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ENZYMIC *O*-METHYLATION OF ISOLIQUIRITIGENIN AND LICODIONE IN ALFALFA AND LICORICE CULTURES

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Key Word Index—*Medicago sativa*; *Glycyrrhiza echinata*; Fabaceae; *O*-methyltransferases; flavonoid biosynthesis; symbiosis; retrochalcone.

Abstract—*S*-Adenosyl-L-methionine (SAM): isoliquiritigenin (2′,4,4′-trihydroxychalcone) 2′-*O*-methyltransferase (CHMT) of alfalfa (*Medicago sativa*) catalyses the formation of 4,4′-dihydroxy-2′-methoxychalcone, which is the most potent inducer of nodulation-genes of *Rhizobium meliloti*, the symbiont of alfalfa which forms nitrogen-fixing nodules. SAM: licodione 2′-*O*-methyltransferase (LMT) is involved in the biosynthesis of a retrochalcone in cultured licorice (*Glycyrrhiza echinata*) cells and has been shown to be induced as a defence response of the cells. Because licodione exists in an equilibrium mixture of tautomeric 2′,4,4′,β-tetrahydroxychalcone (major) and 1-(2,4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-1,3-propanedione (minor), the apparent mode of action of both enzymes is very similar. In this study, cultured alfalfa cells were shown to exhibit rapid and transient increases in the extractable activities of both CHMT and LMT after treatment with yeast extract (YE). Treatment of solution-cultured alfalfa seedlings with YE also resulted in a similar induction of both CHMT and LMT activities in the roots, but no activity was detected in the shoots. These activities were attributed to a single gene product, the CHMT protein, as extracts of *Escherichia coli* transformed with the CHMT cDNA exhibited both CHMT and LMT activities. In contrast, in *G. echinata* cells, LMT was induced after YE treatment, but no CHMT activity was observed. It is concluded that alfalfa CHMT and licorice LMT are distinct enzymes, the former displaying the wider substrate specificity. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

S-Adenosyl-L-methionine (SAM): isoliquiritigenin (2′,4,4′-trihydroxychalcone) 2′-*O*-methyltransferase (chalcone OMT or CHMT) of alfalfa (*Medicago sativa*) catalyses 2′-*O*-methylation of a 6′-deoxychalcone, isoliquiritigenin [1], which is produced by the co-action of chalcone synthase and a reductase found characteristically in the Fabaceae [2–4] (Fig. 1). The product of CHMT, 4,4′-dihydroxy-2′-methoxychalcone, is the strongest inducer of nodulation-genes (*nod*-genes) of *Rhizobium meliloti* so far reported [5]. Thus, CHMT in the roots may play an important role in the symbiosis of *R. meliloti* and alfalfa to form nitrogen-fixing root nodules [6, 7]. The alfalfa CHMT cDNA has been cloned, and CHMT gene expression has been studied in relation to the legume–*Rhizobium* interaction [8].

Licodione OMT (LMT) in cultured cells of another leguminous plant, licorice (*Glycyrrhiza echinata*), transfers a methyl group to the *ortho*-hydroxyl (at C-2′) of licodione [9] (Fig. 1). The product, 2′-*O*-methyllicodione, is a biosynthetic intermediate of a retrochalcone, echinatin. Because licodione exists as an equilibrium mixture of major β-hydroxychalcone (2′,4,4′,β-tetrahydroxychalcone) and minor *ortho*-hydroxy-dibenzoylmethane [1-(2,4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-1,3-propanedione] [10], the apparent catalytic process of CHMT and LMT is very similar, i.e. methyl transfer from SAM to a phenolic hydroxyl group at the *ortho* position to the C_3 unit of the chalcone skeleton. The physiological role of retrochalcone is unknown, but its accumulation and activities of the enzymes of its biosynthesis (including LMT) [11] are transiently enhanced in licorice cells on treatment with substances possessing elicitor activity, e.g. yeast extract (YE). Although the occurrence of retrochalcones of the echinatin type (i.e. oxygen functionalities on C-2, 4, 4′: *cf.* [12]) seems to be restricted to *Glycyrrhiza* species, licodione has additionally been found in cultured alfalfa cells [13, 14].

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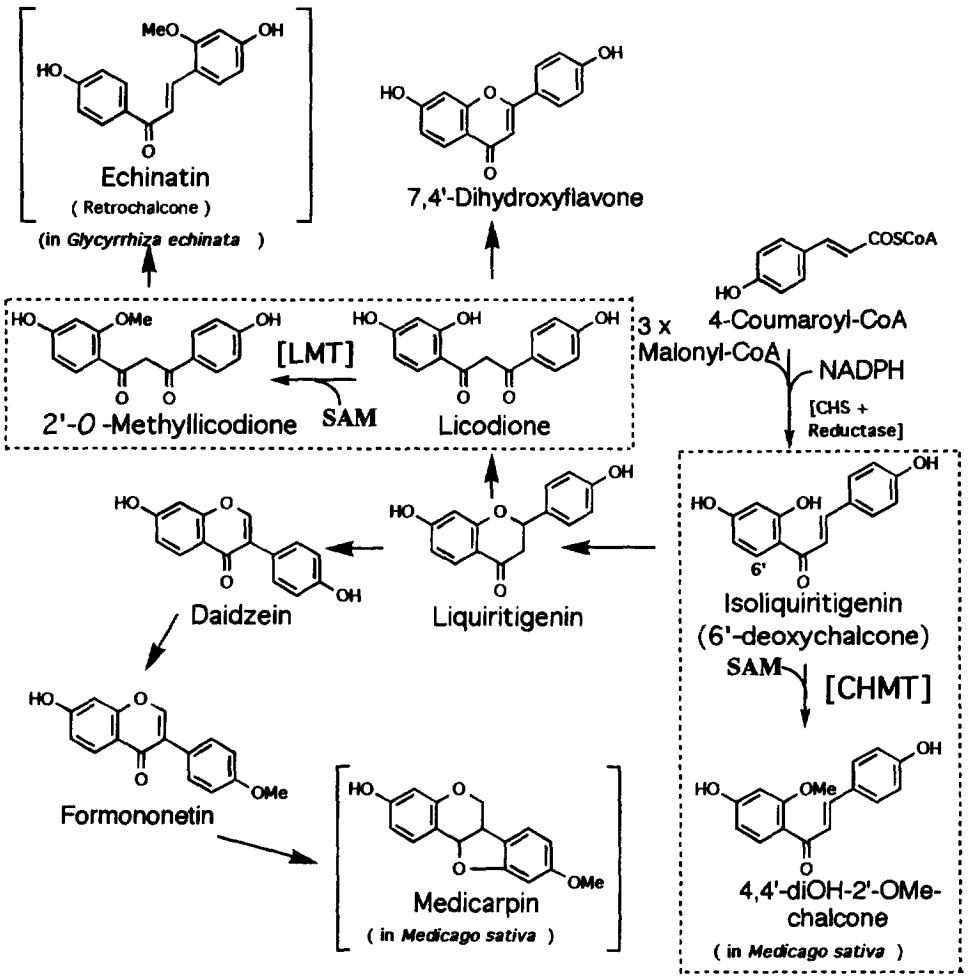

Fig. 1. Biosynthesis of flavonoids in alfalfa and licorice cells.

These facts raise the possibility that CHMT and LMT are the same enzyme. We therefore examined CHMT and LMT activities in cultured alfalfa and licorice cells, and also in alfalfa seedlings after treatment with YE. Our findings are of interest from the viewpoints of substrate specificity of OMTs and their possible functions in plant–microbe interactions.

RESULTS AND DISCUSSION

CHMT and LMT activities in alfalfa

The enzyme preparation from cultured alfalfa (cv. Moapa) cells which had been treated with YE for 24 hr showed methyltransferase activities from SAM to both isoliquiritigenin and licodione. The major products determined by TLC–radiochromatoscanner measurement were 4,4′-dihydroxy-2′-methoxychalcone and 2′-*O*-methyl-licodione from isoliquiritigenin and licodione as substrates, respectively. Thus, cultured alfalfa cells were found, for the first time, to possess LMT activity in addition to the reported CHMT activity. Occasionally, a minor amount of unidentified polar byproduct from the incubation with licodione was observed. Figure 2 demonstrates the changes of CHMT and LMT activities in cultured alfalfa cells when treated with 0.1% (w/v) YE. Both activities rapidly and transiently increased, with the maximum at 24 hr. These enzyme activities were virtually absent from control cultures that were not exposed to YE.

Solution-cultured alfalfa seedlings were also treated with YE. The roots and shoots of the harvested seedlings were separately extracted and enzyme activities were measured (Table 1). The root extracts from non-elicited seedlings exhibited both CHMT and LMT activities, although they were low compared with the values from elicited suspension-cultured cells. Increased activities with both substrates were observed 24 hr after treatment of roots with YE. No activity with either substrate was found in the extracts of shoots. The localization of CHMT in roots is in accordance with previous results [8], and LMT activity has now been found to display the same organ specificity.

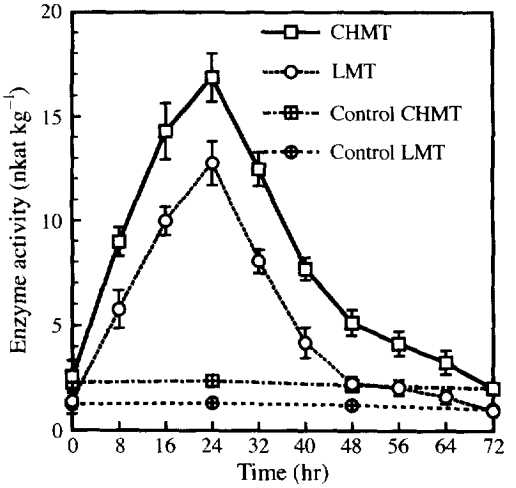

Fig. 2. Activity changes of *O*-methyltransferases in cultured alfalfa cells after treatment with yeast extract (0.1% w/v). Average values and standard deviation from three independent experiments are shown.

Table 1. *O*-Methyltransferase activities in solution-cultured alfalfa seedlings after treatment with yeast extract (0.1% w/v)

Time after YE treatment (hr)	Activity (nkat kg^{-1})			
	Roots		Shoots	
	CHMT	LMT	CHMT	LMT
0	9.74	2.78	0.10	0.12
24	23.15	7.48	0.14	0.13
48	6.56	1.80	0.11	0.12

Heterologously expressed CHMT protein possesses both CHMT and LMT activities

A very interesting question is whether the two OMT activities arise from a single protein, or whether distinct CHMT and LMT proteins exist in alfalfa. The similarity of these activities in terms of the time course of induction, pH dependency (maximum around pH 8; data not shown) and the localization in seedlings strongly suggest the former possibility. In order to confirm this, extracts from *Escherichia coli* transformed with the alfalfa CHMT cDNA in a strong expression vector (pCHMT3) [8] were assayed for OMT activities. The results (Fig. 3) very clearly show that alfalfa CHMT protein overexpressed in bacteria possesses both CHMT and LMT activities.

LMT activity, but not CHMT activity, is found in licorice

Figure 4 shows the activity changes of OMTs in YE-treated licorice cells. LMT activity increased from 16 to 24 hr after the treatment, agreeing with previous observations [11]. In sharp contrast to the situation in

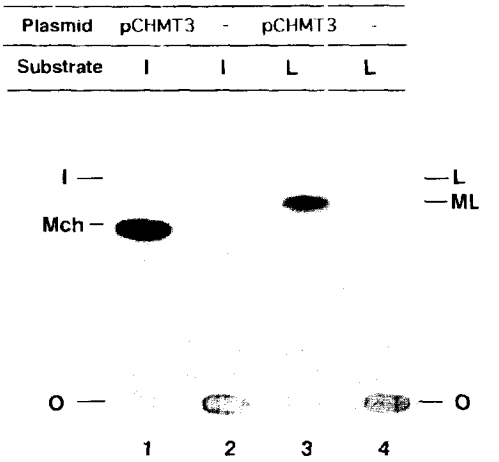

Fig. 3. TLC autoradiogram of the reaction products from isoliquiritigenin (lanes 1 and 2) or licodione (lanes 3 and 4) with [*methyl*-^{14}C]SAM using the extract from *E. coli* [BL21(DE3)] transformed with pCHMT3 (lanes 1 and 3). Lanes 2 and 4 represent the control experiment using the extract from non-transformed BL21(DE3). Abbreviations: I, isoliquiritigenin; L, licodione; Mch, 4,4′-dihydroxy-2′-methoxychalcone; ML, 2′-*O*-methyl-licodione; O, origin.

alfalfa cells, no CHMT activity was observed in either YE-treated or control licorice cells.

It is surprising, in view of the high structural similarity between isoliquiritigenin and licodione, that licorice LMT does not accept isoliquiritigenin as a substrate. Licodione exists in a major (*ca* 70% acetone) enol form (*β*-hydroxychalcone) [10], which is likely to be the substrate for alfalfa CHMT. It is tempting to speculate that licorice LMT may accept the minor keto tautomer as the substrate. The overall molecular shapes of keto and enol forms of licodione would be very different: in the keto form, two benzoyl moieties are attached to a sp^3 carbon (C-2), while the carbon skeleton of the enol tautomer must be planar.

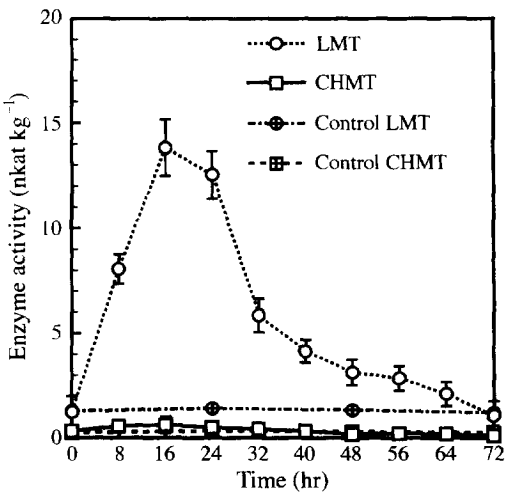

Fig. 4. Activity changes of *O*-methyltransferases in cultured licorice cells after treatment with yeast extract (0.1% w/v). Average values and standard deviation from three independent experiments are shown.

Thus, the active site of an enzyme fitting one tautomer would be very different from the one fitting the other. This point may be tested by designing artificial substrates which are fixed in keto or enol forms.

Physiological considerations

Alfalfa CHMT plays a significant role in establishing root nodules by producing a strong *nod*-inducing molecule which acts by binding to the NodD proteins of *Rhizobium meliloti* [6]. What, then, is the biological significance, if any, of the additional LMT activity possessed by the alfalfa CHMT protein? Licodione is biosynthesized by the action of a cytochrome P450 monoxygenase, licodione synthase [15], initially in a hemiacetal form, which is regarded as the immediate precursor of 7,4′-dihydroxyflavone (Fig. 1). This flavone is a weak inducer of *R. meliloti nod*-genes. Although the LMT product, 2′-*O*-methyl-licodione (or further metabolites, e.g. retrochalcones), has not been detected in alfalfa cells and its (their) *nod*-inducing potential is not known, LMT activity could potentially reduce the formation of the flavone, thus influencing the *nod*-inducing activity of the total flavonoid mixture of alfalfa roots [16, 17].

LMT in *G. echinata* cells has been regarded as a defence gene product, because its activity is increased after the application of the elicitor-active substances [11]. However, isoflavan-type stress metabolites have been reported from other *Glycyrrhiza* species [18, 19], indicating that the genus *Glycyrrhiza* has a distinct isoflavonoid pathway with defensive roles. An isoflavone, formononetin, is constitutively produced in cultured licorice cells used in this study [20]. Because alfalfa CHMT is also induced in response to YE (Fig. 2; ref. [1]), which is a known elicitor of defence genes in alfalfa cells, induction of LMT activity by YE treatment of licorice cells does not necessarily mean that LMT is a defence gene product. Its possible involvement in the symbiosis of *Glycyrrhiza* with soil bacteria should now be investigated.

EXPERIMENTAL

Plant materials. Glycyrrhiza echinata cells were cultured on Murashige–Skoog (MS) agar (0.9%) medium (3% sucrose) with 1 mg l^{-1} IAA and 0.1 mg l^{-1} kinetin. Alfalfa cells (cv. Moapa) were grown on MS medium supplemented with 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin. Callus cultures were maintained in the dark at 25° with subculture at 3-week intervals. Suspension cultures were derived from the callus by transferring the cells into liquid media of the same composition, and cultured on a rotary shaker (120 rpm) in the dark at 25°. The medium for alfalfa seedlings soln culture contained 30 μM $(NH_4)_2SO_4$, 6 μM Na_2HPO_4, 40 μM KCl, 4 μM $CaCl_2$, 3 μM $MgCl_2$ and 0.6 μM $FeCl_3$. The seedlings were grown under 16 hr light/8 hr dark cycle at room temp. for 4 weeks.

Elicitation method. A concd aq. soln of YE (Difco) was filter-sterilized and added to the cell suspension cultures or the soln-culture medium of alfalfa seedlings at a final concn of 1 g l^{-1}.

Enzyme preparation. Freshly collected cells (usually 5 g) were mixed with half the wt of sea sand, 1/10 wt of Polyclar SB-100 (ISP Inc) and the same vol. of 0.1 M K-Pi buffer (routinely pH 8) containing 5 mM EDTA and 2 mM DTT, and the mixt. was homogenized in a chilled mortar. The slurry was filtered through 8 layers of cheesecloth and the filtrate was centrifuged at 10000 *g* for 10 min. The supernatant was treated with Dowex 1 × 2 (1/2 the cell fr. wt) for 20 min to remove endogenous phenolics, and the filtrate was poured through a sintered-glass filter and used as crude enzyme soln.

Enzyme assay. The assay mixt. contained 100 nmol phenolic substrate (licodione or isoliquiritigenin in 10 μl EtOH), 0.47 nmol [*methyl*-^{14}C]SAM (925 Bq in 10 μl dilute H_2SO_4; Amersham) and crude enzyme soln (1 ml). The mixt. was incubated at 30° for 1 hr and, after termination of the reaction by addition of HOAc (20 μl) and carrier samples (100 nmol 2′-*O*-methyl-licodione or 4,4′-dihydroxy-2′-methoxychalcone, each in 10 μl EtOH), it was extracted with 3 ml EtOAc. After vortex mixing (30 sec), the mixt. was centrifuged (2000 *g*, 10 min), and the EtOAc layer (2 ml) was sepd. An aliquot of the EtOAc layer was concd and subjected to silica gel TLC (Merck) with toluene–EtOAc–MeOH–petrol (6:4:1:3) as developing solvent (double development). The spots of carrier samples were visualized under UV, and the location of radioactivity on TLC plates was determined by autoradiography and/or scanning with a TLC radiochromatoscanner (Berthold LB 2820-1 linear analyser). Finally, the radioactive spot was scraped from the plate and the radioactivity measured in a liquid scintillation counter (Beckman LS 5801). Protein content was determined with a protein assay kit (Bio-Rad) and the enzyme activities were expressed in nkat kg^{-1} protein.

Enzyme assay of extracts from E. coli *transformed with pCHMT3.* The enzyme extract was prepd from *E. coli* BL21 (DE3) cells harbouring pCHMT3 [8] (pretreated with 4 mM isopropyl-β-D-thiogalactopyranoside for 3 hr) suspended in 50 mM Tris–HCl buffer (pH 8.0, containing 2 mM EDTA) by treatment with lysozyme (0.01%) and Triton X-100 (0.1%), followed by centrifugation (10000 *g*). The assay mixt. contained 100 nmol phenolic substrate (isoliquiritigenin or licodione), 925 Bq [methyl-^{14}C]SAM and 0.5 ml enzyme soln. Incubation, termination of the reaction, and product detection methods were as above.

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