METABOLISM OF PHENYLALANINE AND CINNAMIC ACID IN TOBACCO CELL LINES WITH HIGH AND LOW YIELDS OF CINNAMOYL PUTRESCINES

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ABSTRACT.—The different yields of cinnamoyl putrescines by two cell lines of *Nicotiana tabacum* were accounted for by the different activities of enzymes involved in their biosynthesis. The metabolism of the precursors phenylalanine and cinnamic acid were compared in these cell lines. The high producing cell line tolerated 10^{-2}M L-phenylalanine without any growth inhibition and incorporated three times more phenylalanine into cinnamoyl putrescines than the low yielding line. The incorporation of exogenously added phenylalanine resulted in a corresponding increase of cinnamoyl putrescines. When phenylalanine levels were increased from $2 \cdot 10^{-3}$ to 10^{-2}M total incorporation was unchanged even in the presence of precursors of the amine moiety. Cinnamic acid was quite toxic to both cell lines and was not directly incorporated into cinnamoyl putrescines. During the first hours of feeding, ethyl acetate-soluble metabolites were dominant which were then converted to water-soluble metabolites. Though most of the added cinnamic acid was finally found as cinnamoyl putrescines, it is concluded that the incorporation did not follow the natural biosynthetic pathway.

The results reported on the influence of added biosynthetic precursors on the yield of secondary compounds by plant cell cultures are quite variable and often contradictory (1). Thus, L-phenylalanine added to the B5-medium of *Coleus blumei* did (2) or did not (3) influence the levels of rosmarinic acid. Or, the final concentration of indole alkaloids in *Catharanthus roseus* cell cultures was positively (4) or negatively (5) effected by the supply of L-tryptophan. There are, of course, many explanations for these different results. A major point of criticism might be the fact that most of the feeding experiments were done with cell lines which were not adequately characterized. Recently, we reported on cell lines of *Nicotiana tabacum* with different yields of cinnamoyl putrescines (6). The 10-fold increased accumulation by cell line TX4 was mainly due to increased activities of enzymes involved in that secondary pathway [e.g. phenylalanine ammonia-lyase (EC 4.3.1.5; PAL), cinnamate 4-hydroxylase (EC 1.14.13.14), 4-coumarate:CoA ligase (EC 6.2.1.12), ornithine decarboxylase (EC 4.1.1.17) and arginine decarboxylase (EC 4.1.1.19)]. A comparison of precursor effects on the levels of cinnamoyl putrescines in the high yielding cell line TX4 and the low yielding cell line TX1 was thought to provide some general clues on the usefulness of feeding experiments for increasing product levels. Here we report on the metabolism of L-phenylalanine and cinnamic acid and their effects on product levels of the variant cell lines.

MATERIAL AND METHODS

PLANT MATERIAL.—Maintenance and some of the characteristics of wild-type cells TX1 and p-fluorophenylalanine(PFP)-resistant cell line TX4 (*Nicotiana tabacum* L.cv.Xanthi) have been described previously (6-10).

FEEDING OF LABELED PRECURSORS.—The indicated amounts of diluted L-phenylalanine-[U-^{14}C] or trans-cinnamic acid-[3-^{14}C] were fed to 5-day-old cultures for the indicated periods under sterile conditions. Radioactivity was monitored by scintillation counting or directly on chromatograms by a Berthold-Silena NIM multichannel TLC-analyzer. The cells were harvested by vacuum filtration and extracted with MCW (methanol-chloroform-water, 12:5:3). The extracts were evaporated to dryness at 30° and redissolved in 10 ml water. The water extract was successively extracted with light petroleum ether and then with ethyl acetate after being acidified to pH 4. The petroleum ether phases containing only traces of radioactivity were discarded. The ethyl acetate phase (only labeled after feeding cinnamic acid) and the water phase were further analyzed for labeled metabolites.

ETHYL ACETATE SOLUBLE COMPOUNDS.—Chromatography of the extract in L1 (toluene-ethyl formate-formic acid, 5:4:1, silica gel) revealed 3-4 major radioactive bands. Two were identified as cinnamic acid and *p*-coumaric acid by co-chromatography. The free cinnamic acids

were scraped off and eluted with methanol. Both cinnamic acids (0.1 mg) when treated with 100 µl MeOH/HCl for 1 h at room temperature gave the methyl esters, which were directly analyzed by a AEI-MS30 mass spectrometer. Cinnamic acid methylester: MS, *m/z* (%) 162(M^+/30), 131(100), 103(85), 77(68). Para-coumaric acid methylester: MS, *m/z* (%) 178(M^+/75), 147(100), 119(35), 91(25). The free cinnamic acids moved at the front with the systems L2 (ethyl acetate-methyl ethyl ketone-formic acid-water, 5:3:1:1, silica gel) and L3 (*n*-butanol-acetic acid-water, 4:1:1, cellulose). Two other radioactive zones had Rf values of 0.9 and 0.55 in system L2. They were also scraped off. Parts of the eluted zones with the lower Rf were hydrolyzed with 2N NaOH at room temperature for 2 h, acidified and extracted with ethyl acetate. Chromatography in L1 revealed cinnamic acid and *p*-coumaric acid. Co-chromatography of the water phase in L3 and detection with aniline phthalate identified glucose as the conjugate of the cinnamic acids. The other part (0.1 mg) was acetylated with 100 µl TFAA at 60° for 30 min. The acetylated samples were analyzed by gc/ms. Separation was achieved on a 15 m x 0.25 mm i.d. fused silica capillary column (J+W California) coated with SE30 on a Perkin-Elmer gas chromatograph F22. Conditions: carrier gas helium 0.65 bar, split ratio 1:30, temperature program 100°-300°, 6°/min, detection by FID. The gc system was coupled to the mass spectrometer AEI-MS30 via an open split connection (11). The spectra were recorded at an electron energy of 24 eV in combination with the data system AEI-DS50.

The TFAA derivative of cinnamoyl glucose ester had the retention index (12) I=2030; MS, *m/z* (%) 694(M^+/7), 319(1.5), 148(51), 147(16), 131(100), 104(26); *p*-coumaroyl glucose ester I=2170; MS, *m/z* (%) 806(M^+/24), 319(7), 260(37), 259(4), 243(100), 216(27).

The zone with the Rf=0.9 gave a positive reaction with the Folin reagent (9) indicating a free phenolic hydroxyl group. An aliquot of the eluted compound was hydrolyzed by 6N HCl for 6 h at 100°. The elution profile from an amino acid analyzer showed aspartic acid as the only amino acid present. The proposed *p*-coumaroyl aspartate was esterified with 100 µl MeOH/HCl for 1 h at room temperature and measured directly by mass spectroscopy: MS, *m/z* (%) 307(M^+/5), 248(4), 160(3), 147(100), 119(10), 91(7). This spectrum corresponded with *p*-coumaroyl aspartate dimethylester. The structure was confirmed by trifluoroacetylation of this sample resulting in following fragmentation: MS, *m/z* (%) 403(M^+/1), 344(12), 243(100), 215(6), 160(11).

WATER SOLUBLE COMPOUNDS.—Analysis and quantification of cinnamoyl putrescines have been described (6,13). Since the absorption spectra of MCW-extracts showed a typical spectrum of caffeoyl putrescine and the ratio of caffeoyl-, feruloyl-, *p*-coumaroyl putrescine was 8:1:1, the quantitative analysis was based on the absorption coefficient of caffeoyl putrescine $\epsilon_{320}=18600$. Chromatographic systems: L4(isobutyl methyl ketone-formic acid-water, 14:3:2, silica gel), L5(*n*-propanol-Tris-HCl ($3\cdot10^{-3}$M, pH 8.0), 7:3, cellulose) or L6(1.5M sodium acetate, IONEX-SA 25-Na).

RESULTS AND DISCUSSION

INFLUENCE OF L-PHENYLALANINE ON CINNAMOYL PUTRESCINE YIELDS.—We recently showed that L-phenylalanine-[U-^{14}C] given as the tracer was more rapidly incorporated into cinnamoyl putrescines by TX4 cells (10). No other metabolites of labeled phenylalanine were detected in MCW-extracts of TX1 and TX4 cells.

The effects of high concentrations of L-phenylalanine on growth and productivity were unknown. Figure 1 shows the influence of increased levels of L-phenylalanine on growth. While a concentration of 50 mg phenylalanine/flask (~700 mg/liter) reduced growth of TX1 cells to 50%, TX4 cells grew without any inhibition on a 100 mg/flask. After 4 days of feeding, both cell lines had taken up similar amounts of the added precursors (table 1). Most of the phenylalanine taken up was found in the MCW-extracts. Chromatography in systems L3–L6 were used to determine incorporation of labeled phenylalanine into cinnamoyl putrescines (table 1). Per flask, TX1 cells incorporated between 2.9 and 3.3 mg phenylalanine into cinnamoyl putrescines, if the medium was supplemented with 20 mg/flask. The total incorporation was only slightly higher (3.4–3.7 mg) at 50 mg phenylalanine. TX4 cells with the increased enzyme activities converted 3 times more phenylalanine into cinnamoyl putrescines (table 1). Increasing the phenylalanine levels from 20 to 50 mg increased the incorporation only from 9 mg to 11.5 mg.

These incorporation data were compared with the total amounts of cinnamoyl putrescines found in the MCW-extracts (table 2). In the presence of 20 mg phenylalanine, the content of cinnamoyl putrescines was roughly doubled by TX1 cells indicating an additional incorporation of 3.0 mg phenylalanine into cinnamoyl putrescines. At 50 mg phenylalanine + 50 mg putrescine/arginine growth was reduced to 50%, but total cinnamoyl putrescines were almost unchanged (10.3

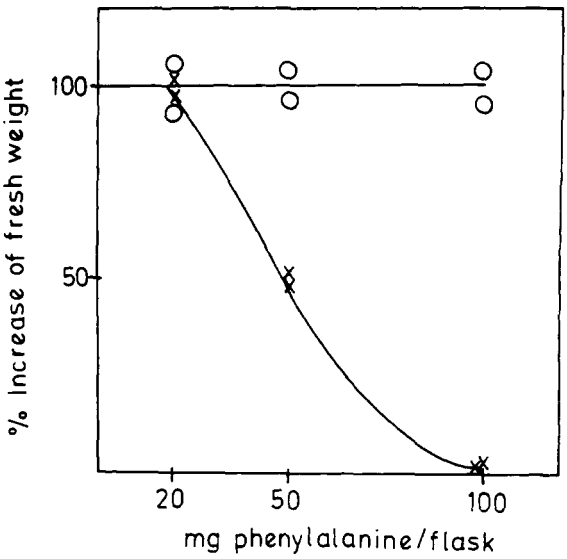

FIG. 1. Influence of feeding increasing levels of L-phenylalanine to 5-day-old cultures of TX1 (×—×) and TX4 (○—○). The initial and final fresh weights of the controls are given in Table 1. Each flask contained 70 ml MS-medium+$2 \cdot 10^{-6}$M 2,4-D.

mg/360 mg dry wt.). The specific conversion of phenylalanine into cinnamoyl putrescine was evidently higher by the growth-inhibited cells. It has often been observed that secondary metabolism is increased when growth ceased (14,15). Thus by suppressing growth of TX1 cells by phosphate limitation, we were able to induce accumulation of cinnamoyl putrescines and the activity of related enzymes (16). Therefore the higher specific incorporation of precursors by growth-

TABLE 1. Distribution of radioactivity after feeding various concentrations of L-phenylalanine (phe), L-arginine (arg) and putrescine (put) to 5-day-old cultures of TX1 (fr. wt.~3.8 g) and TX4 (fr. wt.~4.4 g) for 4 days. At the end the fr. wt. controls of both cell lines were 13.1±0.4 g/flask.

Precursors added +1 μCi L-phe -[U-^{14}C]	% Distribution of total radioactivity in							
	TX1				TX4			
	medium	MCW-extract			medium	MCW-extract		
		total	phe	cipu		total	phe	cipu
control	2.2	18.4	7.2	9.3	2.7	42.4	0.1	36.8
+20 mg phe	2.2	67.4	45.0	14.7	6.3	73.9	20.2	45.1
+20 mg phe+25 mg arg	2.1	71.2	46.5	16.4	3.7	72.9	20.2	44.2
+50 mg phe+60 mg arg	6.5	69.4	55.2	7.4	4.5	70.0	38.9	22.5
+50 mg phe+50 mg put	6.8	72.0	59.3	6.9	5.1	68.3	39.9	23.7

inhibited cells might be due to increased activities of enzymes involved in cinnamoyl biosynthesis.

Feeding of phenylalanine to TX4 increased cinnamoyl putrescines only by 30–40% (table 2). However, this means that between 9 and 12 mg of the added phenylalanine were used for the synthesis of additional cinnamoyl putrescines,

TABLE 2. Determination of cinnamoyl putrescines in 9-day-old cultures of TX1 and TX4 grown in the absence or presence of 20 mg L-phenylalanine per flask ($2 \cdot 10^{-3}$M) for 4 days. The ratio caffeoyl putrescine:feruloyl putrescine:p-coumaroyl putrescine was roughly 8:1:1. Therefore molecular weight (M.W. 250) and molar extinction coefficient ($\epsilon_{320} = 18600$) of caffeoyl putrescine (cipu) were used for calculations.

	TX1		TX4	
	control	+20 mg phenylalanine	control	+20 mg phenylalanine
dry weight (mg)	715	706	720	716
cipu (mg)	4.3	8.8	46.4	63.0
proportion of phenylalanine in cipu (mg)	2.8	5.7	30.1	40.8
phenylalanine in cipu according [^{14}C]-incorporation (mg)	—	2.9	—	9.0

which compared well with the incorporation of label. Arginine, putrescine or ornithine was added to the phenylalanine supplemented media since it was noted that at higher concentrations of phenylalanine the uv-spectra of MCW-extracts were altered in the absence of the putrescine precursors. This indicated the formation of other unidentified cinnamic acid derivatives, which were normally not found in the cell line.

Only a certain amount of the added primary precursors was used for biosynthesis of cinnamoyl putrescines. The degree of incorporation seemed to be dependent upon the activities of enzymes involved in that pathway. Since the incorporation rate was not increasing at the same rate as the precursor levels it seems likely that the activities of the related enzymes were limiting the incorporation.

METABOLISM OF CINNAMIC ACID.—Trans-cinnamic acid proved to be a fairly toxic compound for TX1 and TX4 cells. Feeding of 20 mg per flask of 5-day-old cultures (fr. wt. 4.0 g) killed the cells within 3 hours. After feeding of 5 mg cinnamic acid/flask, most of the radioactivity taken up was first found to be ethyl acetate extractable, while after 48 h most of the radioactivity was water-soluble (fig. 2).

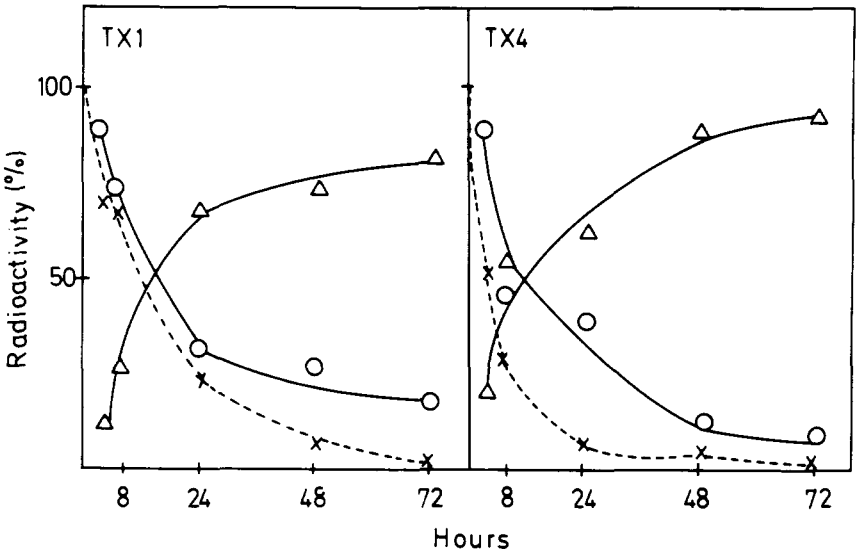

FIG 2. Distribution of radioactivity after feeding 5 mg cinnamic acid-[3-^{14}C] to TX1 and TX4 cells. (×—×) denotes radioactivity left in the medium. Ethyl acetate (○—○) and water (△—△) soluble radioactivity are expressed as percentages of radioactivity of MCW-extracts.

Five labeled compounds were identified from the ethyl acetate extracts by co-chromatography and by gc/ms. Three minor peaks which were only detectable after more than 24 h were not identified. During the first few hours, TX1 cells accumulated most of the radioactivity taken up as free cinnamic acid, while free *p*-coumaric acid was the main metabolite in TX4 cells (table 3). Later on cinnamoyl and *p*-coumaroyl glucose esters were the main components of the ethyl acetate extracts. Gc-analysis showed that the ratio of these esters was roughly 1:1 in both cell lines. Coumaroyl aspartate was always found in higher concentrations in TX4 cells. We were not able to detect the free cinnamic acids, glucose esters or coumaroyl aspartate in TX1 or TX4 cells without feeding cinnamic acid. This means that these compounds were artificially formed due to the exogenous supply of cinnamate. This is in agreement with the fact that they were not detected after feeding phenylalanine.

TABLE 3. Distribution of ethyl acetate extractable radioactivity after feeding 5 mg cinnamic acid-(3-^{14}C) to TX1 and TX4 cells for various time periods. Radioactivity is expressed as percentage of total added radioactivity. Recoveries of radioactivity (medium+MCW-extracts) were between 80–90%. The percentages of ethyl acetate extractable radioactivity of MCW-extracts are given in figure 2.

	Feeding time h	Percentage of total radioactivity in				
		cinnamic acid	*p*-coumaric acid	*p*-coumaroyl aspartate	cinnamoyl/ coumaroyl glucose esters	unknown peaks
TX1	3	12.2	4.9	—	4.2	—
	7	3.9	7.3	—	8.5	—
	24	0.4	1.1	0.3	16.7	1.1
	48	0.2	0.2	1.2	13.1	3.3
	72	0.2	0.2	1.4	6.5	9.9
TX4	3	1.3	17.2	0.3	9.1	—
	7	0.8	4.4	0.7	14.8	—
	24	0.6	1.2	5.2	17.1	0.8
	48	0.4	0.8	1.5	5.0	1.6
	72	0.2	0.3	1.2	2.9	1.8

The course of the distribution of radioactivity (fig. 2) shows that the ethyl acetate soluble compounds were metabolized to water-soluble derivatives. We did not identify all the labeled water soluble metabolites but looked mainly for incorporation into cinnamoyl putrescines. Between at least 15% (TX4) and 25% (TX1) of water-soluble radioactivity was not associated with cinnamoyl putrescines. After hydrolysis with 2N NaOH at room temperature and acidic extraction with ethyl acetate, these percentages were extracted into the organic phase and identified as *p*-coumaric acid. Roughly 70% of the water soluble radioactivity was found in the cinnamoyl putrescines. If the incorporation of cinnamic acid had occurred on the natural biosynthetic pathway, one would have expected to find a ratio of incorporation corresponding to the levels of caffeoyl-, feruloyl-, or coumaroyl putrescine (8:1:1) in the cells. During the first 24 h, however, *p*-coumaroyl putrescine was labeled up to 4 times higher than caffeoyl putrescine. From then on, the label in caffeoyl putrescine increased steadily and was slightly higher after 48 h. This, again, was different from feeding experiments with phenylalanine.

Several reports have demonstrated that exogenously added aromatic acids may undergo conjugation reaction with various moieties (for ref. 17). Formation of glucose esters has been discussed as a detoxification reaction (18,19), but they were also regarded as essential intermediates in hydroxycinnamic acid metabolism (20). Schlepphorst and Barz suggested, from a biochemical point of view, that

formation of esters or amides from energy-rich acyl glucosides appears feasible (17). The amidation reaction in the formation of cinnamoyl putrescines is still unknown. Phosphate esters and CoA-activated acids have been discussed as energy-rich intermediates (21). So far we have failed in our attempts (Berlin, unpublished) to find a hydroxycinnamoyl CoA:putrescine hydroxycinnamoyl transferase activity corresponding to the enzyme involved in the biosynthesis of chlorogenic acid (22). Despite the conversion of cinnamoyl glucose esters into cinnamoyl putrescines, we hesitate to propose glucose esters as natural intermediates in cinnamoyl putrescine biosynthesis. Comparing the differently labeled metabolites after feeding phenylalanine and cinnamic acid, respectively, it appears more likely that the glucose esters are only formed from exogenously added cinnamic acid. These esters may undergo further transacylation reactions as has been shown in the formation of sinapoyl malate (23), benzoyl malate or *N*-phenylacetyl aspartate (17). From this point of view cinnamic acid was only incorporated into cinnamoyl putrescines because the secondary metabolites we were working on were esters, which can also be formed by commonly observed transacylation reactions occurring with exogenously added acids. The formation of cinnamoyl putrescines by the transacylation reaction was quantitatively quite similar in both cell lines, which again did not fit with the different enzyme activities and the different incorporation of phenylalanine by TX1 and TX4 cells (6,9,10).

CONCLUSIONS

Feeding of precursors for increasing yields of natural products will give optimal results only if certain requirements are realized. The precursor should not be toxic to the cells. As shown for L-amino acid resistant TX4 cells (8) this may be overcome by selection for resistance. The cells should be capable of synthesis due to high activities of related enzymes. The precursors should not be substrates for so called "unspecific detoxification reactions" which prevent channeling precursors into the desired biosynthetic pathway.

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