Changes in Amines and Biosynthetic Enzyme Activities in p-Fluorophenylalanine Resistant and Wild Type Tobacco Cell Cultures

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ABSTRACT

The levels of free amines and the activities of their biosynthetic enzymes were measured in a p-fluorophenylalanine resistant Nicotiana tabacum L. cv Xanthi cell line (TX4) which accumulates high levels of cinnamoylamides, and a wild type cell line (TX1). Putrescine in TX1 and spermidine in TX1 and TX4 increased 4-fold by day 4 but declined by day 8 of the culture period. Spermine levels were consistently low, while tyramine was not found in TX1 until day 9 when a gradual rise was noted. Ornithine decarboxylase activity in TX1 and TX4 increased slightly through day 2 but declined gradually thereafter. S-Adenosylmethionine decarboxylase activity remained low throughout the culture period, and tyrosine and arginine decarboxylases in TX1 were very low in activity. In contrast, the activities of tyrosine and arginine decarboxylases were elevated in TX4, but a 3-fold increase in tyramine after a subculture was not accompanied by a rise in tyrosine decarboxylase. However, tyrosine decarboxylase activity did increase during a second rise in tyramine levels in aging cells, late in the culture period. Although significant differences exist in amine levels, between TX4 and TX1, it is unclear how altered amine metabolism relates to p-fluorophenylalanine resistance.

The selection of plant cell cultures resistant to antimetabolites has become a standard procedure for the isolation of biochemically variant cell lines (28). The amino acid analog p-fluorophenylalanine has been used to select a resistant tobacco cell line (TX4) which exhibits enhanced phenylalanine and tyrosine synthesis (21) and accumulates 6 to 10 times more hydroxycinnamoylamides (largely caffeoylputrescine) than wild type cells (TX1) (3–5).

Aliphatic amines and tyramine, together with their hydroxycinnamic amides, have also been found to accumulate in the reproductive organs of numerous plant species, including tobacco (10, 22, 27), and increased levels are associated with a wide range of physiological and developmental responses (15, 25). Cultured cell systems such as the PFP-resistant and sensitive tobacco cell lines allow investigation of biochemical patterns in a controlled environment without developmental influences. While the metabolism of phenylalanine in the TX4 line has been examined (6, 7), and the production of the diamine, putrescine, has been studied (1), clarification of subsequent polyamine biosynthesis and hydroxycinnamoylamide accumulation requires more thorough study of amine metabolism in high and low accumulating tissues. This paper reports on the relationship of the levels of putrescine, spermidine, spermine, and tyramine, and of the activities of their biosynthetic enzymes, to the culture growth cycle of PFP-resistant and wild type Nicotiana tabacum cell suspensions.

MATERIALS AND METHODS

Plant Material. The TX4 culture used in these studies continued to display the traits of PFP-resistance and hydroxycinnamoylamide accumulation (23) initially described by Palmer and Widholm (21). Cell suspensions of TX1 and TX4 (Nicotiana tabacum L. cv Xanthi) were generously provided by Dr. J. Berlin, Braunschweig, Federal Republic of Germany. They were maintained at 24°C in an MX media supplemented with 2 μM 2,4-d and continuous illumination. TX1 and TX4 were subcultured every 7 and 10 d by transferring 1.5 and 2.5 g fresh weight, respectively, to 50 ml of fresh media. Data points represent the means of at least three replicate determinations.

Enzyme Preparation. Cells were collected by vacuum filtration, frozen with liquid N2 in a mortar, and ground to a fine powder. For TDC preparation, 5.0 g of ground cells were extracted in 15 ml of buffer I (0.1 mM sodium phosphate buffer [pH 7.5]) with 5.0 g of Polyclor AT saturated with buffer I. For ADC, ODC, and SDC, 5.0 g of ground cells were extracted in 5 ml of buffer II (0.1 M Tris-HCl [pH 7.5], 10% glycerol [v/v], 15 mM 2-mercaptoethanol, 5 mM EDTA, 1 mM pyridoxal phosphate) with 5.0 g of Polyclor AT saturated with buffer II. The slurries were stirred on ice for 10 min, then squeezed through two layers of Miracloth, and the filtrate was spun for 20 min at 10,000g. The supernatants were brought to 70% saturation with a saturated aqueous solution of (NH4)2SO4 (pH 7.5) and, after stirring for 20 min at 4°C, the solutions were spun for 15 min at 15,000g. For subsequent enzyme assays, the resultant pellets were dissolved in minimal amounts of buffer I for TDC and buffer III (0.1 M Tris-HCl [pH 7.5], 10% glycerol [v/v], 7.5 mM 2-mercaptoethanol, 0.2 mM pyridoxal phosphate) for ADC, ODC, and SDC.

Enzyme Assays. TDC assays were performed in 10-ml centerwell flasks containing 400 μl of 0.4 mM l-[1-14C]tyrosine (5.2 × 104 dpm/assay), 50 μl 1.5 mM pyridoxal phosphate, 50 μl buffer

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2 Abbreviations: PFP; p-fluorophenylalanine; ADC, arginine decarboxylase; ODC, ornithine decarboxylase; SDC, S-Adenosylmethionine decarboxylase; TDC, tyrosine decarboxylase.
I. and 500 \mu l crude enzyme. The standard assay mixture for ADC, ODC, and SDC contained 400 \mu l of either 0.5 mm \text{L-14Carginine} (1.4 \times 10^4 \text{dpm/assay}), \text{L-14Cornithine} (7.7 \times 10^4 \text{dpm/assay}), or \text{L-14Cadenosylmethionine} (7.7 \times 10^4 \text{dpm/assay}), 50 \mu l 1.5 mM pyridoxal phosphate, 50 \mu l buffer III, and 500 \mu l crude enzyme. Assay mixtures were incubated at 30°C for 30 min, then 200 \mu l of phenethyamine:methanol (1:1, v/v) were added to the center well and the reaction stopped by adding 200 \mu l of 5.0 M HCl to the assay mixture. The assay flasks were gently rotated for 60 min and the radioactive contents of the center well were counted in 5.0 ml of toluene-base scintillation fluid.

**Amine Analysis.** Tyramine was extracted from 100 mg fresh weight of cells using 0.2 M HClO4 and, after appropriate dilution with water, measured by reverse-phase HPLC and electrochemical detection (19). Putrescine and polyamines were extracted from 250 mg fresh weight of cells using 3 ml of 5% (w/v) TCA and then benzoylated (24), prior to analysis by reverse-phase HPLC.

**Nucleic Acid and Protein Determinations.** Total nucleic acids were extracted and measured according to the procedures described by Detchon and Possingham (14). DNA was determined by the diphenylamine reaction as described by Burton (9). RNA was calculated as the difference between total nucleic acid and DNA content. Protein levels were measured by the Bradford procedure (8).

### RESULTS AND DISCUSSION

The initial growth rate of PFP-resistant cells was equivalent to that of wild type cells but beyond d 6, during the linear phase of the culture growth cycle, the fresh weight gain in TX4 was slower. As a result, the final tissue accumulation in TX4 cultures was reduced 34% compared to TX1 (Fig. 1).

The free amines identified in both TX1 and TX4 were putrescine, spermidine, spermine, and tyramine. No attempt was made to determine the levels of phenethyamine: in these axochlorophyllous cultures, although its presence has been confirmed in green vegetative tissues of tobacco (22). The tyramine metabolites octopamine and dopamine were not present in the extracts (detection limit: 50 pmol/g fresh weight). Putrescine was the predominant amine in both cultures and showed striking concentration changes during the culture cycle. An increase of 380% in line TX1 and 160% in line TX4 was observed by d 4, followed by equivalent declines over the next 2 to 4 d (Fig. 1). The pattern of change for spermidine was nearly identical to that of putrescine with increases of 460% in TX1 and 260% in TX4 (Fig. 1). Spermine levels, however, remained consistently low throughout the growth cycles of both cell cultures, a feature noted previously for tobacco (22, 25). Free tyramine was not detected in extracts of line TX1 until the cells had reached a stationary phase of growth at d 9. At this same point in the culture cycle, detectable levels of TDC activity in the cell extracts were observed for the first time (Figs. 1, 2). However, Berlin and Widholm (6) had earlier found that 3% of the label from \text{L-14C]tyrosine} fed to \textit{N. tabacum} TX1 cells in log phase was recovered as \text{14CO}_2. We have confirmed their results in 7-d old TX1 cultures, where 1.4% of the label from \text{L-14C]tyrosine} fed to the cultures was recovered as \text{14CO}_2. Since no detectable TDC activity can be extracted from the TX1 culture at 7 d, the observed loss of carbon from the tyrosine skeleton may have occurred through transamination and homogentisate formation.

In contrast to the wild-type culture, TX4 cells contained substantial amounts of tyramine and TDC activity throughout the culture cycle. There was a 280% increase in tyramine content by d 2, followed by a slow decline in levels over the next 6 d (Fig. 1). A second rise in tyramine content, beginning on d 9, corresponded to an increase in TDC activity and the entry of the culture into a stationary phase of growth (Figs. 1, 2). TX4 was noted for having enhanced phenylalanine and tyrosine production resulting from elevated chorismate mutase activity and reduced feedback inhibition by phenylalanine or tyrosine (3). There was no net accumulation of phenylalanine in the culture, however, due to its rapid incorporation into phenylpropanoid (hydroxycinnamoylputrescine) biosynthesis through elevated phenylalanine ammonia-lyase activity (3, 5). The consequences of enhanced tyrosine levels have not been as clearly defined. Berlin and Widholm (6) demonstrated that \text{14C]tyrosine administered to line TX4 was metabolized into CO}_2, proteins, and other insoluble polymeric forms of polyphenols but our results suggest that a substantial portion of the tyrosine pool is decarboxylated directly, resulting in enhanced tyramine levels. Elevated TDC activity and the resulting tyramine production in line TX4 is, however, apparently not an essential element of the PFP-resistance phenotype, since other PFP-resistant tobacco cell lines have been examined which do not possess an elevated TDC activity (MA Walker, BE Ellis, unpublished results). In vitro TDC activity from the TX1 and TX4 cultures was also extremely sensitive to the addition of thiol reagents, with nearly total inhibition observed at 1 mM 2-mercaptopethanol. Since the enzyme extracted from TMV-infected leaves of tobacco is reported to be uninhibited by 2-mercaptopethanol (20), these results suggest that different forms of TDC may exist within \textit{N. tabacum}.

The total putrescine pool (free, as measured here, plus bound as hydroxycinnamoylamine conjugates) is approximately the same size in both TX1 and TX4, but in TX1 putrescine is found largely as a free base while in TX4 it is mainly in the form of conjugates. The supply of aminopropyl moieties for the biosynthesis of larger polyamines appears to be satisfied by the low and

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**FIG. 1.** Changes in free amine content and cell growth in TX1 and TX4 cell cultures. (●) Putrescine; (▲) spermidine; (▲) spermine; (■) tyramine.
and hence in the more rapid metabolic removal of the inhibitor (3). However, we have found that the elevated phenylalanine ammonia-lyase characteristic is accompanied by both enhanced ADC and TDC activity in TX4. This pattern could represent pleiotropic effects of some more distant genetic event involving common regulatory elements. Alternatively, it might represent a more indirect metabolic response to the increased accumulation of phenylalanine metabolites and tyrosine within the PFP-resistant cell. Resolution of these possibilities will probably require a more detailed knowledge of the molecular biology of phenylpropanoid metabolism.

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