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Hydroxycinnamoyl-CoA:Putrescine Hydroxycinnamoyltransferase in Tobacco Cell Cultures with High and Low Levels of Caffeoylputrescine[1]

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ABSTRACT

A new hydroxycinnamoyl-CoA:putrescine hydroxycinnamoyltransferase (PHT) was detected in two variant lines of *Nicotiana tabacum* L. (TX1, TX4) accumulating markedly different levels of caffeoylputrescine. The enzyme accepted only the aliphatic diamines putrescine, cadaverine and 1,3-diaminopropane at a ratio of 100:33:8. Caffeoyl- and feruloyl-CoAs were the best acyl donors. The apparent K_m-values for caffeoyl-CoA and putrescine were near 3 and 10 micromolar, respectively, at the pH-optimum of 10.0. PHT activity was quite similar in low producing TX1 and high producing TX4 cells, while some other biosynthetic enzymes (phenylalanine ammonia-lyase, ornithine decarboxylase) were greatly enhanced in TX4 cells, suggesting that PHT does not catalyze the rate-limiting step in hydroxycinnamoylputrescine formation.

Variant lines of plant cell cultures may accumulate markedly different levels of secondary metabolites under otherwise identical culture conditions. As part of the effort to determine the nature of the biochemical differences between low and highly productive cell lines, enzyme activities involved in the corresponding biosynthetic pathway have been evaluated. The best characterized system in this context seems to be the biosynthesis of hydroxycinnamoylputrescines (HCA-Put[4]) in tobacco cell cultures. Palmer and Widholm (14) described a *p*-fluorophenylalanine-tolerant cell line (TX4) which accumulated 6 to 10 times more phenolics than did the wild-type cells (TX1). The main phenolic was identified as caffeoylputrescine, with smaller amounts of feruloyl- and p-coumaroylputrescine (3). The activities of the enzymes of the phenylpropanoid pathway, and of ornithine and arginine decarboxylases, were distinctly enhanced in high producing TX4 cells compared to TX1 cells (3). A key step missing from the study of this biosynthesis was the enzyme conjugating putrescine to the various HCAs. Enzymic amide formation with HCAs had previously been shown in the cases of conjugation of agmatine (7) and tyramine (12) to be dependent on HCA-CoA thioesters as acyl donors. From these results, we assumed that HCA-Puts might also be formed via the CoA thioesters. Feeding experiments with phenylalanine and putrescine indicated that the conjugating enzyme activity might be present, as with the other biosynthetic enzymes, in higher activity in TX4 cells (2, 4). Thus, TX4 cells seemed to be the best source to use for searching for the enzyme involved in HCA-amide formation. Here we report the detection and some characteristics of a tobacco HCA-CoA:putrescine HCA transferase and compare its activity pattern with those of other enzymes involved in the HCA-Put biosynthesis.

MATERIAL AND METHODS

Plant Material

Origin, maintenance, and some characteristics of tobacco (*Nicotiana tabacum* L.) cell lines TX1 and TX4 have been described (2–5, 14). The initial inoculum (*ca.* 2.5 g/70 mL MX-medium) for the growth, enzyme, and product kinetics was 1:10 dilutions of 10-d-old TX1 and TX4 suspensions.

Chemicals and Substrates

Chemically synthesized caffeoyl-Put was a gift of Dr. Höfle (GBF, Braunschweig). The HCA-CoAs were synthesized by the ester-exchange reaction via *N*-hydroxysuccimide esters (18) as described elsewhere (20). 1-O-*p*-Coumaroyl and 1-*O*-feruloylglucose were isolated from petals of *Antirrhinum majus* according to a method described earlier (19). All other chemicals were of commercial origin.

Preparative Isolation of HCA-CoA:Putrescine HCA-Transferase (PHT)

Eighty g of freshly harvested, linear phase TX4 cells were ground (1–2 min) in a precooled mortar in the presence of liquid nitrogen. After the addition of 20 g Polyclar AT and 500 mL 100 mM Tris-HCl (pH 8.5) containing 1 mM EDTA and 10 mM 2-mercaptoethanol, the homogenate was allowed to stand for 30 min with continuous stirring and then centrifuged for 30 min at 11,000*g*. The enzyme activity was obtained from the precipitate formed between 30 to 80%

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[4] Abbreviations: HCA, hydroxycinnamic acid; Put, putrescine; ODC, ornithine decarboxylase; PAL, phenylalanine ammonia-lyase; PHT, hydroxycinnamoyl-CoA:putrescine hydroxycinnamoyl-transferase; Spd, spermidine; Spm, spermine; Cad, cadaverine.

$(NH_4)_2SO_4$ saturation. The precipitate was redissolved in a minimum volume of K-phosphate buffer (20 mM, pH 7.0) containing 1 mM EDTA and 10 mM 2-mercaptoethanol and was filtered through Pharmacia PD-10 columns. The eluate was dialyzed, centrifuged (20 min at 48,000*g*) and subjected to chromatography on a 15 × 2 cm column of DEAE-Sephacel. The column was washed with 150 mL K-phosphate buffer (20 mM [pH 7.0], 1 mM EDTA, 10 mM 2-mercaptoethanol) before the following gradient was applied: 100 mL 0 to 200 mM KCl in the above K-phosphate buffer at a flow rate of 1 mL/min. Fractions (10 mL) were collected and assayed for PHT activity. Fractions containing high activity were pooled and stored at −20°C.

Enzyme Assay for PHT

The standard reaction mixture contained in a final volume of 50 μL 2 mM Put, 40 μM caffeoyl-CoA, 0.2 mM EDTA, 2 mM 2-mercaptoethanol, and 10 μL protein solution in 100 mM glycine-NaOH buffer (pH 10.0). The reaction was started by the addition of protein, incubated at 30°C for 10 min and stopped by the introduction of 5 μL 6 N HCl. Controls were performed with heat-denaturated protein or omission of protein or putrescine. The enzyme activity was determined by HPLC on a prepacked Nucleosil 120-5 C_{18}-column, 5 μ, 250 × 4 mm. Gradient: linear within 7 min from 20 to 60% B (1.5% H_3PO_4, 20% CH_3COOH, 25% CH_3CN) in solvent A (1.5% H_3PO_4 in H_2O); detection at 320 nm; retention times of caffeoyl-, *p*-coumaroyl-, feruloyl-, and sinapoyl-Put were 6.1, 7.4, 8.1, 8.6 min, respectively; caffeoyldiaminopropane and caffeoylcadaverine were eluted at 5.2 and 7.1 min.

Characterization of PHT Activity

The pH-optimum was determined with the following buffer systems: 100 mM K-phosphate (pH 6.0–7.0), 25–300 mM Tris-HCl (pH 7.0–8.8), 100 mM bicine (*N,N*-bis(2-hydroxyethyl)glycine; (pH 8.0–9.8), 100 mM glycine (pH 8.6–11.4). Apparent K_m and V_{max} (at fixed concentrations of the second substrate) in Tris-HCl (pH 7.8) were graphically determined according to Lineweaver and Burk (11). Product identification was performed by three methods: (a) cochromatography with authentic caffeoyl-Put in HPLC, (b) TLC on microcrystalline cellulose (Avicel) in isopropanol:EtOH:H_2O:CH_3COOH (6:7:6:1) and on silica gel in *n*-butanol:CH_3COOH:H_2O (4:1:1), detection on plates with UV-light and day light after spraying with ninhydrin, and (c) detection of the ^{14}C-labeled product after incubation with 1,4-^{14}C-Put.

Analytical Protein Preparations

For following the pattern of enzyme activities during a growth cycle, 5 g fresh mass of TX1 and TX4 cells was extracted for each enzyme. Preparation of crude protein extracts purified by $(NH_4)_2SO_4$-precipitation and PD-10 filtration for phenylalanine ammonia-lyase (PAL) and ornithine decarboxylase (ODC) has been described (3) and that for PHT was as described above. PHT-activity was measured by HPLC and PAL-activity was assayed photometrically as described (3). The assay conditions for measuring ODC-activity were altered insofar as the 1-^{14}C-ornithine used in the $^{14}CO_2$ absorption assay (3) was replaced by U-^{14}C-ortnithine for a more convenient new assay, originally described as a photometric assay in which Put was extracted into amylalcohol (13). Since the photometric assay gave unacceptably high backgrounds with the crude tobacco cell extracts, U-^{14}C-ornithine was added to the assay and aliquots of extracted labeled Put were directly measured in a scintillation counter.

Protein was measured according to Bradford (8) using bovine serum albumin as standard.

Determination of Metabolites

Extraction and benzoylation of polyamines from 20 mg freeze-dried cells were performed according to Flores and Galston (9). The benzoylated polyamines were quantitated by HPLC-analysis on a Nucleosil 120-5 C_{18}-column with isocratic elution (1.5% H_3PO_4, 45% CH_3CN in H_2O). Retention times for Put, Spd, and Spm were 5.3, 7.1, and 11.2 min, respectively. Caffeoyl-Put was determined as described (17) employing the isocratic HPLC-systems developed by Ponchet *et al.* (16).

RESULTS AND DISCUSSION

Properties of PHT-Activity

Protein preparations from tobacco cell suspension cultures (TX1 and TX4) catalyzed the formation of HCA-Put from HCA-CoA thioesters as acyl donors and free Put as acceptor molecule (Fig. 1). There was no reaction when 1-O-*p*-coumaroyl- and 1-O-feruloylglucoses were tested as possible acyl donors. These esters have been shown to serve as donors for the formation of some O-esters in other systems (1). It should, however, be mentioned that earlier feeding experiments with *p*-fluorophenylalanine and cinnamic acid have shown tran-

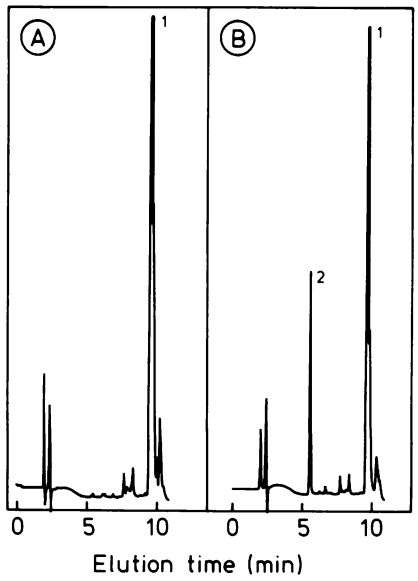

Figure 1. Diagrams of HPLC analyses (0.064 absorbance unit full scale at 320 nm) of PHT assays after 10 min reaction time. A, Omitting Put; B, including Put; 1, caffeoyl-CoA; 2, caffeoylputrescine. For details see "Materials and Methods".

sient occurrence of *p*-fluorocinnamoyl and *p*-coumaroylglucose esters which were then slowly transesterified by TX1 and TX4 cells to the corresponding putrescine derivatives (4, 5).

The enzymic formation of caffeoyl-Put was linear with protein concentration and with time up to 60 min (0.8 μg protein/assay). There was no reaction with heat-denaturated protein. The product caffeoyl-Put was identified by cochromatography (HPLC, TLC) with synthetic and cell culture reference material and by the incorporation of ^{14}C-Put. All the other products were tentatively identified by their chromatographic behavior (HPLC) as compared with the known pattern of HCA-Puts of TX4 cells, with authentic caffeoyl-Put and with the HCA-CoAs. This activity, to be classified as HCA-CoA:Put HCA-transferase (PHT), was partially purified (ninefold) by $(NH_4)_2SO_4$-precipitation and chromatography on DEAE-Sephacel. There was no loss of the initial enzyme activity over a period of 2 months when stored at −20°C. However, thawing and refreezing resulted in 20% loss of activity. The effect of pH on the catalytic properties were tested from pH 6.0 to 11.4. Highest activities with Put as acceptor were found at pH 10.0 with caffeoyl- and sinapoyl-CoAs, at pH 10.3 with feruloyl-CoA, and at pH 8.8 with *p*-coumaroyl-CoA. These rather alkaline pH optima correspond to those described previously for other N-acylating enzymes, the *p*-coumaroyl-CoA:agmatine *p*-coumaroyltransferase (ACT) from barley seedlings (7) and the feruloyl-CoA:tyramine feruloyltransferase (TFT) from tobacco leaves (12).

The substrate specificity of the PHT was examined using the four HCA-CoAs (*p*-coumaroyl-, caffeoyl-, feruloyl-, and sinapoyl-CoAs) as donors and seven amines (Table I) as acceptors. All four HCA-CoAs were utilized, with caffeoyl- and feruloyl-CoA serving as best donors, whereas only putrescine, cadaverine, and 1,3-diaminopropane were converted to acylated products. Whether there are several HCA-CoA-specific enzymes or one enzyme with a broad donor specificity remains to be determined. Examples of strict donor specificities of HCA-transferases have been found (21). Thus, it seems that the enzyme(s) from *N. tabacum* specifically catalyze the acylation of aliphatic diamines with putrescine as best acceptor, which clearly distinguishes the activity described here from those for agmatine (7) and tyramine (12).

All reactions with HCA-CoAs and Put showed typical hyperbolic saturation curves with increasing substrate concentrations and the double-reciprocal plots were linear. The apparent K_m-values for caffeoyl-CoA and Put were found to be near 3 and 10 μM at pH 10.0, and near 33 and 400 μM at pH 7.8 (50% of maximal activity), respectively.

Table I. *Substrate Specificity of the PHT Activity*

Substrates	Relative Activities[a]
Caffeoyl-CoA + putrescine	100
Caffeoyl-CoA + cadaverine	33
Caffeoyl-CoA + diaminopropane	8
Caffeoyl-CoA + spermidine	—[b]
Caffeoyl-CoA + spermine	—[b]
Caffeoyl-CoA + agmatine	—[c]
Caffeoyl-CoA + tyramine	—[b]
Feruloyl-CoA + putrescine	83
Sinapoyl-CoA + putrescine	33
p-Coumaroyl-CoA + putrescine	6[d]

[a] 100 = 225 pkat/mg protein at pH 10.0. [b] Not detected. [c] Formation of caffeoylputrescine due to the conversion of agmatine to putrescine. [d] At pH 8.8 (no reaction at pH 10.0).

Role of PHT Activity in the Biosynthesis of Caffeoyl-Put

The increased formation of caffeoyl-Put by TX4 cells has been attributed to the fact that this cell line was furnished with distinctly higher (total and specific) activities of the enzymes of the phenylpropanoid pathway as well as of ornithine and arginine decarboxylases (3). To ascertain whether the new PHT activity was also greatly enhanced in TX4 cells, its activity pattern in TX1 and TX4 cells was compared during a growth cycle with the activities of two enzymes (*e.g.* PAL, ODC) which seem to exert a regulatory function in HCA-Put biosynthesis. The tendencies in the product accumulations (Put and caffeoyl-Put) (Fig. 2) and the enzyme activities (PAL, ODC) (Fig. 3) compared well with the data obtained 7 years ago (3). The results (Figs. 2 and 3) were not only good proof that true variant lines can be maintained in a stable condition over many years, but were also a good basis for accepting the activity pattern of PHT. In contrast to the enzymes of the 'general phenylpropanoid pathway' (10) and the decarboxylases (ODC, ADC) (3) the levels of PHT activity were quite similar in low producing TX1 and high producing TX4 cells (Fig. 3). In Figure 3 the enzyme activities are given per g fresh mass and the patterns were quite similar when based on mg protein (not shown). Thus, for the overproduction of caffeoyl-Put by TX4 cells, only a limited number of biosynthetic enzyme activities have apparently had to be increased. The detection of rather high PHT activity in TX1 cells as compared to the low PAL activity shows that the regulatory control for the overproduction of HCA-Puts must be exerted by enzymes earlier in the biosynthetic pathway. Induction or enhancement of PAL activity, which includes the coinduction of the other enzymes involved in the HCA-CoA ester formation (3), seems to play an important role in this context, as it diverts the primary metabolite into the secondary pathway and is thus located at a very decisive metabolic site. ODC may have a similar function, as it was also increased in TX4 cells, but this pattern may be complicated by the involvement of ODC in other pathways. Recently, Walker *et al.* (22) detected distinctly higher arginine decarboxylase (ADC) activity in TX4 cells and concluded that putrescine may be formed mainly by ADC rather than by ODC. We therefore also measured ADC activity on several days during the kinetic study. Although ADC activity was enhanced in TX4 cells, we found, as in earlier experiments (3), that ADC activity was never higher than that of ODC.

PHT activity seems to be constitutive in both tobacco cell lines and its expression is not correlated with the ability of a line to form HCA-Puts. It seems likely that only certain enzyme activities have to be increased for the enhanced formation of secondary metabolites. Low levels of a secondary metabolite thus are not necessarily an indicator that such cultures would be poor sources for detecting an enzymic step of that biosynthesis. For example, some cell cultures of *Peganum harmala* unable to synthesize serotonin from trypto-

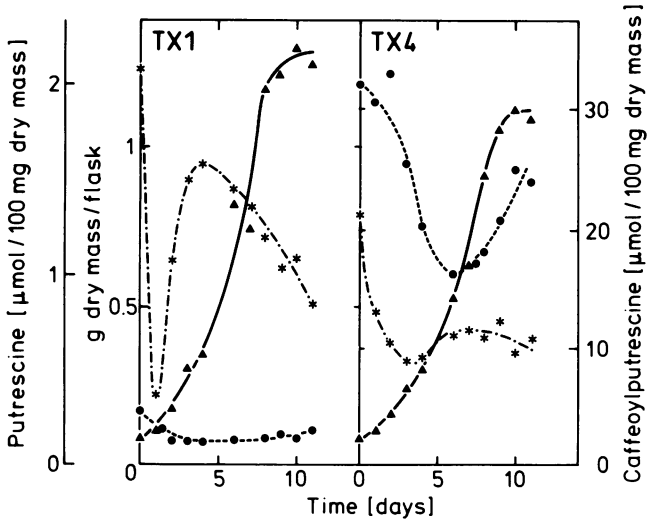

Figure 2. Dry mass (▲), putrescine (*), and caffeoylputrescine (●) content during a growth cycle of TX1 and TX4 cells.

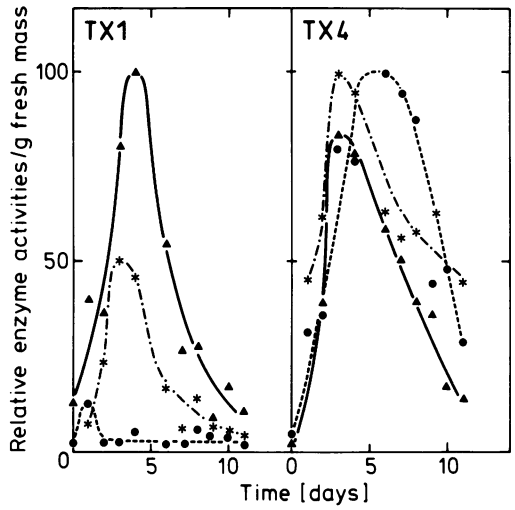

Figure 3. Comparison of relative activities of phenylalanine ammonialyase (●), ornithine decarboxylase (*), and HCA:CoA-putrescine HCA-transferase (▲) in TX1 and TX4 cells. The highest activity/g fresh mass of each enzyme was set at 100% which corresponded to PHT = 1.1 nkat (TX1), PAL = 51 pkat (TX4), and ODC = 5.2 pkat (TX4).

phan, due to the lack of tryptophan decarboxylase, exhibit high tryptamine-5-hydroxylase activity. Thus, serotonin biosynthesis was easily restored by selecting for tryptophan decarboxylase active cell lines (6). Another example, that non- or low-producing cell cultures can express high activity of one or more enzymic steps of a seemingly absent pathway is the specific 12-β-hydroxylation of cardiac glycosides by cultures of *Digitalis lanata* (15).

In conclusion, PHT-activity is a further example that HCA-amide formation is catalyzed via HCA-CoA thioesters and that the corresponding product accumulation patterns of cell culture lines may not necessarily directly reflect the levels of a specific enzymatically catalyzed step within a biosynthetic pathway.

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LITERATURE CITED

1. **Barz W, Köster J, Weltring KM, Strack D** (1985) Recent advances in the metabolism and degradation of phenolic compounds in plants and animals. *In* CF van Sumere, PJ Lea, eds, Annual Proceedings of the Phytochemical Society of Europe. The Biochemistry of Plant Phenolics, vol 25. Claredon Press, Oxford, pp 307–347
2. **Berlin J** (1981) Formation of putrescine and cinnamoyl putrescines in tobacco cell cultures. Phytochemistry **20:** 53–55
3. **Berlin J, Knobloch KH, Höfle G, Witte L** (1982) Biochemical characterization of two tobacco cell lines with different levels of cinnamoyl putrecines. J Natl Prod **45:** 83–87
4. **Berlin J, Witte L** (1982) Metabolism of phenylalanine and cinnamic acid in tobacco cell lines with high and low yields of cinnamoyl putrescines. J Natl Prod **45:** 88–93
5. **Berlin J, Witte L, Hammer J, Kukoschke KG, Zimmer A, Pape D** (1982) Metabolism of *p*-fluorophenylalanine in *p*-fluorophenylalanine sensitive and resistant tobacco cell cultures. Planta **155:** 244–250
6. **Berlin J, Mollenschott C, Sasse F, Witte L, Piehl GW, Büntemeyer H** (1987) Restoration of serotonin biosynthesis in cell suspension cultures of *Peganum harmala* by selection for 4-methyltryptophan-tolerant cell lines. J Plant Physiol **131:** 225–236
7. **Bird CR, Smith TA** (1983) Agmatine coumaroyltransferase from barley seedlings. Phytochemistry **22:** 2401–2403
8. **Bradford MM** (1976) A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254
9. **Flores HE, Galston AW** (1982) Analysis of polyamines in higher plants by high performance liquid chromatography. Plant Physiol **69:** 701–706
10. **Hahlbrock K, Grisebach H** (1975) Biosynthesis of flavonoids. *In* JB Harborne, TJ Mabry, H Mabry, eds, The Flavonoids, Chapman & Hall, London, pp 866–915
11. **Lineweaver H, Burke D** (1934) The determination of enzyme dissoziation constants. J Am Chem Soc **56:** 658–666
12. **Negrel J, Martin C** (1984) The biosynthesis of feruloyltyramine in *Nicotiana tabacum*. Phytochemistry **23:** 2797–2801
13. **Ngo TT, Brillhart KL, Davis RH, Wong RC, Bovaird JC, Digangi JJ, Ristow JL, Marsh JL, Phan APH, Lenhoff HM** (1987) Spectrophotometric assay for ornithine decarboxylase. Anal Biochem **160:** 290–293
14. **Palmer JE, Widholm JM** (1975) Characterization of carrot and tobacco cell cultures resistant to *p*-fluorophenylalanine. Plant Physiol **56:** 233–238
15. **Petersen M, Seitz HU** (1985) Cytochrome P-450 dependent digitoxin 12-β-hydroxylase from cell cultures of *Digitalis lanata*. FEBS Lett **188:** 11–14
16. **Ponchet M, Martin-Tanguy J, Poupet A, Marais A, Beck D** (1982) Separation and quantification of basic hydroxycinnamic amides and hydroxycinnamic acids by reversed-phase high-performance liquid chromatography. J Chromatogr **240:** 397–404
17. **Schiel O, Jarchow-Redecker K, Piehl GW, Lehmann J, Berlin J** (1984) Increased formation of cinnamoyl putrescines by fed-batch fermentation of cell suspension cultures of *Nicotiana tabacum*. Plant Cell Rep **3:** 18–20
18. **Stöckigt J, Zenk MH** (1975) Chemical syntheses and properties of hydroxycinnamoyl-coenzyme A derivatives. Z Naturforsch **30c:** 352–358
19. **Strack D** (1977) Sinapic acid ester fluctuations in cotyledons of *Raphanus sativus*. Z Pflanzenphysiol **84:** 139–145
20. **Strack D, Keller H, Weissenböck G** (1987) Enzymatic synthesis

of hydroxycinnamic acidesters by sugar acids and hydroaromatic acids by protein preparations from rye (*Secale cereale*) primary leaves. J Plant Physiol **131:** 61–67

21. **Ulbrich B, Zenk MH** (1980) Partial purification and properties of *p*-hydroxycinnamoyl-CoA:shikimate-*p*-hydroxycinnamoyl transferase from higher plants. Phytochemistry **19:** 1625–1629
22. **Walker MA, Ellis BE, Dumbroff EB, Downer RG, Martin RJ** (1986) Changes in amines and biosynthetic enzyme activities in *p*-fluorophenylalanine resistant and wild type tobacco cell cultures. Plant Physiol **80:** 825–828