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Stress Responses in Alfalfa (*Medicago sativa* L.)[1]

V. Constitutive and Elicitor-Induced Accumulation of Isoflavonoid Conjugates in Cell Suspension Cultures

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ABSTRACT

The isoflavonoid conjugates medicarpin-3-O-glucoside-6″-O-malonate (MGM), afrormosin-7-O-glucoside (AG), and afrormosin-7-O-glucoside-6″-O-malonate (AGM) were isolated and characterized from cell suspension cultures of alfalfa (*Medicago sativa* L.), where they were the major constitutive secondary metabolites. They were also found in alfalfa roots but not in other parts of the plant. The phytoalexin medicarpin accumulated rapidly in suspension cultured cells treated with elicitor from *Colletotrichum lindemuthianum*, and this was subsequently accompanied by an increase in the levels of MGM. In contrast, net accumulation of afrormosin conjugates was not affected by elicitor treatment. Labeling studies with [^{14}C]phenylalanine indicated that afrormosin conjugates were the major *de novo* synthesized isoflavonoid products in unelicited cells. During elicitation, [^{14}C]phenylalanine was incorporated predominantly into medicarpin, although a significant proportion of the newly synthesized medicarpin was also conjugated. Treatment of ^{14}C-labeled, elicited cells with L-α-aminooxy-β-phenylpropionic acid, a potent inhibitor of PAL activity *in vivo*, resulted in the initial appearance of labeled medicarpin of very low specific activity, suggesting that the phytoalexin could be released from a preformed conjugate under these conditions. Our data draw attention to the involvement of isoflavone hydroxylases during the constitutive and elicitor-induced accumulation of isoflavonoids and their conjugates in alfalfa cell cultures.

Leguminous plants accumulate a wide range of phenolic secondary compounds, including isoflavonoid conjugates, and in many cases the routes of their biosynthesis have been elucidated (6, 7, 13). However, no detailed quantitative analyses are available on the accumulation of many of these compounds in specific parts of the plant or under stress conditions, such as occur following infection or treatment with microbial elicitors. We have recently shown that the pterocarpan phytoalexin medicarpin rapidly accumulates in heterotrophic cell suspension cultures of alfalfa (*Medicago sativa* L.) after treatment with a crude elicitor preparation from the cell walls of *Colletotrichum lindemuthianum*. This accumulation is preceded by induction of the activities of the 11 enzymes involved in medicarpin biosynthesis from L-phenylalanine (5, 18).

The secondary products in these alfalfa cell cultures are similar to those found in alfalfa roots, but different from those of alfalfa leaves (18). We now report the characterization of the three major phenolic compounds, previously named CI, CII, and CIII (18), which accumulate constitutively in alfalfa roots and cell cultures. These metabolites have been identified as glucosidic conjugates of isoflavonoids. We describe the synthesis of these compounds in control and elicitor-treated alfalfa cells and evaluate whether such compounds could act as a preformed precursor pool for the synthesis of phytoalexins, as appears to be the case in soybean (11).

MATERIALS AND METHODS

Cell Cultures and Elicitor Treatment

Cell suspension cultures of alfalfa (*Medicago sativa* L. cv Calwest 475) were initiated and maintained as described (18). Elicitor from *Colletotrichum lindemuthianum* was prepared and used to treat suspension cultured cells according to a previous report (12). Elicitor was added in aqueous solution and an equivalent amount of water added to control cultures.

Identification of Secondary Compounds

Unelicited alfalfa cells (300 g) were harvested by filtration 10 to 14 d after subculture. The cells were homogenized in acetone and the organic phases from three repeated extractions pooled and concentrated under reduced pressure. The residue was dissolved in 500 mL HPLC-grade water (pH 7) and applied to a column (2.5 × 30 cm) packed with Polyamide 6 (J.T. Baker, Phillipsburg, NJ) that had been equilibrated with water. The column was sequentially eluted at 4 mL/min with water, methanol (fraction 1), and methanol + ammonium hydroxide (500:1, v/v) (fraction 2). Fractions 1 and 2 were concentrated under reduced pressure and used for subsequent preparative HPLC. Analytical HPLC was performed as described earlier (9). CI was found in fraction 1, whereas CII and CIII were eluted under basic conditions only (fraction 2). The same system was used for preparative HPLC except that the aqueous phase was acidified with 3% acetic acid instead of 1% phosphoric acid and a 250 × 22.5 mm column (Econosil C18, Alltech) was used with elution at 8 mL/min.

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Fractions containing the compounds of interest (CI, CII, and CIII) were evaporated to dryness and used directly for spectroscopy.

^{1}H NMR spectra were recorded on a Varian XL 400 MHz NMR spectrometer. Electron impact (70 eV) mass spectrometry was conducted on a VG Tritech TS-250 mass spectrometer using direct-probe sample introduction. LSIMS[2] data were obtained on a VG Analytical ZAB2-SE mass spectrometer equipped with a +35 keV Cs^+ primary ion gun operating at 2 μA. Samples for LSIMS were dissolved in a matrix consisting of 1% TFA (EM Science) in 1-thioglycerol (Fluka). All spectra were acquired on a VG 11-250J data system. LSIMS data were recorded in a multichannel analyzer mode and 5 to 10 scans were typically averaged for each spectrum. The determination of methylated sugars by GLC was carried out according to Kamalavilas and Mort (15).

Spectroscopic data for the three compounds are given below:

CI

UV	(70% EtOH) 260, 310 sh
MS	(EI) m/z, 298 (M+), 283, 268, 253
MS	(LSIMS) m/z, 461 $(M + H)^+$

CII

UV	(70% EtOH) 260, 310sh
MS	(EI) m/z, 298 (M+, 100), 283 (13), 268 (58), 253 (12), 166 (22), 151 (7), 149 (8), 132 (15), 117 (14)
MS	(LSIMS) m/z, 547 ($(M + H)^+$, 47), 517 (40), 461 (16), 299 (100), 269 (75)
^{1}HNMR	(δ_H ppm [U^2-DMSO]); 8.32 (1H, C-2); 7.53 (2H, d, J = 8.5, C-2′, C-3′); 7.48 (1H, s, C-5); 7.23 (1H, s, C-8), 6.97 (2H, d, J = 8.5, C-5′, C-6′); 3.89 (3H, s, C-6, O-CH_3); 3.795 (3H, s, C-4′, O-CH_3); 5.12 (1H, 10 Hz, C-1″); 4.2 (2H, C-6″)

CIII

UV	(70% EtOH λ_{max}) 282.5
MS	(EI) m/z 270 (100), 255 (58), 197 (7), 161 (10), 148 (17), 147 (10), 135 (10)
^{1}HNMR	(δ_H ppm [U^2-DMSO]); 8.52 (1H, s, C-6); 7.38 (1H, d, J = 8.3 Hz, C-1); 7.24 (1H, d, J = 8.7Hz, C-7); 6.71 (1H, dd, J = 2.5, 8.3 Hz, C-2); 6.55 (1H, d, J = 2.2, 8.7 Hz, C-8); 6.41 (1H, s, C-10); 5.59 (1H, d, J = 7 Hz, C-11a); 4.86 (1H, d, J = 6.5 Hz, C-1′); 4.28 (1H, m, H-6 eq); 3.69 (3H, s, C-9, O-CH_3)

For determination of malonic acid, CI, CII, and CIII were hydrolyzed (0.1 N KOH, 2 h, room temperature). After neutralization with 0.1 N HCl, the samples were dried under reduced pressure. Malonic acid was determined with *p*-bromphenacylbromide (Pierce, IL, USA) for derivatization followed by HPLC as described (1).

Sugar conjugates were enzymatically hydrolyzed after incubating 20 μL of a 5 mg/mL solution of the unknown compound with 80 μL of enzyme (2 mg/mL) dissolved in phosphate/citrate buffer pH 5.2. Enzymes tested included almond β-glucosidase, cellulase from *Trichoderma viride* (Boehringer) and snail β-glucuronidase (Sigma). After incubation for 20 h at 37°C, reactions were stopped by addition of 5 μL TFA (10% v/v), and 20 μL of reaction mixture was analyzed by HPLC.

Thin-layer electrophoresis was carried out at 500 V on silica gel plates with acetic acid:pyridine:water (0.2:5:95, v/v/v), pH 6.5 as electrolyte.

Labeling Studies

Alfalfa suspension cells (100 mL) 5 d after subculture were incubated with 1.85 MBq L-[U-^{14}C]phenylalanine (Amersham, 19 GBq/mmol) for 60 min. The culture was then divided into two portions. The first (30 mL) was treated with water, the second (60 mL) with fungal elicitor at a final concentration of 50 mg glucose equivalents per L of cell medium. The elicited cells were further divided in two 30-mL batches and one treated with AOPP (final concentration 10 μM). The complete labeling experiment was repeated with 100 μM AOPP. Cells (5-mL batches) were harvested at 0, 2, 4, 8, 12, and 24 h, extracted in acetone, and the extracts concentrated under reduced pressure. The concentrates (50 μL) were analyzed by HPLC (18) with UV detection and the eluates monitored for radioactivity with a Beckman 171 radioisotope detector. Recovery of radiolabel was monitored at all stages by scintillation counting.

RESULTS

Structural Elucidation of Isoflavonoid Conjugates

The three major phenolic metabolites (CI, CII, and CIII) previously observed in alfalfa cell cultures (5, 18) were purified by polyamide column chromatography and preparative HPLC. The purity was checked prior to spectroscopic analysis using different HPLC systems and TLC and was shown to be greater than 95%. Structural data for the three metabolites are given in "Materials and Methods." The two major polar components observed by HPLC, CI, and CII, showed identical UV-spectra with a pronounced maximum at 260 nm. Furthermore, the MS (EI) spectra of CI and CII showed that both compounds produced ions at 298 m/z and gave identical fragmentation patterns. In addition, CII was quite unstable during processing of the extracts and, according to HPLC analysis, appeared to break down to CI. It was therefore concluded that CII was a direct derivative of CI and subsequent structural elucidation therefore focused on CII. In addition to the ion peak at 298 m/z, the MS (EI) spectrum of CII showed the typical Retro-Diels-Alder fragmentation pattern of isoflavones (7, 21). The main fragments of M_r 166 m/z and 132 m/z were assigned as ring A and ring B fragments, respectively. The MS (EI) data were consistent with an isoflavone having one methoxyl group in both rings A and B, and

[2] Abbreviations: LSIMS, liquid secondary ion mass spectroscopy; AOPP, L-α-aminooxy-β-phenylpropionic acid; AGM, afrormosin-7-*O*-glucoside-6″-*O*-malonate; AG, afrormosin-7-*O*-glucoside; MGM, medicarpin-3-*O*-glucoside-6″-*O*-malonate; MG, medicarpin-3-*O*-glucoside; R_t, retention time.

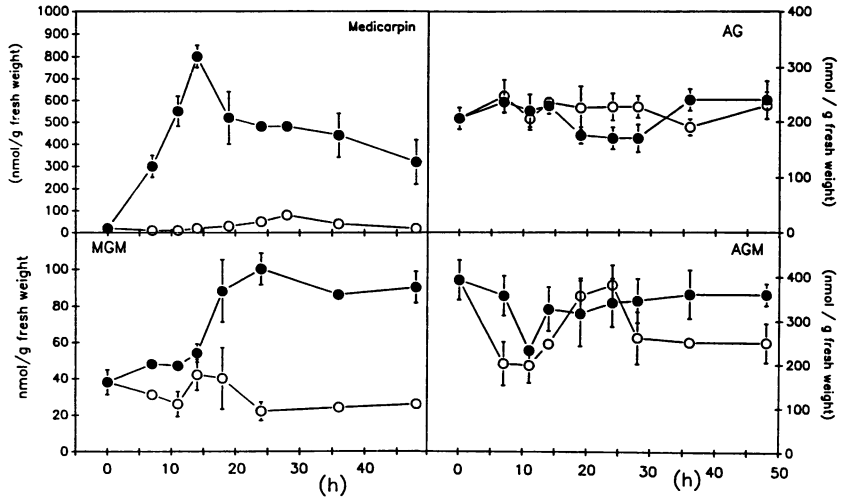

Figure 1. Effect of treatment with fungal elicitor on cellular concentrations of medicarpin, MGM, AG, and AGM. Control treatments (○), elicitor treatments (●).

one hydroxyl group in ring A. The structure of the isoflavone aglycone of CII was confirmed by ^{1}H-NMR and by comparison of structural data with those obtained from commercially available 6,7,4′-trimethoxyisoflavone (Aapin Chemical Ltd., Oxon, England). All spectroscopic data were consistent with the proposal that CII contained afrormosin (4) as the aglycone, although our data do not completely rule out the possibility that the methoxy group could be on the 7 rather than the 6 position of the A-ring. Afrormosin is, however, found in a wide range of legume species (7).

Since CII rapidly broke down to CI, was retained on reversed phase HPLC columns only under acidic conditions (pH 1.5–2.5), and migrated toward the anode during electrophoresis, it was concluded that CII was an acidic conjugate of afrormosin. After methanolysis, the sugar moiety of the conjugate was identified as glucose by GLC (15). In addition, malonic acid was identified as the acid function by derivatization and HPLC analysis. The presence of a malonyl glucoside attached to afrormosin was further confirmed by LSIMS which gave the predicted $(M + H)^+$ peak at 547 m/z, and signals at 461 m/z for the glucoside and 299 m/z for the aglycone. The signal at 5.12 ppm in the ^{1}H-NMR analysis showed that the glucose was in the beta configuration, and the doublet at 4.2 ppm revealed that the malonic acid was esterified through the C6 of the glucose moiety. As a result, CII was identified as AGM. This is the first time that this conjugate has been described as a natural product in plants.

The relationship of CI to CII was further investigated. Chemical hydrolysis by classical methods (21) failed to yield analyzable amounts of the aglycone of either compound. However, both compounds were readily hydrolyzed by almond β-glucosidase to release a moiety which cochromatographed with afrormosin in both cases. The LSIMS spectrum of CI showed a signal at 461 m/z which corresponds to the protonated ion of afrormosin glucoside. As CI could be formed by the *in vitro* breakdown of CII (see above), CI was identified as AG.

The UV and MS(EI) spectra of CIII were identical to those of authentic (±) medicarpin (kindly provided by Dr. W. Barz, Muenster, FRG), and the (−) isomer of medicarpin isolated from elicitor-treated alfalfa cell cultures. The structure of the aglycone was further confirmed by ^{1}H-NMR spectroscopy. The presence of a β-glycosidic proton (δ_H 5.12 ppm, ^{1}H) and the typical spectrum of a sugar conjugate revealed that medicarpin was linked via its hydroxyl group at C3 to a sugar, which was identified by methanolysis/GLC as glucose. All spectroscopic data were identical with those of medicarpin-3-O-glucoside, which has been described from alfalfa roots by Sakagami *et al* (24). However, the presence of an acid function in the conjugate was suggested by the binding of CIII to reversed-phase HPLC columns only in the presence of acids, its elution behavior from the polyamide column, and its migration toward the anode during electrophoresis at neutral pH. The signal at δ_H 4.2 ppm in the NMR-spectrum suggested that glucose was esterified via its C6 hydroxyl group to an acid. The acid function was identified as malonic acid by chemical derivatization and HPLC. CIII was therefore identified as MGM. This is the first time that this compound has been observed as a natural plant product. In addition to MGM, MG was also observed in cell extracts (R_t = 19.5 min, HPLC [9]). MG was a minor component compared to MGM (<10%) and it is possible that MG may have been an artefactual breakdown product of MGM.

Accumulation of Isoflavone Conjugates in Alfalfa Cell Cultures

AGM was the major extractable phenolic compound present in unelicited alfalfa cell suspension cultures, reaching maximum levels 2 to 4 d after subculture (250–500 nmol/g fresh weight). In contrast, AG was present in smaller amounts and accumulated maximally during the first days of the growth cycle (50–75 nmol/g fresh weight). Rapid and careful analysis of cell extracts was required since AG was found at higher concentrations in extracts after standing for several h at room temperature. MGM accumulated to a peak concentration 1 d after subculture (50–100 nmol/g fresh weight) but was also observed at appreciable levels during late stages of the growth cycle (4–7 d).

Addition of fungal elicitor led to a rapid accumulation of

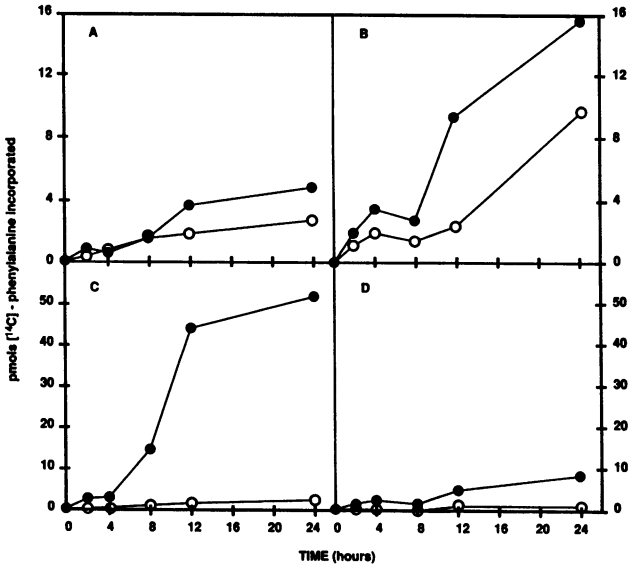

Figure 2. Accumulation of the radiolabeled isoflavonoids AG (A), AGM (B), medicarpin (C), and MGM (D) following treatment of control (○) and elicited (●) alfalfa cells with [^{14}C]phenylalanine.

medicarpin, with maximum levels obtained after 14 h (Fig. 1). As reported earlier (18), approximately 50% of the medicarpin accumulated in the growth medium. Following the maximum of medicarpin accumulation, peak levels of MGM were observed at 24 h (Fig. 1). Thus, MGM accumulated during the period in which free medicarpin was declining (12–48 h), suggesting that the phytoalexin was being actively glucosylated. However, the decline in medicarpin content during this period (800-320 nmol/g fresh weight) was not accompanied by a directly corresponding increase in MGM (40–100 nmol/g), suggesting that the disappearance of medicarpin can only be partially explained by the formation of the malonyl glucoside.

In contrast to MGM, the intracellular concentrations of AG and AGM (Fig. 1) were not affected by treatment of cells with fungal elicitor.

Labeling Studies with [^{14}C]phenylalanine

Cell suspension cultures (elicited or unelicited) were treated with L-[U-^{14}C]phenylalanine (3 nmol/g fresh weight, 60 GBq), sampled over a 24 h period, and analyzed for incorporation of ^{14}C into phenolic metabolites. As determined by HPLC, radiolabel accumulated into nine metabolites of which four could be clearly identified as AG, AGM, MGM, and medicarpin. The remaining five metabolites cochromatographed with minor unknown UV-absorbing fractions. The extent of accumulation of radioactivity into these metabolites depended upon the treatment used. In control cells, the majority of the radiolabel accumulated in AGM (Fig. 2B) and to a lesser extent into AG (Fig. 2A). Incorporation into medicarpin (Fig. 2C) and MGM (Fig. 2D) was very slow and represented less than 0.01% of the applied radioactivity. Incorporation of [^{14}C]phenylalanine into isoflavonoid derivatives was maximal in cultures treated with fungal elicitor. Such cells rapidly accumulated radiolabel in medicarpin (Fig. 2C), which constituted the major radioactive metabolite of the isoflavonoid biosynthetic pathway in elicited cells. Levels of [^{14}C]medicarpin continued to rise over the 24 h period to reach a final value of 50 pmol [^{14}C]phenylalanine incorporated per g fresh weight of cells. Interestingly, the increase in medicarpin synthesis was accompanied by the accumulation of radiolabeled MGM (8 pmol [^{14}C]phenylalanine incorporated/g in 24 h), indicating that up to 16% of the newly synthesized medicarpin was being glycosylated. The incorporation of label into the glycosylated forms of afromosin doubled after 24 h of elicitation (Fig. 2, A and B).

Table I. *Effects of AOPP on Medicarpin Synthesis in Elicited Alfalfa Cell Cultures*

Time Postelicitation	[^{14}C]Medicarpin[a] (per g fresh weight)					
	Elicited			Elicited + AOPP		
h	*dpm*	*nmol*	*dpm/nmol*	*dpm*	*nmol*	*dpm/nmol*
Experiment I (10 μM AOPP)						
0	0	0 (0)	0	0	0 (0)	0
2	860	1 (1)	860	0	5 (4)	0
4	2,160	6 (10)	360	69	8 (10)	9
8	17,020	82 (91)	208	12,015	51 (40)	236
Experiment II (100 μM AOPP)						
0	0 ± 0	1 ± 1	0 ± 0	0 ± 0	3 ± 1	0 ± 0
2	1,071 ± 103	18 ± 1	60 ± 9	137 ± 5	18 ± 3	8 ± 2
4	2,808 ± 407	20 ± 3	140 ± 1	107 ± 17	25 ± 6	4 ± 0
8	11,016 ± 806	82 ± 7	134 ± 24	2,604 ± 105	31 ± 6	84 ± 20

[a] ^{14}C-Medicarpin is expressed as nmol of phytoalexin, and radioactivity (dpm) incorporated from L-[U-^{14}C]phenylalanine, at times shown. Medicarpin was undetectable in control, unelicited cultures over the times shown (0–8 h). At 24 h after addition of label, unelicited cells contained 6 nmol medicarpin per g fresh weight (specific activity 250 dpm/nmol). In experiment I, values for medicarpin determinations given in parentheses represent the results from parallel incubations treated with an equivalent amount of cold phenylalanine to that used in the ^{14}C-studies. In experiment II, the incubations (labeled and unlabeled) were run in duplicate and values are given as means with ± the difference between replicates.

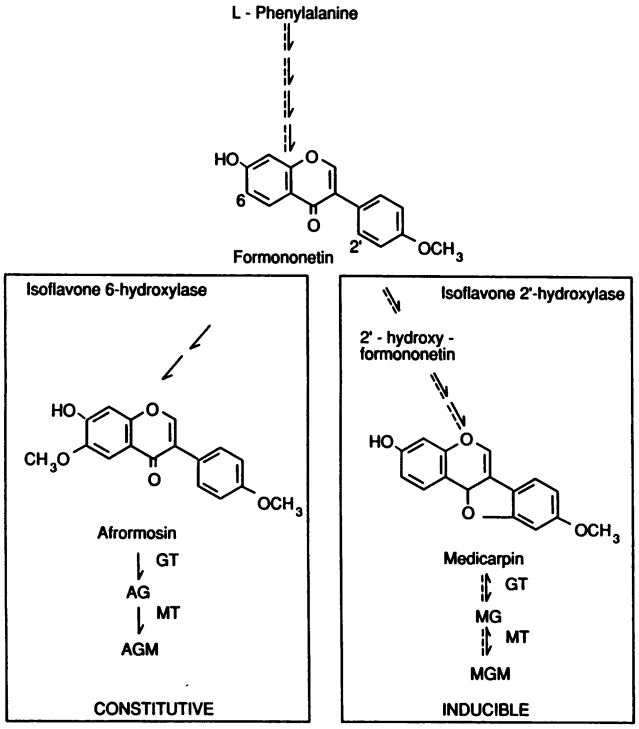

Figure 3. Proposed biosynthetic pathway for isoflavonoid conjugates in alfalfa cell cultures. GT, glucosyl transferase; MT, malonyl transferase.

In chickpea cell suspension cultures, inhibition of the flux through the phenylpropanoid pathway with the potent PAL inhibitor AOPP results in formation of medicarpin from isoflavone glycoside pools (W Barz, personal communication). Treatment of alfalfa cells with elicitor together with 10 or 100 μM AOPP reduced the incorporation of [^{14}C]phenylalanine into isoflavonoids to control levels (data for minus elicitor controls not shown) for the first 4 h of treatment. However, after 4 h, the appearance of radioactivity in AG, AGM, medicarpin, and MGM was only partially inhibited by 10 μM AOPP, and this coincided with the onset of maximal flux of [^{14}C]phenylalanine into the pathway in inhibited cells. The flux into the isoflavonoid pathway was more effectively inhibited by 100 μM AOPP. To determine whether elicited alfalfa cells could potentially mobilize MGM into free medicarpin, changes in the specific activity of [^{14}C]medicarpin were compared in cells treated with elicitor alone or elicitor plus AOPP (Table I). During the first 4 h after addition of 10 or 100 μM AOPP, during which period the inhibitor greatly decreased incorporation from [^{14}C]phenylalanine into medicarpin, similar quantities of medicarpin appeared in cells treated with AOPP and in cells treated with elicitor alone. As the specific activity of ^{14}C in this medicarpin was much lower than in the medicarpin accumulating at this period in the absence of AOPP, we conclude that the medicarpin can originate from a preformed precursor (presumably MGM) in elicited cells in which the flux into the isoflavonoid pathway is blocked. By 8 h, the inhibitory effects of 10 μM AOPP were largely overcome, and the specific activity of medicarpin was then similar in the two treatments. The inhibition was maintained for longer in the presence of 100 μM AOPP.

DISCUSSION

We have identified the three major constitutive phenolic metabolites of our alfalfa cell suspension cultures as MGM, AG, and AGM. These compounds are also found in alfalfa roots, from where medicarpin-9-O-glucoside was previously isolated (24). The malonyl ester linkages in MGM and AGM are particularly labile, and this may account for these compounds not having being described before. Malonyl glucosides of flavonoid derivatives appear to be stored in the vacuole (22). This would allow for the safe sequestration of the aglycones of potentially phytotoxic products during constitutive synthesis under nonstress situations.

A probably pathway for the biosynthesis of the isoflavonoid conjugates is shown in Figure 3. In unelicited cells, the major products are afrormosin conjugates. This suggests that, under such conditions, the first enzyme specific for isoflavonoid phytoalexin biosynthesis, formononetin 2′-hydroxylase, is rate limiting, and that flow occurs instead through the 6-hydroxylase. We have recently shown that isoflavone 2′-hydroxylase activity is virtually undetectable in unelicited alfalfa cells but is induced approximately 5- to 10-fold on treatment with elicitor (18). Our present results suggest that the 6-hydroxylase is probably a separate enzyme whose activity may be less strongly induced in response to elicitor than the 2′-hydroxylase. This point requires further verification, however, as the lack of net accumulation of AGM in elicitor-treated cells in which label from [^{14}C]phenylalanine is incorporated into afrormosin conjugates indicates that the AGM pool turns over fairly rapidly, thus making it diffficult to assess the total flux through the 6-hydroxylase. The use of the term "constitutive" in Figure 3 should not, therefore, be taken to exclude elicitor inducibility. In spite of this constraint, our data draw attention to the potentially important regulatory roles of the Cyt P450 hydroxylases (17, 18) in isoflavonoid accumulation in alfalfa.

In chickpea, the enzymes catalyzing isoflavonoid conjugation have been shown to be constitutively expressed glucosyl and malonyl transferases with high activity which exhibit strict specificity for their respective aglycones/conjugates (3). Our labeling studies suggest that, during the first 4 h of elicitation, a significant proportion of the newly synthesized medicarpin may be converted to the malonyl glucoside (Fig. 2, C and D). After this time, label appeared predominantly in medicarpin itself. After the period of maximum accumulation of medicarpin in the cells, labeling of the conjugate began to increase. Under 'artificial' conditions, in which initial flux from [^{14}C] phenylalanine through the phenylpropanoid pathway was inhibited by an inhibitor of PAL activity, the accumulation during the first 4 h of elicitation of medicarpin of much lower specific activity than that accumulating at this time in cells treated with elicitor alone (Table I), suggests that the conjugate can be converted back to the free aglycone. These data indicate that the synthesis and turnover of the conjugated phytoalexin are processes which may be actively regulated by exogenous stress signals. In chickpea, the conjugated isoflavo-

noids which accumulate are derivatives of precursors of medicarpin rather than the phytoalexin itself (3). In this system, formononetin-7-*O*-glucoside-6″-*O*-malonate can exhibit rapid turnover, whereas the corresponding conjugate of 5-hydroxy-formononetin appears to be metabolically inert (14). Formononetin is not liberated from its conjugate during elicitation or infection with *Ascochyta rabiei* (16) except under artificial conditions where, as in the present work, PAL activity is blocked with AOPP (W Barz, personal communication). It will be of great interest to understand how legume cells activate deconjugation in response to conditions in which phenylpropanoid biosynthetic enzymes are induced but no metabolic flux occurs through the pathway. The induction of glucosyl- and quinate-transferase activities by *trans*-cinnamic acid, the product of the PAL reaction (10, 20), suggests a plausible mechanism involving regulation of enzyme activities/levels by pathway intermediates.

The functions of isoflavonoid conjugates in the intact plant are not clear. In addition to providing protection from metabolic spill-over, conjugation could provide a source of isoflavonoid metabolites which could be mobilized under stress conditions if the availability of carbon sources for phenylpropanoid biosynthesis became rate-limiting, for example in the late stages of an infection which depleted phenylalanine pools. In alfalfa roots, conjugation may prevent medicarpin, formed in response to the mechanical stress of root growth itself or interactions with nonpathogenic soil microorganisms, from inhibiting nodulation through its phytotoxicity to *Rhizobium* species (23) or exhibiting autotoxicity, a possible basis for allelopathy in alfalfa (8). Neither afrormosin nor its isomer alfalone appear to exhibit antimicrobical activity (2, 19). However, afrormosin has been shown to be toxic to soybean loopers (4), and a pool of the conjugated isoflavone could possibly act as a preformed insect deterrent. Whatever their functions, the existence of conjugated isoflavonoids and the enzymatic systems for their synthesis and turnover suggest potential novel means for manipulating defense responses in legumes by genetic engineering.

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