BIOCHEMICAL CHARACTERIZATION OF TWO TOBACCO CELL LINES WITH DIFFERENT LEVELS OF CINNAMOYL PUTRESCINES

JOCHEN BERLIN, KARL-HEINZ KNOBLOCH, GERHARD HÖFLE and LUDGER WITTE

Gesellschaft f. Biotechnologische Forschung m.b.H., Mascheroder Weg 1, D 3300 – Braunschweig, Federal Republic of Germany

ABSTRACT.—A *p*-fluorophenylalanine-resistant tobacco cell line (TX4) and a wild-type culture (TX1) were compared biochemically. The former yielded 6 times more cinnamoyl putrescines than the latter, and the main phenolic compounds of both cell lines were identified as caffeoyl-, feruloyl-, and *p*-coumaroyl putrescine. The increased accumulation by TX4 cells was evidently due to increased enzyme activities of related enzymes, phenylalanine ammonia-lyase, trans-cinnamate 4-hydroxylase, *p*-coumarate-CoA ligase, ornithine and arginine decarboxylases.

In most plant cell cultures, secondary pathways are not well expressed. However, it has been shown that high yielding strains can be obtained from low producing parent cultures by analytical or biochemical selection (1–6). A biochemical characterization of variant strains with different capacities in the synthesis and accumulation of secondary metabolites may help to identify biochemical requirements of high producing strains and to elucidate the enzymology of secondary pathways. The value of plant cell cultures for studying biochemical aspects of secondary pathways has been impressively demonstrated by the progress in the enzymology of flavonoids and indole alkaloids (7–8).

Palmer and Widholm selected a *p*-fluorophenylalanine (PFP) resistant cell line (TX4) from a parent culture of *Nicotiana tabacum* L.cv.Xanthi (TX1) (9). The resistant line accumulated 6 to 10 times more phenolic compounds, and its phenylalanine ammonia-lyase (PAL) activity was considerably increased (10). From feeding of labeled precursors and from their uv-spectra, the phenolic compounds were tentatively regarded as cinnamoyl putrescine derivatives (11). Preliminary results indicated that not only PAL but also activities of other enzymes of the proposed pathway were increased (11). Here we report on a detailed identification of the cinnamoyl putrescines of the tobacco cultures and on the activities of five enzymes involved in the biosynthesis of these compounds in low and high producing cells.

EXPERIMENTAL

PLANT MATERIAL.—Maintenance and some characteristics of wild-type cells TX1 and the PFP-resistant cell line TX4 (*Nicotiana tabacum* L. cv. Xanthi) have been described previously (9,10,12,13). Inoculum size was 1 g fr.wt. per 70 ml MS-medium (14) containing 2 μM 2,4-D. Cells were harvested by vacuum filtration for fresh wt and dry wt determinations and for biochemical analysis.

ISOLATION OF CINNAMOYL PUTRESCINES.—Freeze dried cells were extracted twice with MCW (methanol-chloroform-water, 12:5:3). The combined extracts were separated in two phases by the addition of 3 ml chloroform and 3 ml water to 10 ml MCW-extract. The chloroform-phase was discarded, and the methanol-water phase was concentrated for chromatography in the systems L1 (n-butanol-acetic acid-water, 4:1:1, Whatman 3 MM, cellulose or silica gel tlc), L2 (isobutylmethylketone-formic acid-water, 14:3:2, silica gel tlc) and L3 (n-propanol-water-Tris-HCl 0.1 M pH 8.0, 70:30:1, cellulose). Paper chromatography with L1 separated caffeoyl putrescine (pale fluorescent) from feruloyl putrescine (blue fluorescent) and *p*-coumaroyl putrescine (dark absorbing). The bands were cut out and eluted with 50% methanol. Feruloyl putrescine and *p*-coumaroyl putrescine were further purified from each other by tlc in the systems L3 and L1 on silica gel. L2 was mainly used to check for other impurities.

IDENTIFICATION OF CINNAMOYL PUTRESCINES.—All isolated compounds were compared with chemically synthesized cinnamoyl putrescines (15) as hydrochlorides.

a) *Caffeoyl putrescine.*—The isolated product had an identical uv-spectrum with the synthesized compound (λmax 320, 295, λmin 260 in 50% MeOH), showed a bathochromic shift with NaOH (λmax 365), and a similar shift with $AlCl_3$ (λmax 328, 352 sh), and was ninhydrin positive. Mass spectroscopy of the synthesized, as well as the isolated compound, did not provide a molecular ion nor any typical fragmentation pattern. Therefore

both compounds were refluxed in 2 ml acetic acid anhydride for 10 min. After evaporation to dryness, a mixture of tri-, tetra- and pentaacetyl derivatives was detected by chromatography in ethyl acetate-methanol, 9:1 (silica gel). The mixture was boiled in ethanol for 2 min to give only the triacetyl derivative (Rf=0.2). MS, *m/z* (%) 376 (M^+, 10) 334(8), 317(4), 292(92), 233(16), 163(100). The nmr-spectra of the two triacetyl derivatives were also identical (15).

b) *Feruloyl putrescine.*—The isolated product and the synthetic compound showed identical uv-spectra (λmax 320, 295, λmin 260). The bathochromic shift by alkali (λmax 358) was smaller than for caffeoyl putrescine and the uv-spectra were not altered by $AlCl_3$. The compound was ninhydrin positive. The purified compound (L1, cellulose, silica gel) was directly analyzed by mass spectroscopy. The fragmentation pattern was identical with the synthesized compound and data published by Wheaton and Stewart (16): MS, *m/z*(%) 264 (M^+, 11), 177(83), 145(55), 70(100). The isolated compound and the synthetic compound were also analyzed by gc/ms as trifluoroacetyl derivatives (TFAA, 60°C for 30 min): MS, *m/z*(%) 552 (M^+, 10), 455(4), 273(100), 245(3), 176(15). Gc-conditions: 15m x 0.25 mm i.d. fused silica capillary column coated with SE 30, carrier gas helium 0,65 bar, split ratio 1:30, temperature program 200°-300°C 6°C/min, detection FID. I=2435 according to the Kovats retention index (17).

c) *p-Coumaroyl putrescine.*—The uv-spectra of the isolated compound (L1 Whatman 3 MM; L3 cellulose; L1 silica gel) and the synthesized compound were identical (λmax 302, 289 λmin 245). The bathochromic shift to λmax 340 by NaOH indicated the free phenolic hydroxyl group and the positive ninhydrin reaction the free amino group. Since the ms pattern of the synthetic compound *m/z*(%) 234 (M^-, 3), 147(100), 119(31), 91(27), 70(87) was observed in the mass spectrum of the isolated product only in addition to signals from impurities, the isolated and synthetic compounds were also compared by gc/ms as their trifluoroacetyl derivatives (conditions see above) I=2275. MS, *m/z*(%) 522 (M^+, 3), 425(1), 243(100), 215(5).

QUANTIFICATION OF CINNAMOYL PUTRESCINES.—The absorption coefficients for the hydrochlorides in 50% methanol were for caffeoyl putrescine $\epsilon_{320}=18600$, feruloyl putrescine $\epsilon_{320}=19600$ and p-coumaroyl putrescine $\epsilon_{302}=18750$. The crude MCW-extracts of TX1 and TX4 cells showed uv-spectra typical for caffeoyl/feruloyl putrescine. Since the ratio of isolated caffeoyl-feruloyl-coumaroyl putrescines was roughly 8:1:1 in both cell lines, the quantification from MCW-extracts was based on the ϵ_{320}-value of caffeoyl putrescine. Equivalent levels were found by quantifying cinnamoyl putrescines by the Folin-method (10).

EXTRACTION AND ASSAYS OF ENZYMES.—Extraction and measurement of phenylalanine ammonia-lyase (EC 4.3.1.5; PAL), L-ornithine decarboxylase (EC 4.1.1.17; ODC) and L-arginine decarboxylase (EC 4.1.1.19; ADC) have been described previously (10,13). For the determination of 4-coumarate:CoA ligase (EC 6.2.1.12) and trans-cinnamate 4-hydroxylase (EC 1.14.13.14) 2.5 g (fr.wt.) cells were homogenized with quartz sand and 5 ml 0.1 M Tris-HCl, pH 7.5, containing 7 mM 2-mercaptoethanol and 15% glycerol in the presence of 0.4 g buffer saturated Polyclar AT. After centrifugation (20,000 g, 20 min) the supernatant was chromatographed on Sephadex G-25 equilibrated with the same buffer. From the eluate the activity of 4-coumarate:CoA ligase was measured according to (18) with an altered CoASH concentration of 0.2 mM. Cinnamic acid 4-hydroxylase was measured according to (19) from a microsomal fraction prepared from the enzyme extract after gel filtration by centrifugation at 100,000 g for 70 min. The pellet was resuspended in 0.5 ml 0.1 M Tris-HCl, pH 7.5, containing 3 mM 2-mercaptoethanol. Protein was determined by the Lowry method (20).

RESULTS AND DISCUSSION

IDENTIFICATION AND QUANTIFICATION OF CINNAMOYL PUTRESCINES.—The purpose of our work was to investigate biochemical differences between tobacco cell lines with low (TX1) and high (TX4) accumulation of phenolics. After purification by paper and thin-layer chromatography, the phenolic compounds have now been identified as caffeoyl-, feruloyl- and *p*-coumaroyl putrescines by their uv-spectra, their chemical reactions (e.g. to ninhydrin and Folin-reagent), and their mass fragmentation in comparison with authentic material (see Experimental). In particular, caffeoyl putrescine caused some problems during identification since it was not possible to obtain mass spectra from the isolated product. After chemical synthesis of the compound (15), it was noted that only derivatives of caffeoyl putrescine resulted in typical mass fragmentation patterns. Caffeoyl putrescine is a rather sensitive compound. Thus, the compound was destroyed by chromatography in systems above pH 9 or on silica gel plates. Light caused trans-cis-isomerization (21) and, therefore, often two bands were found in second or third chromatography systems. Due to the lack of an ortho-hydroxyl group, feruloyl- and *p*-coumaroyl putrescine were even stable in basic chromatographic systems like *n*-propanol-ammonium hydroxide solution (7:3) on cellulose and were also eluted from silica gel plates. While we were able to separate the three synthesized compounds on tlc-plates (L3) for direct quantification by a Shimadzu-TLC-scanner,

this was only possible from plant extracts after several purification steps. Separation and quantification of the three compounds as their trifluoroacetyl derivatives by capillary gc proved to be unsuccessful since caffeoyl putrescine gave an unstable product for gc and up to 50% decomposition products were found for feruloyl- and *p*-coumaroyl putrescines. Therefore our quantification was based on the fact that caffeoyl putrescine was the main compound (fig. 1). From the quantities of isolated compounds, a ratio of 8:1:1 (caffeoyl-feruloyl-*p*-coumaroyl putrescine) was calculated. Therefore direct measurement of MCW-extracts at 320 nm was sufficient for our purposes.

We have observed the productivity of TX1 and TX4 cells for more than 6 years. It is now evident that the *p*-fluorophenylalanine resistant cell line TX4 is a very stable line with regard to its resistance and its productivity in the absence of the selective agent. A typical kinetic curve for growth and production over a growth cycle is given in fig. 1; but, as in all cell culture systems, variations were occasionally observed. Thus, TX4 cells had levels between 5–10% and TX1 between 0,3 to 1,5% cinnamoyl putrescines on a dry weight basis over the years. However, the difference between TX1 and TX4 cells always remained 6 to 10-fold. Considering the good growth of TX4 cells (fig. 1) [300 g fresh wt/liter in 20 liters

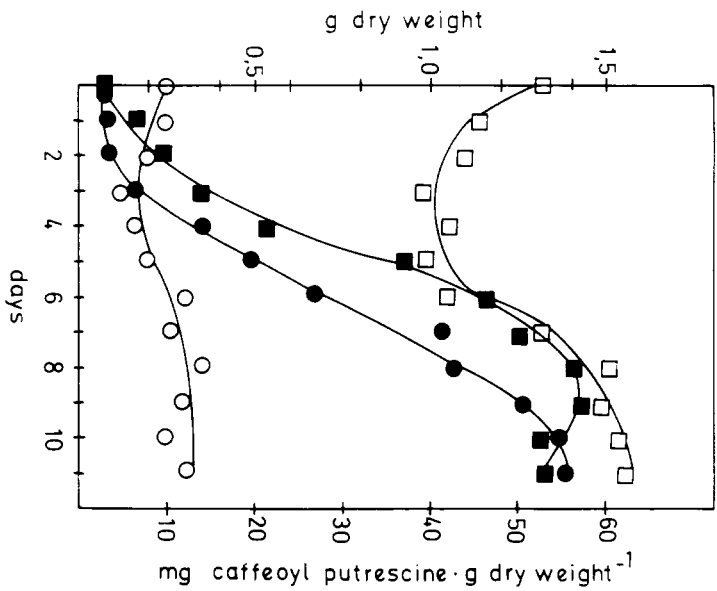

FIG. 1. Growth kinetics of TX1 (●—●) and TX4 (■—■) cells and accumulation of caffeoyl putrescine by TX1 (○—○) and TX4 (□—□) cells.

air-lift fermenter (unpublished)] this line classifies in the list of high producing cell cultures (22) with a yield of caffeoyl putrescine of 0,6–1,0 g/liter. The occurrence of caffeoyl-, feruloyl- and *p*-coumaroyl putrescine in callus cultures of a different *Nicotiana tabacum* variety has already been described (23). More recently such hydroxycinnamic acid amides have also been found in intact plants of tobacco (24). It has been suggested that these amides may play an important role in flowering processes (25) and male sterility (26). The presence of bound putrescine may also be related to the biosynthesis of nicotine in tobacco (23). This alkaloid was not found in our cell lines.

ENZYMOLOGY OF CINNAMOYL PUTRESCINES.—Our main interest was directed to the question of what is the biochemical basis of the increased productivity of

TX4 cells. Therefore the activities of enzymes involved in the phenylpropanoid pathway and those involved in the formation of putrescine were compared in TX1 and TX4 cells (fig. 2). The activities of the first three enzymes of the general phenylpropanoid pathway, according to Hahlbrock the "group I" enzymes (27), were increased manifold in TX4 cells. The changes in activities during the growth cycle indicated a coordinated regulation of these three enzymes. A coordinated behavior of these enzymes has also been noted in several other systems (27). We have recently shown that the conjugate putrescine may be formed via arginine or

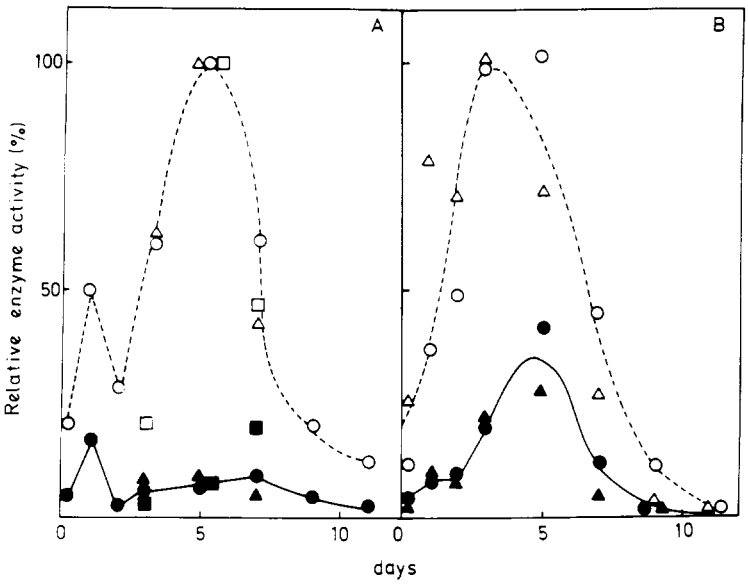

FIG. 2. Comparison of enzyme activities involved in the biosynthesis of cinnamoyl putrescines in TX1 (filled symbols) and TX4 (open symbols).
A) Enzymes of general phenylpropanoid metabolism: phenylalanine ammonia-lyase (○—○), cinnamate 4-hydroxylase (△—△), p-coumarate: CoA ligase (□—□).
B) Enzymes of putrescine biosynthesis: ornithine decarboxylase (△—△) and arginine decarboxylase (○—○).

ornithine decarboxylation (13). The activities of both enzymes were significantly increased in TX4 cells. However, the pattern of ornithine and arginine decarboxylase activities differed from that of the enzymes of the phenylpropanoid pathway. In cell lines accumulating no cinnamoyl putrescine similar changes in the activities of arginine decarboxylase were found (28). The formation of cinnamoyl putrescines by TX1 cells was greatly increased when the cells were transferred to a medium devoid of phosphate and 2,4-D (29). In these medium-induced TX1 cells, the activity of phenylalanine ammonia-lyase, but not of ornithine decarboxylase, showed a close correlation with increased product accumulation compared with non-induced cells (29). Therefore, it is concluded that the activities of the decarboxylases and of the enzymes of the general phenylpropanoid pathway are regulated independently, the former having less influence on the regulation of product formation.

We also tried to find a hydroxycinnamoyl-CoA:putrescine hydroxycinnamoyltransferase in TX1 and TX4 cells. Under conditions allowing conjugation with quinate or shikimate (30,31), we failed to detect such an enzyme in our cell lines. However, feeding experiments with putrescine clearly indicated that TX4 cells should have a higher activity of a conjugating enzyme (13). Thus, one may conclude that the increased accumulation of cinnamoyl putrescines by TX4 cells is due

to the increased activities of many enzymes related to this secondary pathway. From results with the PAL-inhibitor α-aminooxy-β-phenylpropionic acid or the 5-enolpyruvylshikimate-3-phosphate synthase inhibitor glyphosate (32), it can be concluded that no regulatory alterations in phenylalanine supply had occurred in TX4 cells (33). The productivity of cell cultures evidently depends very much on the activity of related enzymes. In TX4 cells all of the tested enzymes were increased considerably. This, however, is not always necessary. We have recently shown that the biosynthesis of indole alkaloids by *Catharanthus* cultures was induced when 10-day-old cells were transferred into an 8% sucrose solution (34). The activity of strictosidine synthase was not enhanced in the induction medium, whereas the activity of tryptophan decarboxylase was increased manifold during the period of rapid alkaloid accumulation (34). Thus, the competence of cell cultures for production may already be achieved if the activity of the first enzyme(s) diverting primary metabolites are increased. If we know that the increased activities of the enzymes leading into the secondary pathways are a prerequisite for high yielding cell lines, one may contemplate biochemical selection systems for high producing strains which may give rise to quite stable high yielding cell lines.

ACKNOWLEDGEMENT

The excellent technical assistance of Ms. A. Zimmer, Ms. H. Gittermann and Mr. D. Pape is gratefully acknowledged.

Received 2 June 1981

LITERATURE CITED

1. M. Tabata, T. Ogino, K. Yoshioka, N. Yoshikawa, and N. Hiraoka, "Frontiers of Plant Tissue Culture 1978" (Ed. T. A. Thorpe), Univ. of Calgary, Canada, p. 213.
2. M. H. Zenk, H. El-Shagi, H. Arens, J. Stöckigt, E. W. Weiler, and B. Deus, "Plant Tissue Culture and Its Bio-technological Application" (Ed. W. Barz, E. Reinhard, M. H. Zenk), Springer-Verlag, Berlin-Heidelberg-New York 1977, p. 27.
3. A. W. Alfermann, D. Merz, and E. Reinhard, *Planta Med. Suppl.*, 1975, p. 70.
4. T. Matsumoto, T. Ikeda, N. Kanno, T. Kisaki, and M. Noguchi, *Agric. Biol. Chem.*, **44**, 967 (1980).
5. T. M. Murphy, C. M. Hamilton, and H. E. Street, *Plant Physiol.*, **64**, 936 (1979).
6. J. Berlin, *Z. Pflanzenphysiol.*, **97**, 317 (1980).
7. K. Hahlbrock and H. Grisebach, *Ann. Rev. Plant Physiol.*, **30**, 105 (1979).
8. M. H. Zenk, *J. Nat. Prod.*, **43**, 438 (1980).
9. J. E. Palmer and J. M. Widholm, *Plant Physiol.*, **56**, 233 (1975).
10. J. Berlin and J. M. Widholm, *Plant Physiol.*, **59**, 550 (1977).
11. J. Berlin and K.-H. Knobloch, *Hoppe Seyler's Z. Physiol. Chem.*, **361**, 219 (1980).
12. J. Berlin and J. M. Widholm, *Z. Naturforsch.*, **34c**, 634 (1978).
13. J. Berlin, *Phytochem.*, **20**, 53 (1981).
14. T. Murashige and F. Skoog, *Physiol. Plant.*, **15**, 473 (1962).
15. G. Höfle, in preparation.
16. R. A. Wheaton and I. Stewart, *Nature*, **206**, 620 (1965).
17. A. Wehrli and E. Kovats, *Helv. Chim. Acta*, **42**, 2709 (1959).
18. K.-H. Knobloch and K. Hahlbrock, *Arch. Biochem. Biophys.*, **184**, 237 (1977).
19. T. Büche and H. Sandermann, *Arch. Biochem. Biophys.*, **158**, 445 (1973).
20. O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
21. J. C. Challice and A. H. Williams, *J. Chromatog.*, **21**, 357 (1966).
22. M. H. Zenk in "Frontiers of Plant Tissue Culture 1978" (Ed. T. A. Thorpe), Univ. of Calgary, Calgary, Canada, p. 1.
23. S. Mizusaki, Y. Tanabe, M. Noguchi and E. Tamaki, *Phytochemistry*, **10**, 1347 (1971).
24. F. Cabanne, J. Martin-Tanguy and C. Martin, *Physiol. Veg.*, **15**, 429 (1977).
25. J. Martin-Tanguy, F. Cabanne, E. Perdrizet and C. Martin, *Phytochemistry*, **17**, 1927 (1978).
26. J. Martin-Tanguy, A. Deshayes, E. Perdrizet and C. Martin, *FEBS Lett.*, **108**, 176 (1979).
27. K. Hahlbrock and H. Grisebach, *Ann. Rev. Plant Physiol.*, **30**, 105 (1979).
28. M. J. Montague, T. A. Armstrong & E. G. Jaworski, *Plant Physiol.*, **63**, 341 (1979).
29. K.-H.Knobloch and J. Berlin, *Planta Med.*, **42**, 167 (1981).
30. B. Ulbrich, J. Stöckigt and M. H. Zenk, *Naturwissenschaften*, **63**, 484 (1976).
31. B. Ulbrich and M. H. Zenk, *Phytochemistry*, **19**, 1625 (1980).
32. N. Amrhein, J. Schab and H. C. Steinrücken, *Naturwissenschaften*, **67**, 356 (1980).
33. J. Berlin and L. Witte, *Z. Naturforsch.*, **36c**, 210 (1981).
34. K.-H. Knobloch, B. Hansen, and J. Berlin, *Z. Naturforsch.*, **36c**, 40 (1981).