA**

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Key Word Index—*Medicago sativa*; Leguminosae; isoflavone 7-*O*-methyltransferase; phytoalexin biosynthesis.

Abstract—Treatment of alfalfa cell suspension cultures with elicitor preparations from baker's yeast or from cell walls of *Colletotrichum lindemuthianum* resulted in a *ca* 200-fold induction of isoflavone *O*-methyltransferase (IOMT) activity. The elicited cultures contained *O*-methyltransferase activity against isoflavone, isoflavan and pterocarpan substrates. These activities could be separated into two distinct fractions by ion-exchange chromatography. The major IOMT activity (IOMT II) was purified to homogeneity by a combination of anion exchange chromatography, hydrophobic interaction chromatography and chromatofocussing. It is a monomeric enzyme of subunit M_r 41 000 which could be photoaffinity labelled with tritiated SAM. IOMT II converted the isoflavone daidzein to its 7-*O*-methyl ether isoformononetin, with K_m values of 20 μM for daidzein and 150 μM for SAM and a pH optimum of 8.5. Both IOMT II and the less abundant IOMT species (IOMT I) exhibited greatest activity with 6,7,4′-trihydroxyisoflavone as methyl acceptor. IOMT I, but not IOMT II, also catalysed the A-ring methylation of the pterocarpan phytoalexin medicarpin. Isoflavone 4′-OMT activity, which is believed necessary for the formation of the B-ring methoxy substituent of medicarpin, was present at very low activity in extracts from the cultures and was only weakly induced by elicitor.

INTRODUCTION

Treatment of alfalfa cell suspension cultures with elicitor preparations from the cell walls of *Colletotrichum lindemuthianum* or from baker's yeast results in the accumulation of the phytoalexin medicarpin (3-hydroxy-9-methoxypterocarpan) and its malonylated glycoside [1, 2]. The other major isoflavonoid identified in the cultures is a malonylglucoside of afrormosin (7-hydroxy-6,4′-dimethoxyisoflavone) or its isomer alfalone (6-hydroxy-7,4′-dimethoxyisoflavone). However, this conjugate is produced constitutively and does not accumulate further in response to elicitor, although labelling studies suggest that its synthesis and turnover may be increased in elicited cells [2]. Accumulation of medicarpin is preceded by large increases in the extractable activities of all the known enzymes involved in its formation from L-phenylalanine [1, 3]. Elicitor strongly induces an activity catalysing the transfer of the methyl group of SAM to the isoflavone daidzein [1]. This reaction yields formononetin, which is assumed to be a precursor in medicarpin biosynthesis. However, the biosynthetic origin of the methoxy group at the 9-position of medicarpin (4′ position, isoflavone numbering) is unclear. Although ^{14}C-labelled formononetin (7-hydroxy-4′-methoxyisoflavone) was efficiently incorporated into medicarpin in $CuCl_2$-treated alfalfa seedlings, daidzein was not [4]. We examine the range of isoflavone *O*-methyltransferase (IOMT) activities induced in elicitor-treated alfalfa cell suspension cultures, and report the purification to homogeneity of the major induced IOMT activity, which surprisingly is a 7-OMT unlikely to be involved in the formation of medicarpin.

RESULTS AND DISCUSSION

OMT activities in elicited alfalfa suspension cultures

In order to determine the range of isoflavonoid-specific OMT activities in elicited alfalfa cell cultures, [$^{14}CH_3$]SAM was incubated with potential substrates and unfractionated, $(NH_4)_2SO_4$-precipitated or partially purified (Mono Q FPLC fractionated) protein preparations from cells treated with elicitor from baker's yeast. Methylated products were partitioned into EtOAc + hexane (1:1), whereas unreacted SAM remained in the aqueous phase. Products were analysed by TLC or HPLC using the systems described in the Experimental (R_f values and elution vols are summarized in Table 1). Mono Q FLPC resolved two IOMT activities (IOMT I and IOMT II) measured with daidzein as substrate (Fig. 1), which were themselves partially resolved from caffeic acid OMT (COMT), the major OMT activity in the alfalfa cultures [5]. The data in Table 2 compare the methylation of a number of isoflavones and related compounds relative to that of daidzein. In crude preparations and the separated IOMT I and IOMT II

*Part 9 in the series 'Stress Responses in Alfalfa (*Medicago sativa* L.)'. For Part 8 see ref. [17].

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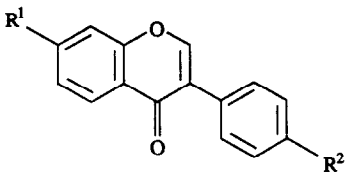
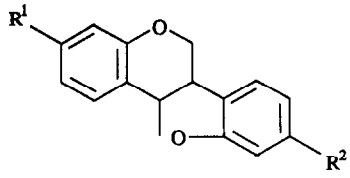
Table 1. Chromatographic behavior of substrates and potential products of isoflavone methylation as determined by TLC and HPLC

Compound*	System†:	TLC (R_f) 1	TLC (R_f) 2	TLC (R_f) 3	HPLC [Elution vol. (ml)] I	HPLC [Elution vol. (ml)] II
Isoflavones:						
Daidzein (1)				0	14.1	9.5
Formononetin (2)		0.50	0.47	0.04	23.2	11.7
Isoformononetin (3)		0.50	0.88	0.30	23.2	20.8
Methyl formononetin (4)		0.60	1.00	ND‡	ND	ND
Pterocarpans:						
3,9-Dihydroxypterocarpan (5)		0.72	ND	0.12	16.6	21.0
Medicarpin (6)		0.79	ND	0.36	27.2	23.2
Isomedicarpin (7)		0.79	ND	0.43	27.2	24.4
Methyl medicarpin (8)		0.90	ND	0.79	38.3	30.6
Unknown methylated product (X)		0.57	1.00	0.50	ND	ND

*Structures:



1 $R^1=R^2=OH$
2 $R^1=OH, R^2=OMe$
3 $R^1=OMe, R^2=OH$
4 $R^1=R^2=OMe$



5 $R^1=R^2=OH$
6 $R^1=OH, R^2=OMe$
7 $R^1=OMe, R^2=OH$
8 $R^1=R^2=OMe$

†See Experimental.
‡ND: Not determined.

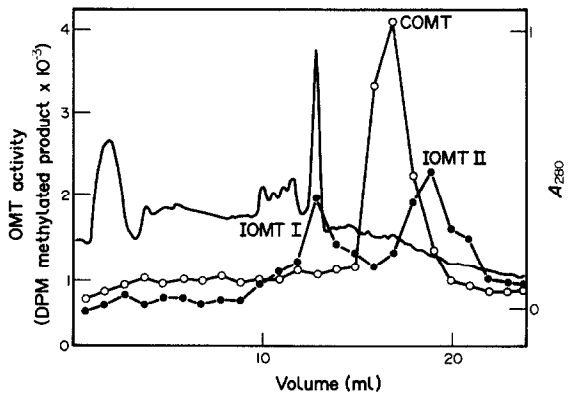


Fig. 1. Mono Q FPLC of extracts from alfalfa cells treated for 16 hr with yeast elicitor. ——$=A_{280}$ nm, O—O = COMT activity, ●—● = IOMT activity.

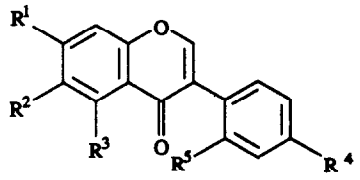
fractions, the most effective substrate for *O*-methylation was 6,7,4′-trihydroxyisoflavone. The IOMT I fraction methylated all isoflavones and pterocarpans tested, whereas IOMT II was virtually inactive against most mono-methylated isoflavones and the two pterocarpans. TLC or HPLC under basic conditions (systems 3 and II respectively in Table 1) could resolve formononetin and isoformononetin, the two potential products of the mono-*O*-methylation of daidzein. Using crude or partially purified enzyme preparations, the predominant product was shown to be isoformononetin, with production of formononetin representing only 4% of the methylating activity (Fig. 2). The major IOMT activity in the alfalfa cell extracts is therefore an isoflavone 7-OMT rather than the 4′-OMT required for the biosynthesis of medicarpin. Although the position of methylation was not determined for the other substrates tested in Table 2, 7-*O*-methylation is probable in each case. No dimethylation of substrates was observed where tested (with daidzein or medicarpin).

When either crude or $(NH_4)_2SO_4$-precipitated protein extracts from elicited cells were incubated with daidzein and [$^{14}CH_3$]SAM and the reaction products analysed by TLC in solvent 1, two radioactive metabolites were sometimes resolved. In addition to material which co-chromatographed with formononetin/isoformononetin an unknown methylated product (X) could be observed running at a slightly higher R_f value (Table 1). After purification of the enzyme preparation through DEAE-Sepharose, X was no longer observed as a reaction product, and it was, therefore, concluded that it is

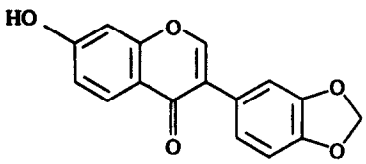
Table 2. Substrate specifities of IOMT activities from elicited alfalfa suspension cultures

Substrate*						% Activity†		
						Total extract	IOMT I	IOMT II
Isoflavones:	R^1	R^2	R^3	R^4	R^5			
Daidzein	OH	H	H	OH	H	100	100	100
Genistein	OH	H	OH	OH	H	101	87	50
Prunetin	OMe	H	OH	OH	H	73	139	19
6,7,4'-Trihydroxy isoflavone	OH	OH	H	OH	H	251	577	178
Pseudobaptigenin	OH	H	H	Methylene dioxy	H	ND‡	ND	3
Formononetin	OH	H	H	OMe	H	0	63	5
Biochanin A	OH	H	OH	OMe	H	2	66	6
Isoformononetin	OMe	H	H	OH	H	3	ND	2
2'-Hydroxyformononetin	OH	H	H	OMe	OH	3	ND	55
Pterocarpans:								
3,9-Dihydroxypterocarpan						6	110	3
Medicarpin						0	150	4
Isoflavan:								
Vestitol						ND	ND	149

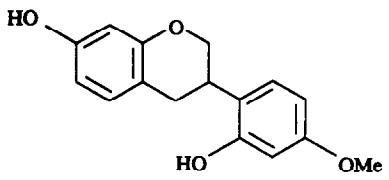
*Structures of substrates:



General isoflavone structures



Pseudobaptigenin



Vestitol

†Activities were determined in duplicate and are given relative to the *O*-methylation of daidzein (100%). The total extract, IOMT I and IOMT II contained 93, 15 and 35 fkat of activity per assay respectively.

‡ND: Not determined.

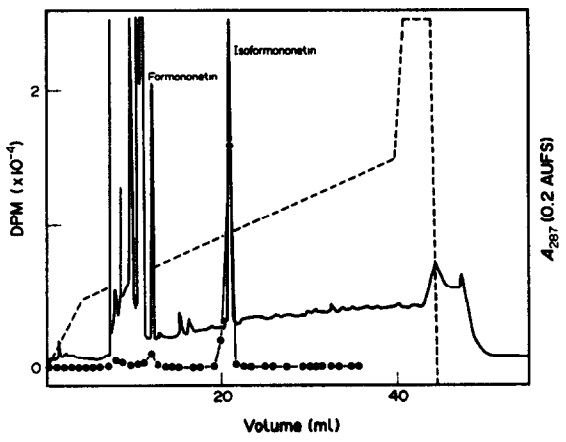


Fig. 2. HPLC profile (system II, see Experimental) of UV absorbance at 287 nm and radioactivity (●—●) of reaction products formed by incubating daidzein and [$^{14}CH_3$]SAM with a crude extract from alfalfa cells treated for 48 hr with yeast elicitor. This preparation contained negligible levels of activity against endogenous phenolic substrate(s). Aufs = absorption units full scale, --- = solvent gradient (see Experimental).

probably a methylation product of an endogenous phenolic compound which bound to extracted proteins.

Alfalfa suspension cultures were treated with yeast elicitor and $(NH_4)_2SO_4$-precipitated extracts from the cells assayed for IOMT (4' and 7-specific) activities, and for the formation of the unknown methylated product X, using two-dimensional TLC of radiolabelled products in solvents I and II. All three OMT activities were negligible in control cultures (~1 nkat kg^{-1} protein) and remained at this level throughout the 48 hr period in untreated flasks (data not shown). Elicitor treatment resulted in increases in the extractable activities of all three methyltransferase reactions, as assayed at pH 8.5 (Fig 3). The most marked elicitation was seen for the isoflavone 7-OMT, which was induced 200-fold within 12 hr of elicitor treatment. In contrast, the isoflavone 4'-OMT activity only increased 10-fold over the 48 hr period. The methyl transferase which catalysed the formation of the uncharacterized product X was rapidly induced, by 80-fold, within 4 hr of elicitor treatment. Its apparent specific activity remained elevated for up to 16 hr and then slowly declined, such that in some preparations from cells treated for 48 hr with elicitor this activity was no longer detectable. It is not clear whether this apparently transi-

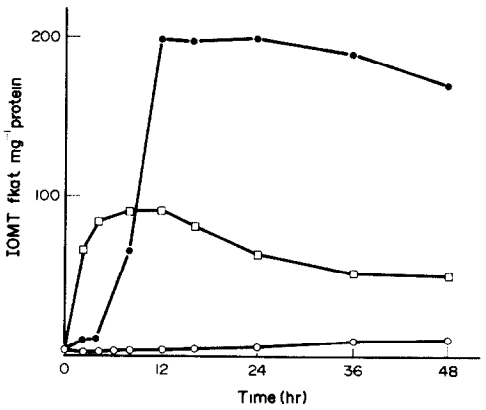


Fig. 3. Induction of 4'-IOMT (○—○), 7-IOMT (●—●) and the activity catalysing the formation of the unidentified methylated product X (□—□) in alfalfa cell cultures (cv Apollo) treated with yeast elicitor.

ent induction is caused by regulation of enzyme level/activity or merely reflects changes in the levels of endogenous substrate. In either event, the rapid induction of this methyl transferase activity warrants further study.

Changes in the proportions of the IOMT I and IOMT II fractions during elicitation were determined by analysing $(NH_4)_2SO_4$ precipitated cell extracts by Mono Q FPLC (data not shown). The fold-induction of IOMT I was approximately half that observed for IOMT II (17-fold), and by 48 hr post-elicitation IOMT I was only a minor component (*ca* 6%) of the total IOMT extractable activity of the cells. IOMT I activity only increased after a lag of 8 hr, whereas a nearly 10-fold increase in IOMT II activity was observed during this period.

Microsomes were prepared [3] from freshly harvested suspension cultures of alfalfa which had been treated with fungal elicitor for 16 hr. Of the total IOMT activity present in the post-10 000 *g* supernatant (371 nkat kg^{-1} protein), 89% of the activity was recovered in the cytosol and only 1.2% in the microsomal pellet. We therefore conclude that the bulk of IOMT activity is not integrally membrane bound, in contrast to the localization of COMT in callus cultures derived from Douglas fir [6].

Purification of IOMT II

Mixed alfalfa cell culture batches of cv Apollo and cv Calwest 475, elicited by exposure to a cell wall preparation from *Colletotrichum lindemuthanium*, were used as source for purification of IOMT II. In preliminary studies, five-day-old seedlings of 17 cultivars of alfalfa were also screened as potential sources of enzyme. Seedlings were treated with $CuCl_2$ (an elicitor of phytoalexin accumulation in intact plants [4, 8]) and IOMT activity determined. Although considerable variation in the induced IOMT activity was observed between cultivars, none of the seedling sources proved to be a better source of the enzyme than elicited cell cultures.

The results of a typical purification are shown in Fig. 4, and are summarized in tabular form in Table 3. A proportion of the enzyme activity was irreversibly lost by precipitation with $(NH_4)_2SO_4$, and concentration by this method was therefore avoided, if possible, during subsequent steps in the purification. IOMT activity (with daidzein as substrate) eluted from DEAE-Sepharose as a minor peak (IOMT I) between 50 and 70 mM $(NH_4)_2SO_4$ and a major peak between 90 and 110 mM $(NH_4)_2SO_4$ (IOMT II) (Fig. 4a). Further purification of IOMT I was not attempted. IOMT II was applied to an HPLC-HIC column and the activity eluted as a single major hydrophobic peak with a shoulder of lower hydrophobicity (Fig. 4b). This chromatographic procedure gave a good-fold purification and could also be used to concentrate dilute enzyme preparations. Purification of IOMT II was concluded by chromatofocussing and Mono Q anion exchange FPLC (Fig. 4c, d). Using the above procedures, the enzyme activity was enriched 362-fold with a 4.5% recovery. Due to the instability of the enzyme activity, it was necessary to complete the final three chromatographic steps within a 12 hr period.

In protein purifications which used extracts from cells treated with elicitor from baker's yeast, IOMT II could be resolved by Mono Q FPLC into two forms, IOMT IIA and IOMT IIB, which eluted at 19 and 21 ml respectively and which contained equivalent amounts of enzyme activity (data not shown). In Fig. 4d, showing the Mono Q separation of IOMT from cells treated with elicitor from *Colletotrichum lindemuthianum*, IOMT IIB appears as a shoulder eluting after the major peak of IOMT IIA.

Properties of IOMT II

Purified IOMT II preparations from yeast elicitor-treated cells were photoaffinity labelled with [C^3H_3]SAM [8] and analysed by SDS-PAGE. The gels were silver stained and the ^3H-labelled OMT present identified by fluorography (Fig. 5). As determined by silver staining, both IOMT IIA and IOMT IIB preparations contained a single major polypeptide of M_r 41 000. Photoaffinity labelling confirmed that the 41 000 protein was an OMT, and no other tritiated peptides were observed.

The M_r of alfalfa IOMT II (41 000) corresponded closely to the 43 000 photoaffinity labelled peptide present in purified preparations of hydroxymaackiain *O*-methyltransferase (HMKMT) from peas [7]. However, the native M_r of HMKMT was determined to be 66 000 and there was some uncertainty as to whether the enzyme was composed of one or more subunits. DEAE-purified preparations of IOMT II were analysed by Superose 6 FPLC, which indicated an apparent native M_r of 42 200. IOMT II is therefore catalytically active as the monomer. The M_r of IOMT corresponded exactly to that determined for COMT from alfalfa [5], and the two activities co-eluted from gel-filtration columns. It is also similar to the M_rs of three *o*-diphenol OMTs from tobacco [8] and of a SAM: 3'-hydroxy-*N*-methyl-(*s*)-coclaurine-4'-OMT involved in isoquinoline alkaloid biosynthesis in *Berberis* spp. [9], but is very different from that reported for the IOMT from suspension cultures of chickpea (110 000) [10].

The isoelectric points of IOMT IIA and IIB were determined by HPLC-Mono P chromatography to be 4.9 and 4.3 respectively. Chromatofocussing of HMKMT in pea extracts indicated that this OMT was present as two isoenzymic forms of pI 5.2 and 4.9 [8].

IOMT II activity had a pH optimum of 8.5 in Tris–HCl and 8.7 in glycine–NaOH buffer. These values correspond closely to the pH optima determined for

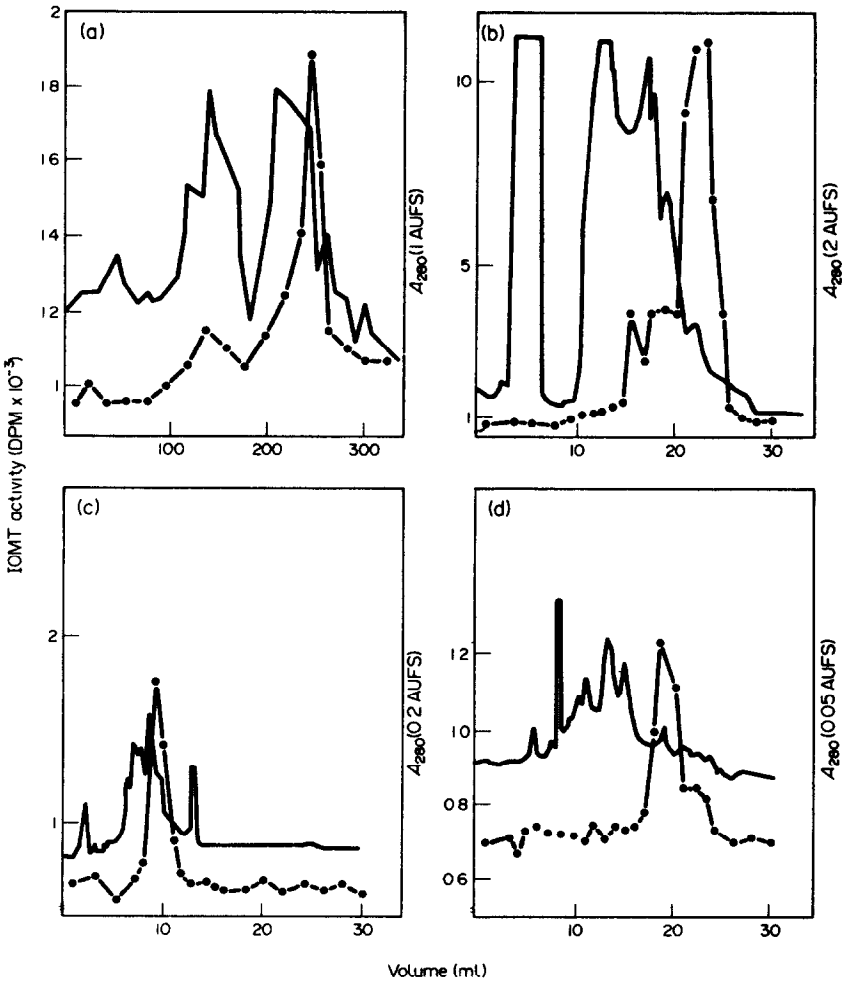


Fig. 4. Purification of IOMT II from elicited alfalfa suspension cultures by (a) chromatography on DEAE-Sepharose, (b) hydrophobic interaction HPLC, (c) chromatofocussing pH 7–4 (FPLC) and (d) Mono Q anion exchange FPLC. The unbroken lines show absorbance at 280 nm and the data points (●—●) IOMT activity. Note that activity was determined by measuring the total radioactivity which partitioned into a toluene-based scintillant from fractions which were incubated with daidzein and [$^{14}CH_3$]SAM (hence high backgrounds).

Table 3. Purification of isoflavone 7-O-methyltransferase activity from elicited alfalfa cell suspension cultures

Purification step	Protein (mg)	Specific activity (μkat kg^{-1} protein)	Purification (-fold)	Recovery (%)
10000 *g* supernatant	373	0.06	1	100
$(NH_4)_2SO_4$ precipitate, 45–80% saturation	226	0.04	0.7	40
DEAE-Sepharose CL-6B anion exchange	21	0.8	14	75
Hydrophobic interaction-HPLC	1.7	5.0	91	38
Mono P chromatofocussing	0.5	8.1	146	18
Mono Q anion exchange	0.05	20.1	362	4.5

chickpea IOMT in these buffers (8.6 and 9.0 respectively) [10]. Enzyme activity was not affected by inclusion of 5 mM Mn^{2+}, Mg^{2+}, or EDTA in the assay mixture, nor by 1 mM azide or cyanide; however, as reported for other OMTs, IOMT II activity was inhibited by *p*-chloromercuribenzoate (43% inhibition at 1 mM). Activity was increased by up to 39% in the presence of MeSH, DTT or dithioerythritol at concentrations of 10–20 mM. Similarly, the stability and activity of IOMT from chickpea [10] and HMKMT from peas [8] was enhanced by sulphydryl reagents. IOMT was routinely assayed in the presence of 5 mM EDTA and 20 mM MeSH.

IOMT activity was stabilized in the presence of EDTA and thiol-containing reagents. When stored in assay

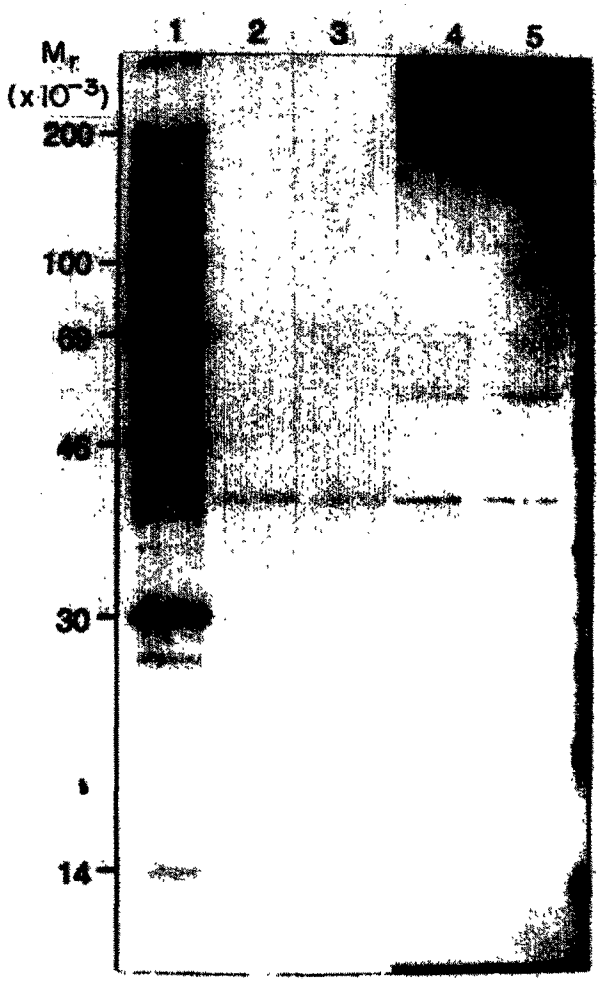


Fig. 5. SDS-PAGE analysis of purified IOMT IIA and IOMT IIB as determined by fluorography of photoaffinity labelled enzyme (lanes 2 and 3 respectively) and by silver staining (lanes 4 and 5 respectively). M_r markers (^{14}C) are shown in lane 1.

buffer, a 200-fold purified IOMT II preparation had a half-life of 60 hr at 4° (4 hr in the absence of EDTA and MeSH). Freezing and storage at −70° (for up to 2 months) of purified IOMT II in assay buffer resulted in a 27% loss in activity which could be greatly reduced by the addition of 10% (v/v) glycerol (4% loss).

IOMT II was most active towards isoflavones with a free 7-hydroxy function with daidzein, 6,7,4′-trihydroxyisoflavone and the isoflavan vestitol the preferred substrates. Methoxylated isoflavones or pterocarpans were poor substrates for IOMT II, except for the case of 2′-hydroxyformononetin which was, however, less actively methylated than its corresponding isoflavan vestitol. IOMT II did not catalyse the methylation of apigenin, kaempferol, flavonoids mono-hydroxylated in the 2′,3,4′,5, or 7 positions, flavanones such as naringenin or coumestans such as coumestrol. The evidence, therefore, suggests that IOMT II is an isoflavone 7-*O*-methyltransferase rather than a 4′-*O*-methyltransferase. This was confirmed by analysing the reaction products at each stage of the enzyme purification.

Although the isoflavone 7-*O*-methyltransferase could be purified away from the very low level of isoflavone 4′-*O*-methyltransferase activity present in crude extracts, it was not possible to completely remove all COMT activity from IOMT II. Thus, final preparations (after the 5 purification stages shown in Table 3) always contained COMT activity up to a maximum of 21% of the IOMT

activity. Although COMT in alfalfa has no activity toward isoflavones [5], we tried to determine whether COMT activity was integral to the IOMT activity or was present as a contaminant. A DEAE-Sepharose-purified preparation of IOMT II was passed through an affinity column of α-carboxy-linked SAH at pH 7.5 [5]. Under these conditions, IOMT is recovered in the unbound protein fraction in quantitative yield (101.7%) and with a 1.3-fold purification, whereas 90% of the COMT activity binds to the matrix. Using this procedure as part of the overall purification, it was possible to reduce COMT activity in the final preparation to 11% of the IOMT activity. However, it was never possible to completely remove COMT using any of the chromatographic procedures tested. We propose that COMT activity is not integral to IOMT, but that the two enzymes co-purify because of their similarities with respect to M_r, hydrophobicity and ionic charge. This close similarity is further supported by the results of peptide mapping studies [5].

IOMT II preparations which had been purified 150-fold by a combination of DEAE-Sepharose chromatography, passage through an SAH-affinity column to remove as much COMT as possible, and HIC-HPLC, were used to determine the kinetic properties of the 7-*O*-methyltransferase. These preparations contained IOMT IIA and IOMT IIB forms, and 12% COMT activity. From double reciprocal plot analysis of initial rate data, K_m values for daidzein and SAM were determined to be 20 and 150 μM respectively. SAH was a competitive inhibitor with respect to SAM with a K_i of 35 μM. These results are consistent with those observed for IOMTs from chickpea [10] and lupin [11], although the K_m values for both substrates are higher than reported for HMKMT from peas [8]. The IOMTs of alfalfa, chickpea and lupin show high affinities for their phenolic substrates and relatively low affinities for SAM. In contrast, the COMT iso-forms in alfalfa have very low K_ms for SAM and higher K_ms for their phenolic substrate [5]. These kinetic differences between the OMTs suggest that whereas COMT may be regulated largely by SAM/SAH ratios, IOMTs may be rate limited by the availability of their isoflavone substrates. Other potential isoflavone substrates of IOMT, such as 6,7,4′-trihydroxyisoflavone, were not characterized with respect to their kinetic constants. However, the K_m value for daidzein was consistent with this compound serving as one potential endogenous substrate of IOMT II in elicited alfalfa cultures.

Role of the isoflavone 7-O-methyltransferase in the elicitation response in alfalfa

In the present studies, isoflavone 4′-*O*-methyltransferase activity was only present at very low levels, was very unstable, and was only weakly induced by elicitor. This low induction is perhaps surprising, as medicarpin (the product of a 4′-*O*-methylated isoflavone) is the major isoflavonoid accumulating in the elicited alfalfa suspension cultures [2]. It is possible that the conditions for extraction and assay of the 4′-OMT activity were not optimal, as initial optimization of assay conditions with respect to buffer components and pH measured total rather than position-specific *O*-methylation. The situation is complicated, however, by an earlier demonstration that radiolabelled daidzein is not incorporated into medicarpin in $CuCl_2$-treated seedlings of alfalfa, whereas formononetin is a good precursor [4]. On this basis, it was suggested that methylation of the 4′-position of isoflavones was an integral part of the aryl migration reaction catalysed by isoflavone synthase [4]. However, isoflavone synthase activity from elicited alfalfa cell suspension cultures does not lead to a methylated product [3]. Previous studies of daidzein *O*-methyltransferase activities [10, 12] did not demonstrate conclusively the position of methylation, and cannot, therefore, be taken as indicating the presence of a 4′-*O*-methyltransferase activity.

The stages at which *O*-methylation might occur during the biosynthesis of isoflavonoids in alfalfa are summarized in Fig. 6. We conclude that 7-*O*-methylation of daidzein is not a biosynthetic step in the formation of medicarpin as isoformononetin is a very poor substrate for 2′-hydroxylation (data not shown). A most likely role for the IOMT II described in the present paper is in the synthesis of the isoflavones alfalone or afrormosin. Afrormosin was previously identified as a constitutively formed metabolite in alfalfa cell suspension cultures, accumulating as its glucoside and malonyl glucoside [2]. Its isomer alfalone is difficult to distinguish from afrormosin, and could also have been present [13]. *O*-Methylation in the 6 or 7 positions of 6,7,4′-trihydroxyisoflavone, the preferred substrate *in vitro* for IOMT I and IOMT II, would lead to the A-ring substitution pattern of afrormosin or alfalone respectively.

Further work is necessary to determine whether IOMT II has 6-OMT activity (necessary for a role in afromosin synthesis) and whether 6,7,4′-trihydroxyisoflavone is a true substrate *in vivo* in alfalfa. Labelling studies have suggested a route to afrormosin from formononetin via texasin (6,7-dihydroxy-4′-methoxyisoflavone) [14]. Whereas elicitor strongly induces the accumulation of medicarpin in the alfalfa cultures, afrormosin/alfalone glucoside and malonylglucoside levels remain unaffected [2]. However, elicitor treatment does lead to a significant increase in incorporation of ^{14}C-phenylalanine into the glucosides [2], and it is therefore possible that synthesis and turnover of these compounds are stimulated on elicitation, the increased synthesis being associated with the increase in IOMT activity. In alfalfa callus cultures, alfalone levels are inducible by treatment with a fungal naphthoquinone [13].

It is clear that elicitor-induced isoflavone *O*-methyltransferase activities in alfalfa catalyse a number of reactions whose relation to phytoalexin synthesis is not yet clear. It is now necessary to re-examine the biosynthetic origin of the 4′-methoxyl group which is a common substituent on stress-induced isoflavonoids in the leguminosae.

EXPERIMENTAL

Chemicals and plant material. All biochemicals were purchased from Sigma except flavonoids which were obtained from Apin (Abingdon, UK). 4′-Hydroxy-7-methoxyisoflavone (isoformononetin) was synthesized by reacting a MeOH soln of daidzein (in molar excess) on ice with CH_2N_2 in Et_2O. After 15 min the reaction mixt. was blown to dryness under N_2 and the isoformononetin purified from daidzein by reversed-phase prep. HPLC using an octadecyl packing and MeOH + H_2O (9:11) as mobile phase. 7,4′-Dimethoxyisoflavone (methylformononetin) and methyl medicarpin were prepared by reacting MeOH solns of formononetin and medicarpin respectively with excess CH_2N_2

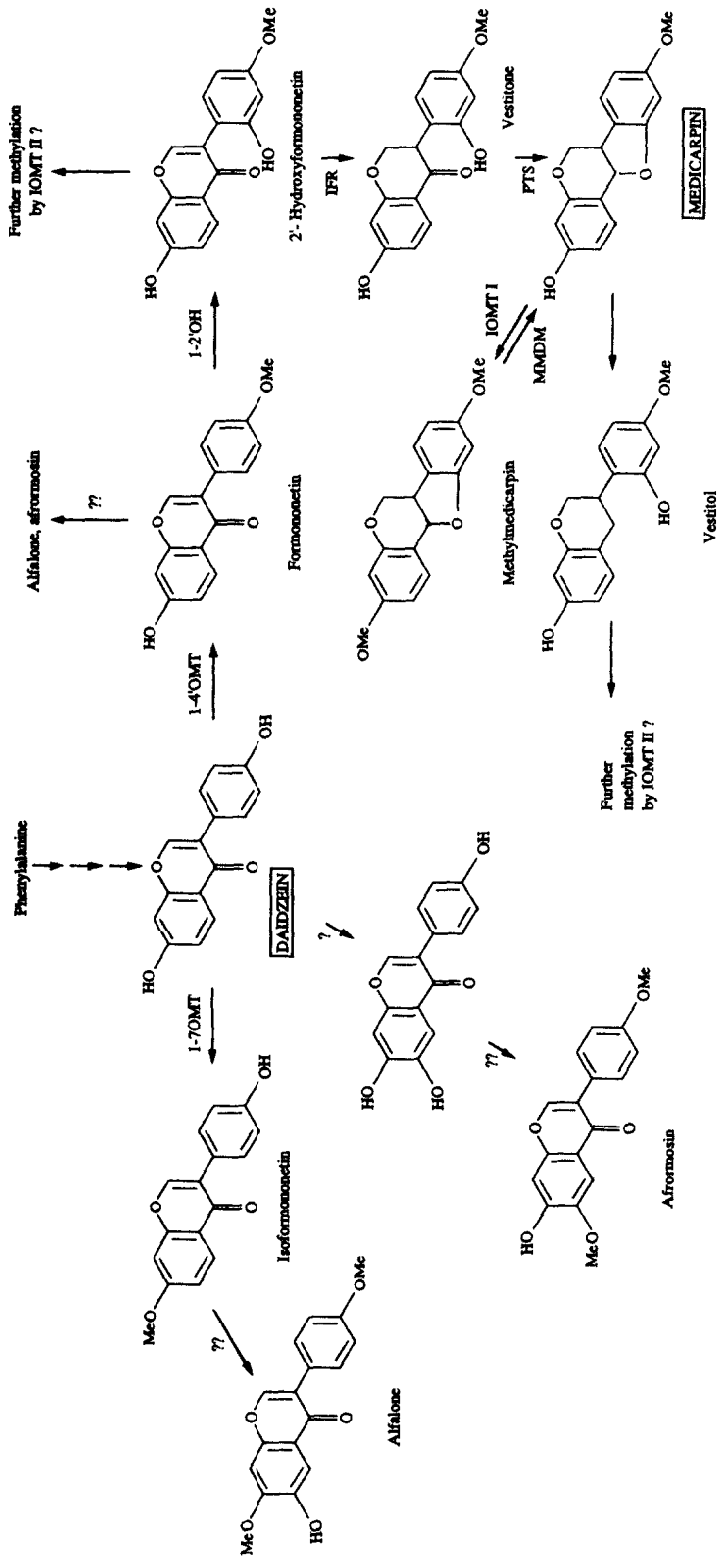


Fig. 6. Potential pathways of isoflavonoid biosynthesis in alfalfa cell suspension cultures, indicating putative stages for *O*-methylation. 1-4'OMT = isoflavone 4'-*O*-methyltransferase; 1-7OMT = isoflavone 7-*O*-methyltransferase; 1-2'OH = isoflavone 2'-hydroxylase; IFR = isoflavone reductase; PTS = pterocarpan synthase; MMDM = methyl medicarpin demethylase (an activity de-methylating the A-ring methoxy substituents of both methylmedicarpin and isoformononetin is present in the alfalfa cultures, data not shown). Note that this scheme is based on the presence of enzyme activities rather than precursor feeding studies. The latter suggest that formononetin is a natural precursor of afrormosin [14].

at 25° for 24 hr. The identities of isoformononetin and methyl formononetin were confirmed by EI-MS. *S*-Adenosyl [$^{14}CH_3$]-L-methionine (1665 MBq mmol^{-1}) was obtained from ICN (Costa Mesa, CA) and *S*-adenosyl [C^3H_3]-L-methionine (3.15 TBq mmol^{-1}) and ^{14}C-methylated protein standards from Amersham (Arlington Heights, IL).

Suspension cultured cells of alfalfa cvs Apollo and Calwest 475 were initiated and maintained as described previously [1]. Cells were treated with an elicitor preparation, derived from either *Colletotrichum lindemuthianum* or baker's yeast [15] at a final concn of 50 μg glucose equivalents ml^{-1}, when the plant cultures were in mid-logarithmic growth.

Enzyme assays. IOMT and COMT were assayed as described previously [1]. For purification and characterization studies (other than determination of kinetics) the sensitivity of the assay was increased by reducing the concn of [$^{14}CH_3$]SAM to 16 μM and increasing the specific activity to 603 KBq μmol^{-1}. For assay of frs during enzyme purification, the procedure of Preisig *et al.* [8] was used to measure the incorporation of radiolabel into the product. For all other assays, and for the identification of products, the reaction mixt. was partitioned into EtOAc + hexane (1:1) and, following concn, analysed by TLC or HPLC. TLC analysis on silica gel plates (Kieselgel 60 F_{254}, Merck) used the following solvents: 1, petrol–EtOAc–MeOH (10:10:1); 2, $CHCl_3$–MeOH–NEt_3 (8:1:1); 3, $CHCl_3$–NEt_3 (9:1). Identification of products was based on co-chromatography of radioactivity, as determined from autoradiography, with the UV absorbance of authentic reference compounds on the TLC plate. HPLC analysis was carried out on Octadecyl analytical columns (250 × 4.6 mm, 5 μm packing, J. T. Baker, Phillipsburg, NJ) using the gradient system (1% H_3PO_4–MeCN) described previously [16] (system I), or a gradient from solvent A to solvent B of 20% B (5 min), 20–60% B (45 min) and 100% B (1 min) eluted at 0.8 ml min^{-1}, (system II). Solvent A was H_2O adjusted to pH 10 with NEt_3 and solvent B was THF. The eluant was monitored at 287 nm. Identification by HPLC was based on the coincidence of UV absorbing standards and radioactivity collected in frs.

Enzyme purification and characterization. Frozen alfalfa cells (500 g) which had been treated for 16 hr with elicitor from the cell walls of *Colletotrichum lindemuthianum* were allowed to thaw in 1 l of 0.2 M Tris–HCl pH 7.5 containing 5 mM EDTA, 28 mM MeSH and 10 mM diethyldithiocarbamate, and homogenized first with a Waring blendor and second with a Brinkman Polytron. After filtration through 20 μm nylon mesh, the filtrate was centrifuged at 10000 *g* for 30 min. The supernatant was stirred at 4° for 15 min with 200 g of pre-washed Dowex 1 and 100 g polyvinylpolypyrrolidone. After filtration the solution was adjusted to 1 mg ml^{-1} with respect to streptomycin sulphate and recentrifuged. The supernatant was fractionated with $(NH_4)_2SO_4$ between 45 and 85% saturation and the pellet collected by centrifugation at 17000 *g* for 30 min. The pellet was resuspended in 80 ml of 10 mM Bis–Tris–HCl pH 6.8 containing 1 mM DTT and 0.5 mM EDTA (buffer A) and dialysed at 4° for 4 hr against two 2 l changes of buffer A. After clarification, the sample was applied to a column (bed vol. = 100 ml) of DEAE-Sepharose CL-6B and, after extensive washing, the bound activity eluted at 0.6 ml min^{-1} with a 500 ml linear gradient of 0–0.2 M $(NH_4)_2SO_4$ in buffer A. Frs (10 ml) containing enzyme activity were pooled and adjusted to 2 M with respect to $(NH_4)_2SO_4$. After filtration (0.45 μm filter) the protein samples were applied to a hydrophobic interaction HPLC column (4.6 × 150 mm, Hydropore, Rainin Instrument Co., Inc., Woburn, MA) and the activity eluted at 1 ml min^{-1} with an inverse gradient of 2 M $(NH_4)_2SO_4$ in 0.1 M K-Pi pH 7 to 50 mM K-Pi pH 7.2. Frs (1 ml) containing activity were re-equilibrated on gel filtration columns (PD 10, Pharmacia) in 25 mM Bis–Tris–propane–HCl pH 7.0 and injected in 1 ml aliquots onto a Mono P HR 5/5 column (Pharmacia). Bound activity was eluted at 1 ml min^{-1} with 18 ml of a 10% (v/v) soln of Polybuffer 74 (pH 4) and 0.5 ml frs collected. The most active frs were desalted on PD-10 columns in 20 mM Bis–Tris–propane–HCl pH 7.0 (buffer B) and 1 ml samples applied to a Mono Q HR 5/5 FPLC column (Pharmacia). The bound protein was washed at 1 ml min^{-1} with buffer B (5 ml) and then eluted with a 20 ml gradient of 0–75 mM $(NH_4)_2SO_4$ in buffer B.

For determination of native M_r, protein samples (0.3 ml) were injected onto a Superose 6 HR 10/30 column (Pharmacia) and eluted with 0.1 M Tris–HCl pH 7.5, 0.15 M NaCl at 0.3 ml min^{-1}. The column was calibrated with aldolase (158000), bovine serum albumin (67000), ovalbumin (45000) and chymotrypsinogen A (25000). For analysis of crude protein preparations by Mono Q FPLC, $(NH_4)_2SO_4$ ppts were taken up in buffer B containing 75 mM $(NH_4)_2SO_4$ and passed through a QMA SepPak (Waters Associates) prior to desalting in buffer B and injection. In all cases the recovery of enzyme activity in frs from the columns used for purification and analysis was improved by the addition of 10% 2 M Tris–HCl pH 8.5 containing 50 mM EDTA and 280 mM MeSH. Photoaffinity labelling of OMT preparations was carried out in microtitre plates essentially as described by Preisig *et al.* [8]. Samples were separated on SDS-PAGE gels (10%) and analysed for protein content by silver staining and for radiolabelled peptides by fluorography [8]. Protein content of solns was determined by dye-binding assay (BioRad).

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