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METABOLISM OF PHENYLALANINE AND TYROSINE IN TOBACCO CELL LINES RESISTANT AND SENSITIVE TO *p*-FLUOROPHENYLALANINE

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Key Word Index—*Nicotiana tabacum*; Solanaceae; suspension cultures; amino acid analogs; mutants; aromatic amino acids; polyphenols.

Abstract—Chromatography of soluble polyphenols of *p*-fluorophenylalanine-sensitive and -resistant tobacco cells revealed that the 10-fold increased level found in the resistant line was largely due to the accumulation of two unidentified polyphenols. The uptake of Phe-[U-^{14}C] and Tyr-[U-^{14}C] by the resistant line was *ca* 10% that by the sensitive line. About 90% of the phenylalanine-[^{14}C] which was taken up by both cell lines could be accounted for as free phenylalanine in protein, soluble polyphenols or CO_2. The fate of Tyr-[^{14}C] was similar to that of phenylalanine except that the incorporation was into insoluble polymeric forms of polyphenols rather than into soluble polyphenols. The resistant line incorporated 9-times more phenylalanine-[^{14}C] into soluble polyphenols than did the sensitive line. The different ^{14}C-aromatic amino acid accumulation and incorporation patterns noted with the two cell lines indicates that there are different active pools. Differential uptake rates by the two cell lines might affect the distribution of the absorbed amino acid among the different pools.

INTRODUCTION

A *p*-fluorophenylalanine (PFP)-resistant tobacco cell line was found to accumulate at least 6-times more polyphenols than the PFP-sensitive wild type [1]. The chorismate mutase of the resistant line showed a lessened feedback control by phenylalanine (Phe) and tyrosine (Tyr), which should increase the synthesis rates of both amino acids, but only the free Tyr pool was increased [1]. A 10–20 times higher phenylalanine ammonia lyase (PAL) activity was subsequently detected in the resistant line, and this increased activity evidently diverted the over-synthesized Phe into polyphenols [2]. In the present paper we compare the fate of exogenously supplied Phe and Tyr in the PFP-resistant and -sensitive tobacco cell lines.

RESULTS

Chromatographic analysis of the polyphenols extracted from both the PFP-resistant and -sensitive tobacco cell lines showed that the spectrum of polyphenols was similar, but the quantities were much higher in the resistant line. Apparently two compounds make up the bulk of the polyphenols present in the resistant line. In system 1 (Fig. 1, zone 5) both compounds had a very similar R_f value while in system 2 the two were well separated (R_f 0.5 and 0.65). Chromatography in several other systems did not reveal more than two major bands. The ratio of the two compounds in chlorogenic acid equivalents was 5:3 in favor of the compound with the lower R_f value in system 2. Both compounds fluoresced blue under UV and had maximal *A* at 315 nm (lower R_f in system 2) and 305 nm, respectively. Co-chromatography with authentic chorogenic acid showed that neither of the accumulated compounds was chlorogenic acid. Further studies are needed to identify these compounds.

Incubation with phenylalanine-[U-^{14}C]

The metabolism of Phe-[^{14}C] was studied to determine if the observed increase in polyphenols [1] in the resistant cell line was due to a higher conversion of Phe into polyphenols by the higher activity of PAL [2]. After incubating for 1 hr with Phe-[^{14}C] the sensitive line (Fig. 2) had absorbed 60% of the total radioactivity from the medium, while twice as many resistant cells had taken up only 8%. Thus the Phe uptake rate was at least 10-times higher in the sensitive line. The lower uptake rate by the resistant line, as measured by growth inhibition, was also found for other amino acids (threonine), their analogs (5-methyltryptophan), cinnamic acid and 5-methylindole (unpublished results). Nitrate uptake, however, was similar in both cell lines [2].

Due to the widely different uptake rates, the data given in Table 1 are listed as the percentage of the absorbed radioactivity that was found in the CO_2 released, the methanol–chloroform–water (MCW) extract, and in protein. The amount of $^{14}CO_2$ released was similar for both cell lines (6 to 10%). When the protein fraction was hydrolyzed *ca* 95% of the solubilized radioactivity was found in Phe and only 0.1 to 0.2% remained in the residue. The percent incorporation of Phe into protein was slightly lower in the resistant line as noted before [1]. However, the amount of extractable protein in both cell lines was always similar during the growth cycle and leucine incorporation into protein was comparable when both lines had the same free leucine pool size [2]. The greatest differences between cell lines can be seen in the MCW-extracts (Table 1 and Fig. 1). Phe was taken up

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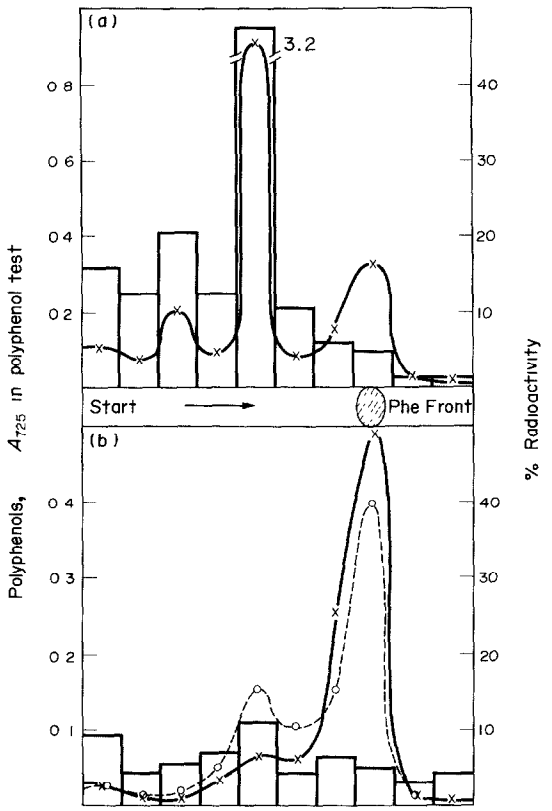

Fig. 1. Chromatography in system I of MCW extracts of PFP-resistant (a) and PFP-sensitive tobacco cells after 3 hr feeding of 1 μCi Phe-[U-^{14}C]. The bars represent zones scraped from the chromatograms with zone 1 on the left and denote the polyphenol content while the solid lines show the percent distribution of the radioactivity of the MCW extract The dashed line in (b) shows the percent distribution of radioactivity after a 1.5 hr turnover period (see Experimental). At least 6000 dpm (with a recovery of more than 80 %) were chromatographed.

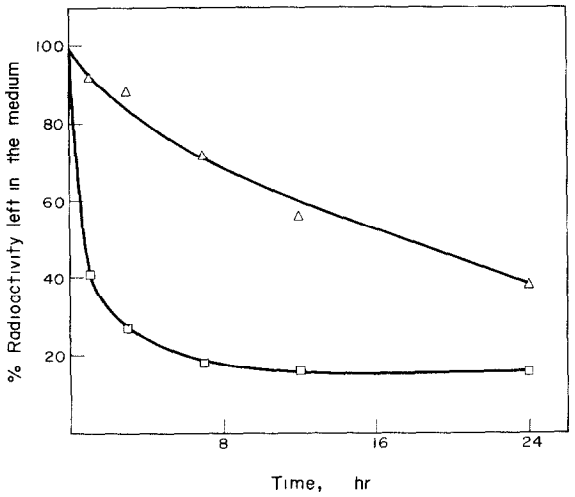

Fig. 2. Uptake of 1 μCi Phe-[U-^{14}C] monitored as radioactivity left in the medium by 1.3 g PFP-sensitive (□—□) and 2.5 g PFP-resistant tobacco cells (△—△) for 24 hr. Weights were determined at the time of harvest

rapidly by the sensitive line and accumulated. The amount taken up should not, however, cause a significant change in the free Phe pool since the total amount of Phe-[^{14}C] added represented *ca* 1 % of the measured free Phe of the cells. If after the 3 hr uptake period, the sensitive cells were rinsed and incubated for a further 1.5 hr without labeled Phe, the total MCW-extract and free Phe radioactivity decreased by 18.7 and 31.9 % respectively (Fig. 1). The radioactivity in the polyphenols (Zone 5), however, increased by 40.6 %. This turnover experiment was not conducted with the resistant line since very little radioactivity was found in free phenylalanine after a 3 hr incubation period (Fig. 1). This was apparently due to the reduced absorption and the more rapid conversion of the Phe-[^{14}C] into the accumulated MCW soluble phenolic compounds (Fig. 1).

Table 1. Metabolism of 1 μCi Phe-[U-^{14}C] added to PFP-sensitive and resistant tobacco cells for various times. Fresh weights of cells per flask were 1.3 and 2.5 g at time of harvest for sensitive and resistant cells, respectively. The experiments were performed on the 4th day following inoculation

	% Radioactivity absorbed			% of MCW extract-radioactivity	
Time (hr)	CO_2	MCW* extract	protein hydrolysate	phenylalanine	zone 5 (Fig 1)
Sensitive					
1	6.9	44.4	48.7	70.6 [31.3]†	4.6 [2.0]†
3	7.0	37.8	55.2	65 5 [24 7]†	6 2 [2 3]†
7	9.0	37.9	53.1	55.8	9 7
12	9 1	37.6	53.3	73 3	9 1
24	10.1	20 1	69 8	21.6	39.4
Resistant					
1	7.3	53.6	39.1	25.3 [13.5]†	32.4 [17.4]†
3	8.8	48 3	42.9	20.0 [9.7]†	45.2 [21 8]†
7	9.0	54.0	37.0	10.8	51 7
12	5.8	48 3	45.0	17.3	54 6
24	6 8	44 8	48.4	12 3	68 9

† The numbers in brackets denote percent of radioactivity absorbed * MCW, methanol chloroform–water.

Table 2. Percent distribution of radioactivity after 24 hr feeding of 1 μCi Tyr-[U-^{14}C] to 2 g sensitive and 3.2 g resistant cells, respectively. The cell fresh weights were determined after the incubation

	Sensitive	Resistant
Medium	13.8	36.7
CO_2	2.5 [3.2]*	2.8 [4.6]
MCW	16.6 [21.4]	11.1 [18.3]
Protein	51.0 [66.2]	42.4 [69.8]
Residue	7.0 [9.2]	4.4 [7.3]
Recovery	90.9	97.4

* Numbers in brackets denote present distribution of the radioactivity taken up.

Incubation with tyrosine-[U-^{14}C]

The lessened feedback control of the chorismate mutase of the resistant line apparently resulted in the 3-fold increase of free Tyr in this line [1]. In order to detect any differences in Tyr metabolism, Tyr-[^{14}C] was fed as described for Phe-[^{14}C]. The reduced absorption by the resistant line was similar to that found with Phe (Fig. 2). The $^{14}CO_2$-production found in a 24 hr feeding study (Table 2) was lower than that found with Phe but was similar for both cell lines (3.2 and 4.6% of that taken up). Dougall and Shimbayashi [3] found that 3.6% of added Tyr was converted to CO_2 by tobacco callus in 24 hr. The radioactivity of the MCW insoluble fraction (protein hydrolysate plus residue) of the sensitive line contained 76.6% as Tyr (resistant line 80.5%). Chromatography of the protein hydrolysate showed smearing of the radioactivity except for the tyrosine peak.

After 24 hr the MCW-extract of the sensitive line contained 8.8% as Tyr while the resistant line contained 21.3% indicating that Tyr was still being taken up by this line. The sensitive, but not the resistant line, had depleted the Tyr from the medium within this period. In a 3 hr feeding experiment with the sensitive line, where Tyr was not depleted from the medium, 85.4% of the MCW radioactivity was in Tyr. Thus the sensitive line can accumulate both Tyr-[^{14}C] and Phe-[^{14}C]. Upon incubation for 1.5 hr in medium without Tyr-[^{14}C], 39.9% of the total radioactivity and 42.1% of total Tyr-[^{14}C] was lost from the MCW fraction. Thus most metabolites of Tyr are alcohol insoluble, while Phe was incorporated into soluble polyphenols which remained in the MCW extract. The radioactivity other than Tyr in the MCW extract from both cell lines, was distributed uniformly across the chromatogram (TLC and ion exchange columns of the amino acid analyzer).

DISCUSSION

The Phe-[^{14}C] and Tyr-[^{14}C] which is taken up by both cell lines can be incorporated into protein, converted to CO_2 or remain as free amino acid. The Phe-[^{14}C], however, can also be converted to soluble polyphenols, but not insoluble polymetric forms, while Tyr-[^{14}C] can be converted to insoluble or soluble polymeric polyphenols (polyphenol oxidase products). There was no indication of Tyr incorporation into any homogeneous MCW soluble polyphenolic constituent in agreement with work reported earlier by Ibrahim *et al.* [4] with *Pyrus communis* leaf discs. The *Pyrus* system did, however, incorporate Phe into phenolic compounds [5].

From 9 to 12% of the Tyr-[^{14}C], which was incorporated into the MCW insoluble fraction in 24 hr by both cell lines, remained in the residue after hydrolysis (Table 2). Chromatography of the hydrolysate showed smearing of radioactivity except for the Tyr peak. A similar smearing was seen with the MCW soluble fraction. These results indicate that Tyr was metabolized by tyrosinase (polyphenol oxidase) [6]. Phe-[^{14}C] was not metabolized in this way since only 0.1 to 0.2% was found in the residue. In experiments where added shikimate-[^{14}C] was converted largely to Phe and Tyr, 7 to 8% of the radioactivity in the MCW insoluble fraction remained in the residue (unpublished). Thus it appears that both endogenously synthesized and exogenously supplied Tyr, but not Phe, was polymerized by polyphenol oxidase. Dougall and Shimbayashi [3] found that Tyr-[^{14}C] was incorporated into a similar acid resistant material by tobacco callus which was later shown not to be lignin [7].

The resistant line incorporated 9-times more of the absorbed Phe into soluble polyphenolics when a continuous supply of Phe is provided (Table 1, Fig. 2). This compares well with the 6 to 10-times higher levels of polyphenols and the 10-times higher PAL activity found in this line [2]. The exact turnover rate of Phe cannot be determined by feeding labeled Phe, since the localization of the Phe is unknown, but comparisons between the two lines can be made. In the resistant line after 1 and 3 hr, respectively (Table 1), 86.5% and 90.3% of the absorbed Phe-[^{14}C] was converted or incorporated into other compounds, compared to 68.7% and 74.3% in the sensitive line as determined from the amount of free Phe-[^{14}C] remaining. This indicates that the resistant line has a higher turnover rate, which would be in accordance with the lessened feedback control of the chorismate mutase by Phe [1] and the increased incorporation into soluble polyphenols (Table 1, Fig. 1). However, when one considers the sp. act. of the Phe pools in both lines, measured as radioactivity taken up and left as free Phe, one would assume a 2.5 times higher turnover rate for the resistant line after 1 and 3 hr (Table 1). Nine times more Phe-[^{14}C] is incorporated into polyphenols by the resistant line, but that is only 20% of the absorbed Phe which is metabolized. This means a 20 to 25% higher turnover rate in the resistant line would be sufficient to synthesize the increased polyphenols in that line.

A question can also be raised concerning why only the sensitive line accumulates absorbed radioactivity as the free amino acid. Similar results were noted when the precursor shikimic acid-[^{14}C] was fed and only the sensitive line accumulated high amounts of ^{14}C in free Phe and Tyr (unpublished). We could not detect a difference in Tyr metabolism between the cell lines, but Tyr-[^{14}C] likewise accumulated to higher levels in the sensitive line. The percent incorporation of Phe into protein is always lower in resistant line than in the sensitive line [1] although it is known that extractable protein [2] and Phe levels in protein are comparable in both lines. At least two possibilities might explain these results. The different rate of uptake of compounds by both lines might alter the flow of the absorbed compound into the different cellular pools or the differing rates of activity of the pools might also alter this flow. The resistant line should have a very active polyphenol synthesis Phe pool, while this pool should be less active in the sensitive line. At least 20 to 30% of the radioactivity taken up will enter the polyphenol synthesis pool while the other Phe-[^{14}C]

taken up might go to pools for protein synthesis or storage. In the sensitive line most of the radioactivity will be transported to the protein synthesizing pool. This would explain the reduced per cent incorporation noted in the resistant line, because in this line the sp. act. of Phe in this pool would be lower. Both the involvement of the uptake process in the fate of the aromatic amino acid and the presence of different active pools might explain our results.

Rapidly growing resistant cells contain about 8 to to 10 μmol per g fr. wt of Phe equivalents in polyphenols [1] and about 5 to 7 μmol Phe per g fr. wt in protein. Free Phe is only *ca* 0.06 μmol per g fr. wt [1] which is negligible compared with the amounts of Phe in polyphenols and protein. Thus there should be more Phe-[^{14}C] incorporated into the polyphenols than into protein, but measurements show that incorporation into polyphenols was about one-half of that into protein when either exogenous Phe-[^{14}C] or shikimate-[^{14}C] were used. There are several possible explanations of this anomaly. (1) The phenolic test may not give a true equivalence to Phe content since the phenolics being measured have not been identified; (2) the polyphenols might be more stable than protein in these cells; and (3) the cell population is not homogeneous so that some might be synthesizing phenolics while others relatively more protein.

The greatly reduced uptake found for most compounds by the PFP-resistant lines could make this line a valuable experimental system for studying uptake phenomena.

EXPERIMENTAL

Suspension cultures of tobacco pith (*Nicotiana tabacum* L. cv Xanthi) were grown as described [8] in 50 ml liquid medium and were the cell lines described previously [1, 2].

Feeding experiments. Cells in log phase (4–6 days old) were incubated with L-phenylalanine-[U-^{14}C] (414 mCi/mmol, NEN) and L-tyrosine-[U-^{14}C] (360 mCi/mmol, NEN) for specified periods. $^{14}CO_2$ release was measured as described [9] and uptake was monitored by counting an aliquot of filtered medium. For 'turnover studies' cells were collected after the uptake period on miracloth, rinsed with H_2O and divided into equal parts. One part was extracted immediately, while the other was transferred to unlabeled conditioned medium obtained from cells of the same age. After incubation these cells were harvested and extracted with MCW MeOH–$CHCl_3$–H_2O, 12·5:3, and divided into a $CHCl_3$ layer and a H_2O–MeOH layer [10]. The $CHCl_3$ contained only 5–7% of the MCW radioactivity. Chromatography of the H_2O–MeOH layer was performed on Si gel plates in system 1 (Methylisobutylketone–HOAc–H_2O, 14·3·2) and cellulose layers in system 2 (*n*-BuOH–HOAc–H_2O, 4:1·1). Zones (2 cm) were scraped off and counted directly or were eluted with 50% MeOH. MeOH extracted polyphenols were determined with the Folin reagent [11] and aliquots were counted in a Packard scintillation spectrometer. Tyr-[^{14}C] in the MCW-extract was measured after separation by amino acid analyzer. The residue of the MCW extraction was hydrolyzed for 24 hr in 6 M HCl in an autoclave at 120° in sealed glass containers in a N_2 atmosphere. The extract was filtered (protein hydrolysate) and the ^{14}C in Phe or Tyr was measured by counting chromatograms developed in system 1. The washed hydrolysate residue collected on the filter paper was burned in a sample oxidizer and counted.

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