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ALFALFA CELL CULTURES TREATED WITH A FUNGAL ELICITOR ACCUMULATE FLAVONE METABOLITES RATHER THAN ISOFLAVONES IN THE PRESENCE OF THE METHYLATION INHIBITOR TUBERICIDIN

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Key Word Index—*Medicago sativa*; Leguminosae; isoflavones; flavones; flavanones; retrochalcones; phytoalexin response.

Abstract—Alfalfa cell suspension cultures accumulating isoflavonoid phytoalexins in response to exposure to a fungal elicitor were treated with cycloleucine, sinefungin or tubericidin; these three compounds disrupt transmethylation reactions in cells directly, or by altering the accumulation of *S*-adenosyl-L-methionine or *S*-adenosyl-L-homocysteine, respectively. As determined by measuring the reduction of the dye 2,3,5-triphenyltetrazolium, all compounds were equally toxic to the cells, but tubericidin was the most effective compound at inhibiting the accumulation of the phytoalexin medicarpin and the incorporation of radioactivity from L-[methyl-^{3}H]-methionine into phenolic metabolites. The specificity of the inhibition by tubericidin was confirmed by showing that the compound was an effective inhibitor of *S*-adenosyl-L-homocysteine hydrolase both *in vivo* and *in vitro*. In the presence of tubericidin, elicitor-treated alfalfa cell cultures turned yellow and this was associated with a reduction in the synthesis of isoflavonoid phytoalexins and the concomitant accumulation of the retrochalcone licodione, together with lesser amounts of 7,4′-dihydroxyflavone and 7,4′-dihydroxyflavanone. These results suggest that the methylation inhibitor tubericidin selectively inhibits the accumulation of isoflavones, with the flavone and licodione accumulating as alternative phytoalexins. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

When legumes, such as alfalfa (*Medicago sativa* L.), are challenged with fungal pathogens they accumulate appreciable concentrations of antibiotic isoflavonoid phytoalexins. In alfalfa the major phytoalexin is the pterocarpan medicarpin and its biosynthesis and further metabolism has been the subject of several detailed metabolism studies [1–3]. The commonly accepted pathway to medicarpin proceeds via phenylpropanoid, chalcone, flavanone and isoflavone intermediates (Fig. 1), with the majority of the individual biosynthetic enzymes described and in many cases the corresponding genes cloned [4]. However, one point of ambiguity which remains concerns the identity of the precursor, which is 4′-*O*-methylated. Since formononetin (7-hydroxy-4′-methoxyisoflavone) is an excellent precursor of medicarpin synthesis [2] and accumulates to significant concentrations in alfalfa as formononetin 7-*O*-glucoside-6″-*O*-malonate [5], the 4′-methoxyl group must be introduced prior to this biosynthetic intermediate. The specificity of methyltransferases in alfalfa also suggests that methoxylation does not occur with chalcone and flavanone intermediates earlier on in the pathway [6]. Instead, it is commonly accepted that the methoxyl group is introduced by the 4′-*O*-methylation of daidzein (Fig. 1) [7]. However, several lines of evidence suggest that this reaction may not occur. Firstly, although based on TLC analysis the early literature suggested that daidzein, or its glycosides, are present in both healthy and infected alfalfa, using HPLC we have been unable to identify this isoflavonoid in plants or cell cultures under any conditions [8]. Secondly, although radiolabelled 2′,4,4′-trihydroxychalcone and formononetin, the intermediates situated before and after daidzein in the proposed pathway, respectively, were excellent precursors of medicarpin, the incorporation of radioactive daidzein into the phytoalexin was unaccountably poor [2]. Finally, the elicitor-inducible isoflavone *O*-methyltransferase with activity toward daidzein in both alfalfa [6] and other legumes [9] catalyses the methylation of the 7-hydroxyl group of daidzein rather than the 4′-hydroxyl group, and the corresponding 4′-*O*-methyltransferase has not been identified. While these observations

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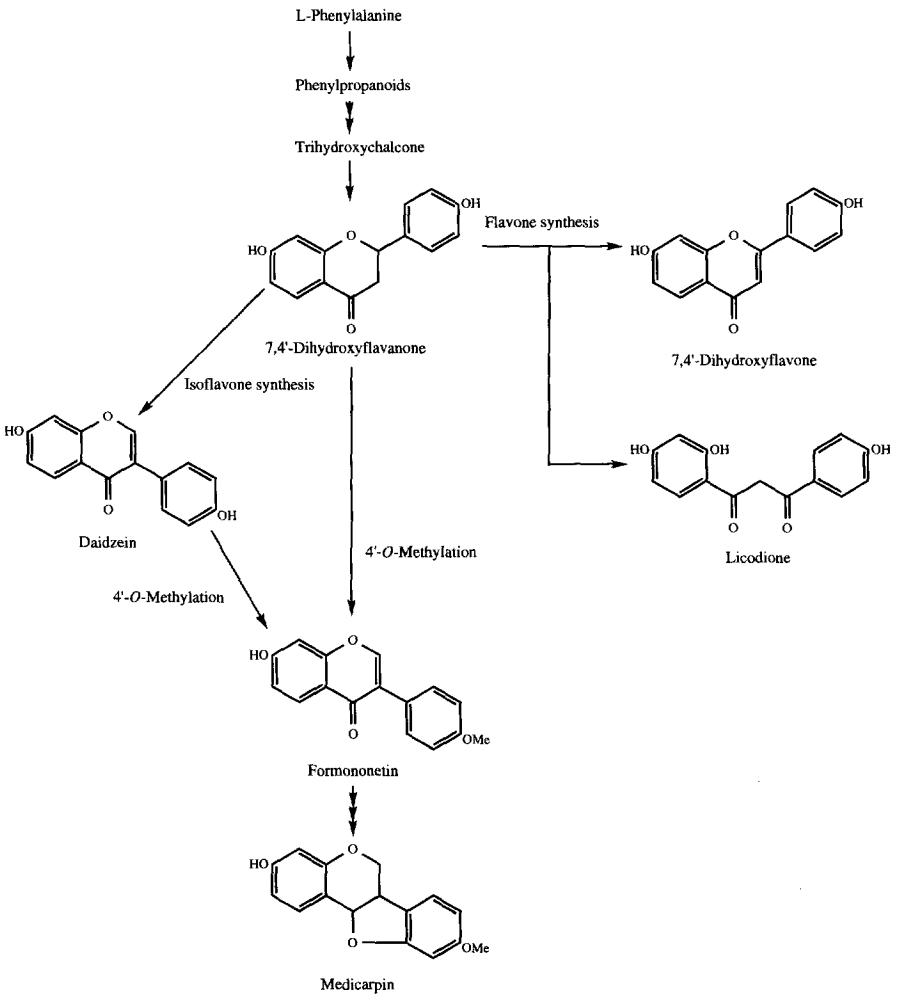

Fig. 1. Overview of medicarpin synthesis showing the stages at which the 4'-methoxylation could occur and the effect of inhibiting isoflavone synthesis on the formation of 7,4'-dihydroxyflavone and licodione.

do not disprove the proposed reaction sequence they do suggest that the timing and role of methylation in the biosynthesis of medicarpin is not fully evaluated.

We have previously determined that treatment of alfalfa cell cultures with fungal elicitors results in a four-fold increase in the methylation reactions, with the majority of this increased flux being directed into the synthesis of methylated isoflavonoid phytoalexins [10]. This increase in methylation was also associated with the accumulation of *S*-adenosyl-L-methionine (SAM), the source of the methyl groups, and *S*-adenosyl-L-homocysteine (SAH) the reaction product formed following the loss of methyl group from SAM [11]. The elicitor-mediated increase in the methylation of isoflavonoids suggested that this would be an ideal system to identify the stage at which methyl groups became incorporated into the precursors of isoflavonoid phytoalexins by using inhibitors of methylation reactions. Three methylation inhibitors, cycloleucine, sinefungin and tubericidin, each with differing modes of action, were selected for such a study [12]. Cycloleucine reduces the synthesis of SAM by inhibiting SAM synthetase [13]. Sinefungin is an analogue SAH and acts directly as a competitive inhibitor of SAM-dependent methyltransferases [12], while tubericidin (7-deazaadenosine) is an inhibitor of SAH hydrolase, causing SAH to accumulate and in turn inhibit methyltransferases [14]. We now report that the most effective inhibitor, tubericidin, caused alfalfa cell cultures exposed to fungal elicitors to inhibit isoflavone accumulation in preference to the biosynthetic precursor 7,4'-dihydroxyflavanone and flavone and retrochalcone shunt metabolites (Fig. 1).

RESULTS AND DISCUSSION

Specificity and effectiveness of methylation inhibitors

Before using the methylation inhibitors to disrupt metabolism selectively in elicitor-treated cell cultures it was desirable to determine their efficacy in inhibiting both methylation reactions and the accumulation of phytoalexins. Methylation reactions in alfalfa cell cultures were monitored by determining the incorporation

of radioactivity from L-[methyl-^{3}H]-methionine into phenolic metabolites, which could be partitioned into ethyl acetate [10], and the accumulation of medicarpin was determined by HPLC [7]. In the absence of inhibitors, treatment with elicitor for 8 hr resulted in a major increase in medicarpin content and the incorporation of [^{3}H]-methyl groups from methionine into the phenolic fraction (Table 1). When used at a final concentration of 100 μM all the inhibitors reduced both medicarpin accumulation and incorporation of radioactivity into phenolics in the order tubericidin > sinefungin > cycloleucine. When used at a concentration of 10 μM only tubericidin was shown to be an effective inhibitor.

It was then important to determine whether these inhibitory activities were due to inhibition of target enzymes or to non-specific toxic effects. The toxicity of the inhibitors was determined by monitoring the reduction of the dye 2,3,5-triphenyltetrazolium to red formazan. The dye accepts electrons from the mitochondrial electron transport chain and gives a measure of the respiration and vitality of plant cell cultures [15]. All three inhibitors reduced the formation of formazan to a similar degree, suggesting that they were all equally toxic to the cells (Table 1). These results demonstrated that the sensitivity of the methylation reactions in elicitor-treated alfalfa cells by tubericidin could not be accounted for by a general inhibition in the respiration rate alone. Instead, they suggest that tubericidin was acting to reduce transmethylation by inhibiting SAH hydrolase. Further evidence for the inhibition of SAH hydrolase by tubericidin was obtained by determining the effect of the inhibitor on SAH concentrations *in vivo*, using HPLC [11]. Cells exposed to just elicitor for 8 hr contained 0.35±0.1 μg of SAH g^{-1} fr. wt of cells (mean±S.D., $n = 3$), while in the presence of tubericidin the cells contained 1.4±0.2 $\mu g\, g^{-1}$. The activity of tubericidin as an inhibitor of SAH hydrolase was then confirmed by assaying the enzyme in crude extracts from untreated alfalfa cell cultures in the presence of various concentrations of tubercidin. As demonstrated in animals [14] tubericidin was an effective inhibitor of SAH hydrolase from alfalfa with an I_{50} of 3 μM, again confirming that tubericidin was a selective inhibitor of methylation reactions.

Tubericidin was, therefore, used in all subsequent inhibition studies at a concentration of 10 μM to minimize toxicity. The reasons for the lower inhibitory activities of cycloleucine and sinefungin compared with tubericidin were not determined.

Effect of tubericidin on inducible flavonoid synthesis in elicitor-treated alfalfa cell cultures

During the screening for the optimal inhibitor treatment it was observed that the cells and media of elicitor-treated cultures turned visibly yellow when the methylation of phenolics was inhibited by 10 μM tubericidin or 100 μM tubericidin or sinefungin. To identify this yellow product, cell cultures were treated with elicitor for 8 hr in the presence and absence of 10 μM tubericidin, and the cell extracts were analysed by HPLC (Fig. 2). In the cells treated with elicitor only, the dominant metabolites were the phytoalexin medicarpin and its conjugate medicarpin 3-*O*-glucoside-6″-*O*-malonate (MGM) (Fig. 2(A)). In the medium of these cultures the dominant UV-absorbing metabolite was also identified as medicarpin, with the elicited medium containing 102±15 μM medicarpin (mean±S.D., $n = 3$) as compared with 2±0 μM in the control medium. Cultures pretreated with tubericidin contained reduced amounts of medicarpin and MGM (Table 2). However, the inhibitor-treated cells contained three additional metabolites not normally observed in elicitor-treated cells (Fig. 2(B)), which were termed methylation inhibitor products (MIPs). MIPs 1 and 2 were relatively minor metabolites eluting from the HPLC column at 18.2 and 19 min, respectively, while the predominant metabolite was MIP 3, eluting at 21 min. A similar profile of MIPs was also determined in the medium of elicited cultures treated with tubericidin, but these compounds were not observed in the cells or medium of the inhibitor-treated cells in the absence of elicitor (data not shown). These results suggested that MIP accumulation resulted from the inhibition of methylation reactions involved in either

Table 1. The effect of methylation inhibitors on the reduction of 2,3,5-triphenyltetrazolium to red formazan, the accumulation of the phytoalexin medicarpin and the incorporation of radioactivity from [methyl-^{3}H]-methionine into phenolic metabolites in alfalfa cell cultures treated with fungal elicitor

Treatment	Inhibitor (μM)	Formazan ($A_{465}\, g^{-1}$ fr. wt)	Medicarpin (nmol g^{-1} fr. wt)		% Administered ^{3}H in EtOAc	
			Cells	Medium	Cells	Medium
No elicitor	0	0.15±0.03	2±0	10±2	8±1	4±0
Elicitor	0	0.19±0.01	102±12	80±11	40±3	9±2
+ cycloleucine	10	0.17±0.00	89±13	84±7	35±2	10±3
	100	0.13±0.02	60±5	51±4	26±0	8±2
+ sinefungin	10	0.14±0.05	94±14	80±11	34±9	10±5
	100	0.08±0.04	59±2	44±10	21±3	8±2
+ tubericidin	10	0.16±0.01	32±6	6±0	12±3	3±1
	100	0.12±0.05	10+0	4+1	4+1	2+1

Values represent the means of triplicate determinations ±S.D.

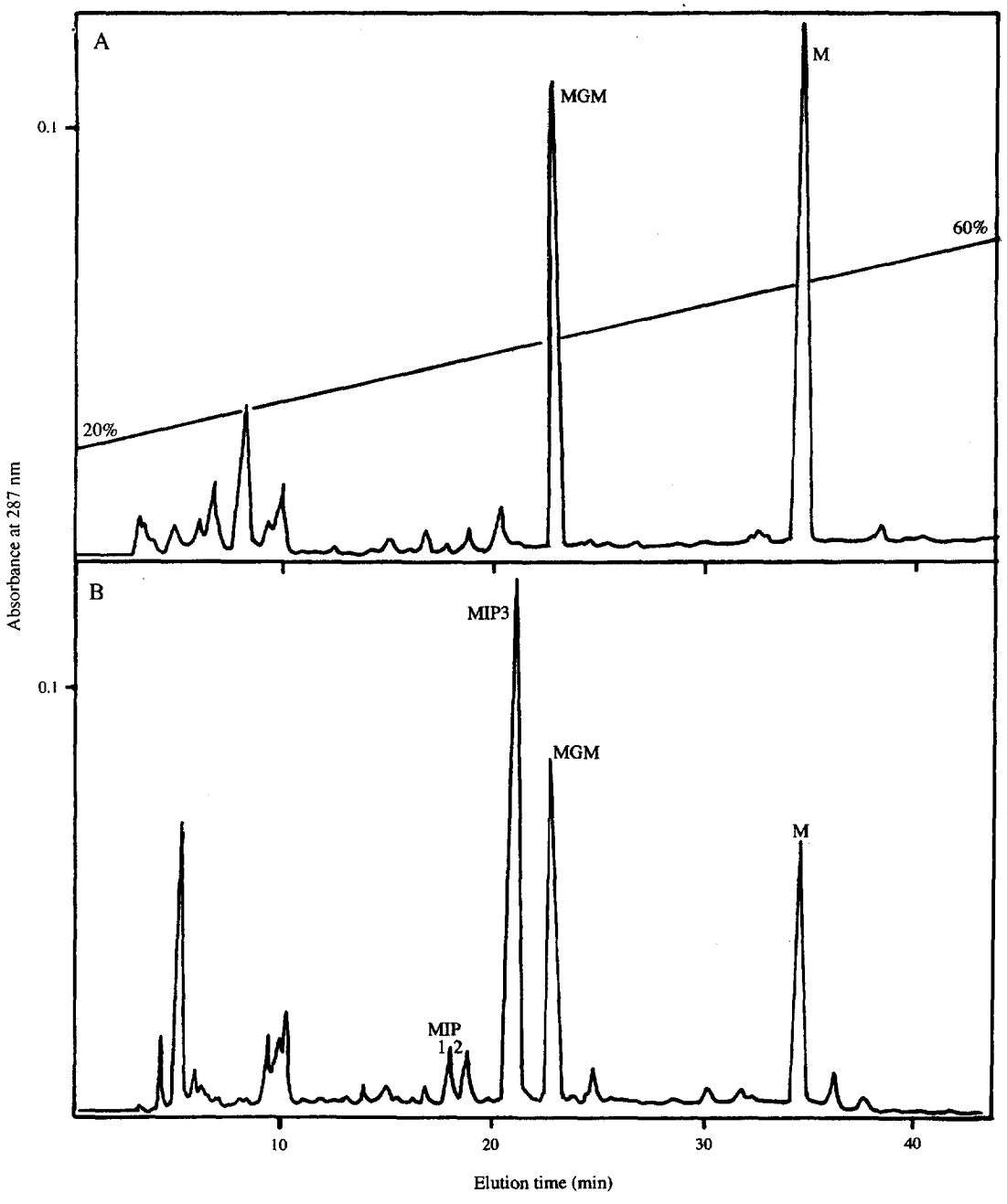

Fig. 2. HPLC separation of UV-absorbing metabolites in extracts from (A) cell cultures treated with elicitor for 8 hr, and (B) cells treated with elicitor for 8 hr after preincubation with 10 μM tubericidin for 12 hr. The increasing percentage of acetonitrile used to elute the compounds is shown in box A.

the synthesis or metabolism of inducible isoflavonoid phytoalexins.

The MIPs were purified by a combination of HPLC and TLC and identified by co-chromatography with available standards and from their UV absorbance and ^{1}H NMR spectra with reference to published data [16, 17]. The minor metabolite MIP 1 was identified as 7,4′-dihydroxyflavanone, an intermediate of isoflavonoid synthesis in alfalfa [7], which does not normally accumulate in elicited cultures [8]. MIP 2, which was yellow, was identified by UV spectroscopy and co-chromatography as 7,4′-dihydroxylflavone, a flavone normally observed as a minor constitutive metabolite of alfalfa leaves [5, 8]. Careful examination showed that this metabolite also accumulated to low concentrations in elicitor-treated alfalfa cultures in the absence of tubericidin (Table 2). From its UV and ^{1}H NMR spectra, MIP 3, which was bright yellow, was identified as the retrochalcone licodine (1,3-diphenylpropanedione). Its identify was further confirmed by its spontaneous isomerization to 7,4′-dihydroxyflavone in the presence of acid [17]. Since the

Table 2. Effect of the methylation inhibitor tubericidin (10 μM) on the accumulation of flavonoid metabolites in elicitor-treated alfalfa cell cultures

Metabolite	Time* (hr)	Concentration (μM)			
		−Inhibitor		+Inhibitor	
		Cells	Medium	Cells	Medium
Medicarpin	8	80±16	102±21	26±2	25±4
7,4′-Dihydroxyflavanone	8	ND	ND	36±7	28±4
7,4′-Dihydroxyflavanone	8	11±4	16±4	27±4	29±7
Licodione	8	ND	ND	101±11	102±5
Medicarpin	24	210±43	40±12	44±12	13±5
7,4′-Dihydroxyflavanone	24	ND	ND	35±0	12±7
7,4′-Dihydroxyflavanone	24	21±3	15±6	72±10	20±4
Licodione	24	ND	ND	124 + 23	15 + 9

Values refer to the means ±S.D. ($n = 3$); ND = none detected.
*Time after addition of elicitor.

quantitative analysis of these compounds by HPLC used acid in the mobile phase it is possible that some of the 7,4′-dihydroxyflavone determined in the cells and medium was formed as an artefact during chromatography.

Having identified the MIPs it was then of interest to determine their rates of accumulation, as compared with that of medicarpin, in elicitor-treated cultures treated with and without tubericidin (Table 2). Over a 24 hr period following addition of elicitor it appeared that tubericidin treatment had diverted, rather than reduced, flux through the flavonoid/isoflavonoid branch of the phenylpropanoid pathway with the increased accumulation of licodione and flavone and flavanone products more than compensating for the decrease in isoflavonoid phytoalexin synthesis. At all time points, licodione was the major UV-absorbing metabolite in the inhibitor-treated cells, but the relative proportions of the flavone and flavanone increased with time.

Methylation and the regulation of flavonoid synthesis in alfalfa during the elicitation response

Our results demonstrate that the inhibitor tubericidin, while apparently acting as a selective inhibitor of transmethylation reactions rather than a general toxin, reduced the accumulation of isoflavonoids in elicitor-treated alfalfa cells and caused an accumulation of licodione, 7,4′-dihydroxyflavone and 7,4′-dihydroxyflavanone. The accumulation of 7,4′-dihydroxyflavone and licodione presumably resulted from the build of 7,4′-dihydroxyflavanone, since studies in cell cultures of *Glycyrrhiza echinata* have shown that the flavone and licodione are synthesized from 7,4′-dihydroxyflavanone following C-2 hydroxylation by a cytochrome P450 monooxygenase [18]. The 2-hydroxyflavanone intermediate then undergoes either 2,3-dehydration to give the flavone, or opening of the hemiacetal ring to give licodione (Fig. 1). Interestingly, licodione has previously been determined in alfalfa cell cultures treated for 24 hr with yeast extract [19] or following treatment with a fungal naphthoquinone [17]. However, in our cell lines, licodione does not accumulate as part of the normal phytoalexin response when using a yeast cell wall elicitor, though small amounts of 7,4′-dihydroxyflavone, which could be derived from licodione, can be observed.

The reason for the accumulation of 7,4′-dihydroxyflavanone following treatment with an inhibitor of methylation reactions is unclear. In *Pueraria lobata*, where the conversion of flavanones into isoflavones has been studied in some detail, 7,4′-dihydroxyflavanone is first converted into 2,7,4′-trihydroxyisoflavanone by a P450 mixed function oxidase [20]. This intermediate then undergoes dehydration to yield daidzein. These processes do not involve concommitant methylation. Similarly, a microsomal preparation from soybean could convert 7,4′-dihydroxyflavanone directly into daidzein [21] and in alfalfa microsomal preparations can catalyse the conversion of naringenin into genistein [4]. Therefore, the isoflavone synthase in alfalfa must be broadly similar to that determined in *P. lobata* and soybean. One possible explanation for the accumulation of 7,4′-dihydroxyflavanone in the inhibitor-treated cells is that tubericidin inhibits the isoflavone synthase reaction. When an isoflavone synthase preparation from the microsomal fraction of elicitor-treated cells was assayed for the conversion of [^{14}C]-naringenin into [^{14}C]-genistein the enzyme activities were similarly low in the presence (0.15 ± 0.02 nkat g^{-1} protein, mean±variation in duplicates) and absence (0.13 ± 0.03 nkat g^{-1} protein) of 10 μM tubericidin. Thus, tubericidin does not directly inhibit the isoflavone synthase, though the possibility that it does so indirectly cannot be discounted.

Since labelling studies in alfalfa have shown that daidzein is a poor precursor of medicarpin there has been a suggestion that isoflavone synthesis and 4′-*O*-methylation occur together *in vivo* (Fig. 1) [2]. Interestingly, Crombie and Whiting have suggested that methylation appears to be part of the mechanism of isoflavone rearrangement during rotenoid biosynthesis [22]. This is a much closer relationship than we could propose for the biosynthesis of medicarpin in alfalfa at

this stage. However, the intimacy between isoflavone synthesis and methylation is reinforced by the accumulation of formononetin, but not daidzein, in this species [8]. In other legumes, such as *P. lobata* and soybean, methylation and isoflavone rearrangement are clearly decoupled, as daidzein and its derivatives accumulate as significant metabolites in the cell [20, 21].

In the case of the phytoalexin, response in alfalfa regulatory control by methylation may be of particular interest, as both 7,4′-dihydroxyflavone and licodione are strongly antifungal [17]. The switching from inducible isoflavonoid to flavonoid synthesis might, therefore, be expected to alter significantly the spectrum of antibiotic activities associated with the phytoalexin response, and may suggest a new strategy for improving disease resistance in legumes.

EXPERIMENTAL

Studies with cell cultures. Suspension cultures of alfalfa cv. Europe were treated in mid-logarithmic growth with a yeast cell wall elicitor prepns at a final concn of 150 μg glucose equiv ml^{-1} medium [5]. For studies with methylation inhibitors, aq. solns (0.5 ml) of sinefungin, cycloleucine and tubericidin (Sigma) were added to 50-ml cultures to give a final concen of 10 μM or 100 μM 12 hr prior to addition of elicitor. At timed intervals, cells were harvested by vacuum filtration, and phytoalexins accumulating in cells and medium quantified by HPLC [7]. For cell viability studies, 50-ml cultures were centrifuged under aseptic conditions and then resuspended in 50 mM Na-Pi buffer (pH 7.5) containing 4 mg ml^{-1} 2,3,5-triphenyltetrazolium chloride 12 hr prior to adding elicitor. The cultures were then incubated in duplicate for 8 hr and the cells (1 g) extracted with 5 (v/w) 95% EtOH by heating at 60° for 10 min. The absorbance of the formazan in the extract was then determined at 485 nm. To correct for any non-specific colour developments, elicitor-treated cultures incubated in the absence of dye were also extracted and their absorbances at 485 nm determined.

For radiolabelling studies, at timed intervals after elicitor treatment, 5 ml of the cultures were aseptically transferred to a sterile 50-ml centrifuge tube and treated with 335 pmol (25 μl) [methyl-^{3}H]-methionine (2.79 TBq mmol^{-1}, Amersham International). The tubes were incubated for 2 hr on an orbital shaker at 25° before harvesting the cells by centrifugation (900 *g*, 10 min, room temp.). The supernatant was assayed for incorporation of radioactivity into phenolic metabolites by partitioning (×2) 1 ml samples against (0.5 ml) EtOAc. The EtOAc phases were pooled and assayed for radioactivity by scintillation counting. Cells were assayed for incorporation of radioactivity into phenolics by homogenizing the cells in 4 (v/w) ice-cold Me_2CO using a Polytron homogeniser (Kinematica, Lucerne, Switzerland), followed by 4 (v/w) Me_2CO–H_2O (1:1). The combined extracts were then concd to the aq. component, diluted to 1 ml with H_2O, then partitioned with EtOAc and assayed for radioactivity as described for the medium.

Enzyme assays and metabolite analysis. SAH hydrolase activity was determined in untreated cell cultures in mid-logarithmic growth phase by monitoring formation of *S*-[^{14}C-adenosyl]-L-homocysteine from [^{14}C]-adenosine and DL-homocysteine as described in ref. [23]. Microsomal prepns from cells treated with elicitor for 12 hr were assayed for isoflavone synthase activity with [^{14}C]-naringenin as substrate [7]. Protein content of the enzyme assays was determined using a dye binding assay with gamma-globulin as standard protein as recommended by the manufacturer (BioRad). SAH concns in alfalfa cell cultures were determined by HPLC [11].

Analysis and identification of MIPs. Cultures pretreated with 10 μM tubericidin and then exposed to elicitor for 12 hr were used for isolation of MIPs. MIPs accumulating in the medium were partitioned (×2) against EtOAc and the H_2O present in the combined organic phase removed with Na_2SO_4. The extract was then concd to dryness under red. pres. Cells were ground to a powder under liquid N_2 and then sequentially extracted (×2) with 4 (v/w) Me_2CO and once with 4 (v/w) Me_2CO–MeOH (1:1). Crude extracts were then concd and partitioned between H_2O and 2 vol. EtOAc. The organic phases were then processed as described for the medium. Concd extracts were redissolved in a minimal vol. of MeOH and applied to a reversed phase HPLC column in 100 μl lots using the system described previously [5]. MIPs 1–3 were collected manually and the MeCN present removed under a stream of N_2 prior to partitioning the remaining aq. residue against an equal vol. of EtOAc. After concn under a stream of N_2 the organic frs were then applied to analyt. TLC plates coated with silica gel containing fluorescent indicator (Merck) and developed in $CHCl_3$–MeOH (19:1). Purified flavonoids were recovered from the plates by removing the silica gel and eluting the compounds with MeOH. Identification of the compounds was based on UV spectroscopy in comparison with published spectra [16, 17]. Confirmation of the identity of MIP 2 as 7,4′-dihydroxyflavone was obtained by its co-chromatography (TLC and HPLC) with the authentic flavonoid (Apin Chemicals, Oxon, U.K.). The identities of MIP 1 (7,4′-dihydroxyflavanone) and MIP 3 (licodione), for which no reference standards were available, were further confirmed by dissolving the compounds in Me_2CO-d_6 and analysing them by ^{1}H NMR using a Varian VXR-400S 400-MHz spectrometer. In both cases their spectra were found to be consistent with that reported for the same compounds characterized previously in alfalfa cell cultures [17]. Having identified the MIPs they were then used to calibrate the HPLC for subsequent quantification of theses metabolites in crude extracts.

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REFERENCES

1. Dewick, P. M. and Martin, M., *Phytochemistry*, 1979, **18**, 591.
2. Dewick, P. M. and Martin, M., *Phytochemistry*, 1979, **18**, 597.
3. Martin, M. and Dewick, P. M., *Phytochemistry*, 1980, **19**, 2341.
4. Dixon, R. A., Choudhary, A. D., Dalkin, K., Edwards, R., Fahrendorf, T., Gowri, G., Harrison, M. J., Lamb, C. J., Loake, G. J., Maxwell, C. A., Orr, J. and Paiva, N. L., in *Phenolic Metabolism in Plants*, eds H. A. Stafford and R. K. Ibrahim. Plenum Press, New York, 1992, p. 91.
5. Tiller, S. A., Parry, A. D. and Edwards, R., *Physiologia Plantarum*, 1994, **91**, 27.
6. Edwards, R. and Dixon, R. A., *Phytochemistry*, 1991, **30**, 2597.
7. Edwards, R. and Kessmann, H., in *Molecular Plant Pathology. A Practical Approach*, Vol. 2, eds S. J. Gurr, M. J. McPherson and D. J. Bowles. IRL Press, Oxford, U.K., 1992, p. 45.
8. Edwards, R. and Parry, A. D., in *Biotechnology in Agriculture and Forestry; Medicinal and Aromatic Plants VII*, Vol. 28, ed. Y. P. S. Bajaj. Springer, Heidelberg, Germany, 1994, p. 328.
9. Barz, W. and Welle, R., in *Phenolic Metabolism in Plants*, eds H. A. Stafford and R. K. Ibrahim. Plenum Press, New York, 1992, p. 139.
10. Edwards, R., Parry, A. D., Gregory, A. C. E., Tiller, S. A. and Daniell, T. J., *Acta Hort.*, 1994, **381**, 214.
11. Edwards, R., *Phytochemical Analysis*, 1994, **6**, 25.
12. Lawrence, F. and Robert-Gero, M., in *Protein Methylation*, eds W. K. Paik and S. Kim. CRC Press, Boca Raton, FL, 1990, p. 305.
13. Small, D. H., Carnegie, P. R. and Anderson, R. McD., *Neuroscience Letters*, 1981, **21**, 287.
14. Chiang, P. K., Guranowski, A. and Segall, J. E., *Archives of Biochemistry and Biophysics*, 1981, **207**, 175.
15. Towill, L. E. and Mazur, P., *Canadian Journal of Botany*, 1974, **53**, 1097.
16. Mabry, T. J., Markham, K. R. and Thomas, M. B., *The Systematic Identification of Flavonoids*. Springer, New York, 1970.
17. Kobayashi, A., Yata, S. and Kawazu, K., *Agriculture and Biological Chemistry*, 1988, **52**, 3223.
18. Otani, K., Takahashi, T., Furuya, T. and Ayabe, S., *Plant Physiology*, 1994, **105**, 1427.
19. Kirikae, Y., Sakurai, M., Furuno, T., Takahashi, T. and Ayabe, S., *Bioscience and Biotechnical Biochemistry*, 1993, **57**, 1353.
20. Hakamatsuka, T. and Sankawa, U., *FEBS Letters*, 1993, **271**, 219.
21. Hagmann, M. and Grisebach, H., *FEBS Letters*, 1984, **175**, 199.
22. Crombie, L. and Whiting, D. A., *Tetrahedron Letters*, 1992, **33**, 3663.
23. Poulton, J. E. and Butt, V. S., *Archives of Biochemistry and Biophysics*, 1976, **172**, 135.