Pergamon

PII: S0031-9422(97)01098-4

Phytochemistry, Vol. 48, No. 1, pp. 79–84, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

LYSINE DECARBOXYLASE TRANSGENIC TOBACCO ROOT CULTURES BIOSYNTHESIZE NOVEL HYDROXYCINNAMOYLCADAVERINES

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(*Received in revised form* 12 *November* 1997)

Key Word Index—*Nicotiana tabacum*; Solanaceae; transgenic root cultures; gene of lysine decarboxylase; anabasine; cadaverine; hydroxycinnamoylcadaverines; hydroxycinnamoylputrescines.

Abstract—Expression of a bacterial lysine decarboxylase (under the control of the 35S-promoter of the cauliflower mosaic virus and fused to the coding sequence of the small subunit rbcS transit peptide) in tobacco root cultures can affect at least two different secondary pathways. The overproduction of cadaverine by a line with high lysine decarboxylase activity led not only to greatly enhanced accumulation of the minor alkaloid anabasine but also to the formation of hydroxycinnamoylcadaverines, new metabolites which have not yet been described as natural constituents of tobacco. The transgenic root culture was changed into a suspension culture and also plants were regenerated from the root culture. The differences in the metabolic fate of cadaverine in the different morphological states are described. The advantages of systems like this for studying metabolic engineering of plant secondary pathways is discussed.

INTRODUCTION

Cadaverine supply is the major factor limiting anabasine biosynthesis in tobacco root cultures [1, 2]. This limitation can be overcome by engineering a bacterial lysine decarboxylase gene (*ldc*) into tobacco cells. Some of the cadaverine overproducing transgenic lines accumulated anabasine levels of up to 0.5% of dry mass [3, 4], while the alkaloid was hardly detectable in control cultures. Cadaverine overproduction might also affect the pattern of hydroxycinnamoyldiamines (HCAs). Cultured cells of tobacco usually accumulate hydroxycinnamoylputrescines (HCPs) [5, 6]. Hydroxycinnamoylcadaverines (HCCs) have never been described as natural constituents of tobacco. However, the absence of HCCs might only result from the lack of, or low level of cadaverine in tobacco. When cadaverine was fed to tobacco suspension cultures HCCs were indeed formed [7]. This finding was in agreement with the fact that the conjugating enzyme hydroxycinnamoyl-CoA : putrescine hydroxycinnamoyltransferase accepts cadaverine as substrate [7–9]. Therefore, it was speculated that genetically engineered overproduction of cadaverine may lead to the formation of novel HCCs and thus the pattern of two secondary pathways may be affected by the expression of a bacterial *ldc* gene in tobacco root cultures.

RESULTS

Accumulation of hydroxycinnamoylputrescines (HCPs) and hydroxycinnamoylcadaverines (HCCs) in ldc-*transgenic root cultures*

The extracts of all *ldc*-transgenic root cultures were regularly inspected for their HCAs with the aim of detecting lines with unusual patterns that indicated the presence of HCCs. As a by-product of this search, we obtained information on the levels of HCPs in these root cultures. With the exception of line SSR-68-2* the patterns of all 30 *ldc*-transgenic root cultures analysed were almost identical, showing the accumulation of the 3 typical HCPs of tobacco (caffeoylputrescine *ca* 50–70%, 4-coumaroylputrescine *ca* 10–15%, and feruloylputrescine *ca* 20–40%). However, the specific contents of the HCPs of the various lines differed by up to a factor of 3, as shown for the levels of caffeoylputrescine in Table 1. This variation could not be explained as a direct consequence of the

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Table 1. Specific content of caffeoylputrescine in several independently established *ldc*-transgenic root cultures of *N. tabacum cv Samsum* [4]. All cultures were grown for 14 days in liquid WM-medium. The initial inoculum was 1 g/35 ml, the final fresh mass (dried between paper towels) was between 5–7 g. The absolute values of some lines varied up to 50% when repeatedly analysed during a period of one year

Line	Caffeoylputrescine (mg/g dry mass)
SSR68-1	1.6
SSR68-2*	2.4
SSR68-4	3.2
SSR68-5	0.9
SSR68-6	2.0
SSR68-8	2.5
SSR30-5	1.5
SSR30-6	1.0
SSR30-11	2.3
SSR30-12	1.7

expression of the bacterial enzyme or cadaverine overproduction. Indeed 29 of the 30 lines showed no evidence for the accumulation of measurable amounts of HCCs at the standard conditions of sample preparation. The different amounts of the HCPs in the various lines could also not be explained by the fact that their specific content was not constant over a 4-week growth period but decreased greatly in older cultures. At the time of measurement, during the linear growth phase, the specific HCP contents of the various lines were quite stable. When some lines were analysed repeatedly in independent experiments performed during one year, total levels of one line varied only by 20–50%. Indeed, the capacity for HCP formation seemed to be line specific. For example, the control culture SSgus always contained lower levels of HCPs than line SSR68-2* (Fig. 1). Thus, the substantial differences of HCPs of the various root cultures should be regarded as unspecific alterations occurring during any transformation event.

As an example of a line with an unchanged HCA composition, the HPLC pattern of line SSgus is shown in Fig. 1. Line SSR68-2* represented the only line with a significantly different HCA pattern (Fig. 1). This line was known to express the highest LDC activity (*ca* 30 pkat/mg protein) of all root cultures and to accumulate the highest levels of cadaverine (*ca* 6 mg/g dry mass) and anabasine (*ca* 5 mg/g dry mass) [4]. In addition, this line belonged to the group of root cultures with a good capacity for HCP biosynthesis (Table 1). Both factors, high levels of cadaverine and a good biosynthetic potential for HCPs, seemed to be needed for high HCC formation. From day 7–14 of a culture period, SSR68-2* contained between 3–4 mg HCCs/g dry mass (caffeoylcadaverine 40–50%, feruloylcadaverine 50–60%, 4-coumaroylcadaverine less than 5%).

Identification of the hydroxycinnamoylcadaverines

As reference material was only partially available, we performed a number of experiments to prove the identity of the HCCs. The location of the HCPs was verified by spiking the samples with authentic material from previous studies and with extracts of the tobacco cell suspension culture TX4 [5] which contains a 20- to 40-fold higher content of HCPs than SSR68-2*. The location of the hydroxycinnamoylcadaverines was confirmed by adding high levels of cadaverine to the cultures. When 10 mg cadaverine was fed to an initial inoculum of 0.25 g/35 ml, after 14 days all extracts of HCAs from transformed root cultures of *N. tabacum cv Samsum* contained 3 novel peaks corresponding to caffeoyl-, 4-coumaroyl- and feruloylcadaverine. The ratio of the 3 HCCs of one line was very similar to the corresponding ratio of the 3 HCPs. In contrast to other cultures, these 3 HCC peaks were present in SSR68-2* without feeding of cadaverine (Fig. 1). When cadaverine was fed to SSR68-2* the HCC peaks greatly increased (Table 2). Due to the high LDC activity, lysine was rapidly decarboxylated to cadaverine by SSR68-2*, while this was not the case in SSgus. Consequently, lysine feeding to SSR68-2* also greatly enhanced the HCC peaks, while in SSgus traces, at best, of HCCs were found (Table 2).

To demonstrate that the novel compounds contained the cadaverine moiety, we performed feeding experiments with uniformly labelled ^{14}C-lysine and ^{14}C-cadaverine. The tracer experiments fully confirmed the feeding experiments with the inactive substrates. All 3 HCCs were labelled by both tracers in the SSR68-2* root culture expressing the engineered LDC activity (Table 3), while in extracts of SSgus radioactivity was only detected in HCC peaks when ^{14}C-cadaverine was added to culture medium (data not shown).

The conclusions from the incorporation data were further confirmed by spiking cell extracts with authentic feruloylcadaverine which co-chromatographed with the HCC peak tentatively identified to be this compound (Fig. 1). Thus, the increased levels after feeding of large amounts of cadaverine, the differential response of SSgus and SSR68-2* to the feeding of lysine, the incorporation of radioactivity from lysine and cadaverine into the compounds and the co-chromatography all support the conclusion that the novel compounds are HCCs.

Change of the root culture in suspensions and plants

One advantage of working with tobacco root cultures is that they can be changed into morphologically undifferentiated cells or that they can be regenerated to whole plants. When SSR68-2* was grown in the presence of 2 μM 2,4-D, the roots began to curl after 5–10 days and were covered with callus after 20 days. At this stage the alkaloid content decreased by 90%,

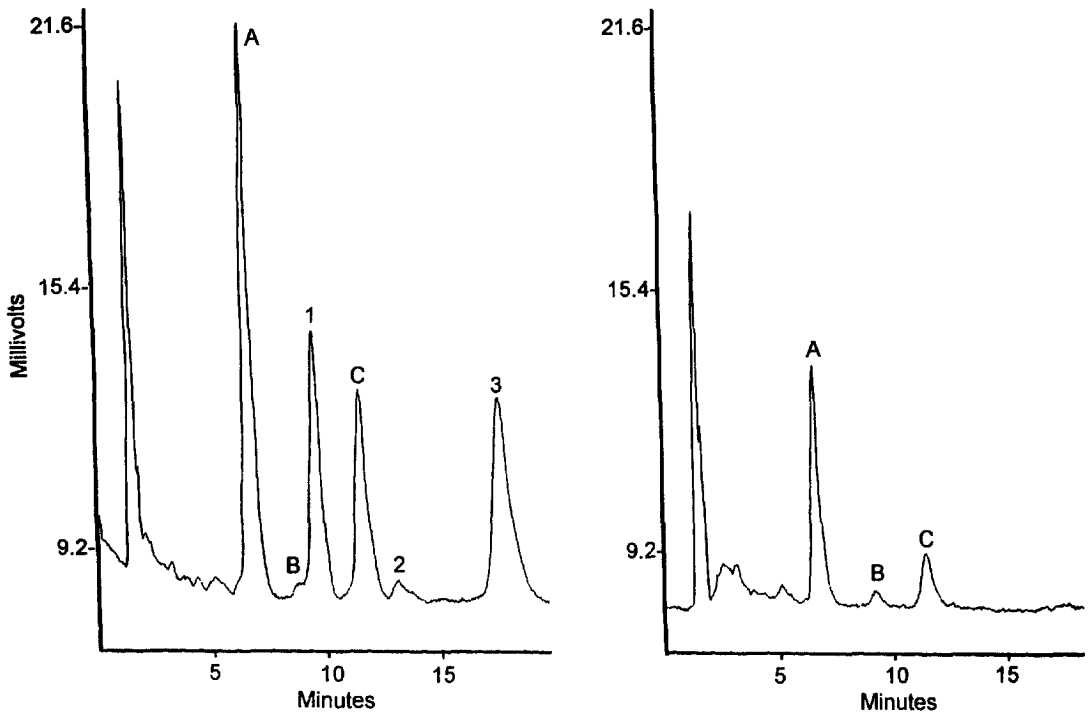

Fig. 1. HPLC pattern of hydroxycinnamoyldiamines of SSR68-2* (left) and SSgus (right) root cultures. **A** = caffeoylputrescine, **B** = 4-coumaroylputrescine, **C** = feruloylputrescine; **1** = caffeoylcadaverine, **2** = 4-coumaroylcadaverine, **3** = feruloylcadaverine. As identical amounts of extracts of 14-day-old cells of both lines were chromatographed, the chromatograms give also an impression of the different potential of the cell lines for HCA formation.

Table 2. Percentage changes of hydroxycinnamoyldiamines after feeding L-lysine and cadaverine, respectively, to the *ldc* transgenic root culture SSR68-2* and the control culture SSgus. The cultures were grown for 14 days on medium with 10 mg L-lysine or 10 mg cadaverine. The peaks areas of the various HCAs in HPLC runs of untreated controls were set at 100. Only for caffeoylputrescine the peak areas were also expressed in mg/g dry mass. The controls of SSR68-2* and SSgus contained 2.8 and 0.9 mg caffeoylputrescine/g dry mass, respectively. As HCCs are absent in control cultures of SSgus the given values represent the **fold**-increase of the lowest recognized peak area

Hydroxycinnamoyl diamines	SSR68-2*		Ssgus	
	+lysine	+cadaverine	+lysine	+cadaverine
			% of control	
Caffeoylputrescine	−37.3	−45.2	+8.3	+4.3
Caffeoylcadaverine	+83.1	+154.8	traces	+12-fold
Coumaroylputrescine	traces	traces	traces	traces
Coumaroylcadaverine	+368	+286	traces	3-fold
Feruloylputrescine	−4.3	−21.1	+25.3	−45.8
Feruloylcadaverine	+156	+218	+2-fold	+23-fold

while cadaverine, HCP and HCC levels remained similar or were slightly higher. The specific LDC activity was reduced to 10–15 pkat/mg protein compared with 30–40 pkat in root cells. After three growth cycles, rapidly growing suspension cultures were obtained. Root cultures were recovered from these suspensions even after 12 passages on 2,4-D when the phytohormone was removed from the medium for 2–3 passages. Table 3 indicates that the formation of HCCs was not greatly affected when the culture had changed from the root into a morphologically undifferentiated state, while the decreased labelling of the alkaloid fraction (Table 4) showed the repression of alkaloid formation in suspension cultures. When the alkaloid fraction was analysed by HPLC roughly 90% of the label in the roots was found in anabasine,

Table 3. Incorporation of radioactivity into hydroxycinnamoylcadaverines after feeding of 2 μCi L-[U-^{14}C]-lysine diluted with 1 mg inactive L-lysine to a root and a callus suspension culture of SSR68-2* for 4 days. An aliquot of the crude acidic cell extract was chromatographed by HPLC and the fractions were measured by scintillation counting. The sum of all fractions contained roughly 75% of the injected radioactivity. The fraction "unbound" contained lysine, cadaverine and anabasine

Fraction (min)	Root culture Bq	total radioactivity(%)	Suspension culture Bq	total radioactivity (%)
Background	0.66	—	0.66	—
Unbound (2–3 min)	1110	86.7	763	81.7
Caffeoylcadaverine (12–14 min)	65	5.1	95	10.2
4-Coumaroylcadaverine (17–18 min)	7.3	0.6	5.8	0.6
Feruloylcadaverine (24–27 min)	85	6.6	433	4.6

Table 4. Distribution of radioactivity after feeding 2 μCi L-[U-^{14}C]-lysine diluted with 1 mg inactive L-lysine to a 4-day-old root and suspension cultures of the *ldc*-transgenic line SSR68-2* for 4 days and 3 days, respectively. Initial inoculum was 0.25 g/10 ml medium. The root and suspension cultures had absorbed 79.7% and 69.3%, respectively, of the added radioactivity. The H_2SO_4 cell extracts contained 40.4 and 40.1% of the absorbed radioactivity

Percentage of extractable radioactivity	SSR68-2* grown as root culture	suspension culture
Anabasine	3.9	0.4
Cadaverine	26.8	22.5
Lysine	37.1	34.7
Hydroxycinnamoylcadaverines	12.3	16.2

while only a trace amount of the radioactivity of the corresponding extract from the suspension was located there.

When the roots were maintained on agar medium, shoot formation (on the average one or two shoots/plate) was often observed at sites with some callus-type growth around. Shoots were rooted in water and afterwards grown in soil. Leaves of plantlets grown in soil for 8 weeks were analysed for LDC activity, cadaverine, HCAs and alkaloids. The LDC activity in the leaves varied from plant to plant. Typically values of 10–30 pkat/mg protein were found, but could also be as low as 1–2 pkat/mg. The levels of anabasine were so low that they could not be quantified. HCAs levels were greatly reduced to 20–40% of those found in root and suspension cultures. Though the levels of cadaverine were doubled in some leaves (1% dry mass) compared with the root cultures, HCCs were not detected. In view of the very variable results of individually regenerated plants from shoots which appeared spontaneously on the agar plate, attempts were made to obtain seeds from the regenerated plants. In contrast to other regenerated plants of *ldc*-transgenic root cultures most flowers of SSR68-2* withered before producing seeds. Up to now, seeds from only a single capsule were obtained which showed very low germination and growth. This cannot be explained as an effect of overproduction of cadaverine, as the highest cadaverine overproducer of all *ldc*-transgenic plants [10] provided very viable seeds.

DISCUSSION

It has clearly been shown that the engineering of a bacterial *ldc* gene into tobacco cells leads not only to the overproduction of cadaverine and the promotion of the minor alkaloid anabasine to a major one [3, 4] but can also result in the formation of novel HCCs, compounds closely related to the natural HCP constituents. The analyses have concentrated on these two groups of compounds because there was sufficient information available that cadaverine supply was the limiting factor for the formation of anabasine and HCCs. However, we cannot exclude the possibility that other pathways/constituents might have also been affected in the root and suspension cultures, or would be affected in differentiated plants.

The establishment of *ldc*-transgenic root cultures overproducing compounds such as cadaverine, anabasine or hydroxycinnamoylcadaverines does not seem to be attractive at first view. As these secondary products are of no commercial value, the absolute levels reached are, indeed, of minor importance. However, this system provides a convenient and perhaps powerful model for studying factors important for the metabolic engineering of pathways in higher plants. What are the advantages of this system? The experimental results show that levels of secondary products can vary and may fluctuate substantially, especially in cell cultures. Table 1 gives an impression of the variation of levels of major products among individually established root cultures. To demonstrate unequivocally that increased levels of nicotine or caffeoylputrescine, for example, are due to metabolic engineering requires substantial effort. It has been reported that nicotine and HCP levels were increased 2-fold in

N. rustica root cultures over-expressing a yeast ornthine decarboxylase [6]. However, when levels of major compounds such as nicotine and HCPs may vary by a factor of 2–3 in independently established cultures transformed with the same vector (Table 1, [4]), one should obtain much greater increases to be sure that the engineered enzyme rather than natural variation is the reason for the overproduction. We encountered the same problem caused by high and variable basic levels of anabasine when we analysed the effect of cadaverine overproduction in root cultures of *N. glauca* [11], where the alkaloid is a major constituent. In *N. tabacum cv Samsum* the levels of the target compounds anabasine and HCCs are lacking or so low that any measurable increase can be related to the action of the engineered enzyme. Additional evidence that the observed product increases result from the action of the engineered enzyme can be obtained by feeding substrate and product of the engineered enzyme to the transgenic cultures (Table 2) [4]. This method should work whenever the product (here cadaverine) has been clearly identified as a limiting factor.

The availability of a system where the normal variation of product levels does not interfere with the interpretation of the metabolic consequences of an engineered step is useful for tackling a number of open questions for manipulating plant secondary pathways. For example, the importance of correct protein targeting or tissue specific expression on the biosynthetic potential of transgenic tobacco cultures or plants could be studied. The fact that cadaverine can be channelled into different pathways, whose metabolic activities are under developmental control, would allow the mechanism by which the cells regulate the distribution of a common precursor between two pathways to be examined. How to overcome not only the first but also the next rate-limiting step(s) of a pathway could be explored. Our studies have clearly identified the second rate limiting step of both pathways—the supply of lysine. How should a vector be constructed to allow optimal expression of the two genes so that the product of the first engineered step can be used by the second engineered enzyme? In conclusion, the overproduced metabolites anabasine and HCCs might become excellent indicators for many, but of course not all, questions related to nicotine and HCP formation in tobacco. In contrast to cadaverine, putrescine supply does not seem to be a limiting factor of both pathways. Cadaverine seems to enter the alkaloid pathway at a different site from putrescine. Consequently, *ldc*-transgenic cultures are not suitable for identifying regulatory controls of the entry of putrescine into the nicotine pathway.

EXPERIMENTAL

Plant material

Establishment and maintenance of the transgenic tobacco root cultures have recently been described [4]. The cultures were grown in liquid WM-medium (=B5-medium with 2.2 mM phosphate and 4% sucrose) on a rotary shaker. The root cultures were changed into a suspension culture after 2–3 growth periods of 3 weeks in MS-medium with 2 μM 2,4-D. Such suspension cultures could be returned to the root state when transferred back to the WM medium for 2–3 growth periods. Root cultures of both lines often formed shoots when maintained on solid phytohormone-free B5-medium, especially at sites where the roots were covered by brownish callus material. The shoots were removed and rooted in H_2O before being grown in pots with soil. After 8–12 weeks the plants flowered and in one case poorly-germinating seeds were obtained.

LDC activity measurements

Assay conditions and measurement of LDC activityusing L-[U-^{14}C]-lysine and extraction of ^{14}C-cadaverine into pentan-1-ol have been described [4].

Cell extraction and determination of metabolites

Freeze-dried cells (25 mg) were extracted in 2.5 ml 0.1 N H_2SO_4 in a test tube under stirring. The centrifuged supernatant was directly used for the measurement of the HCAs by HPLC (Column: Lichrospher 100 RP-18 (5 μm) 250 × 4; Solvent: 95% H_2O (1% CH_3COOH and 0.5% tetrabutylammoniumhydrogensulfate) + 5% MeOH; Flow rate: 1.5 ml/min; Detection: 310 nm). Calibration was performed with authentic caffeoylputrescine provided by Dr Höfle, GBF. For alkaloid determination 1 ml of the alkalinized extract was extracted twice with 1 ml EtOAc, the organic phase was conc. and taken up in MeOH for HPLC analysis (Column: Lichrospher 100 RP-18 (5 μm) 250 × 4; Solvent: H_2O : CH_3 COOH: tetrahydrofuran 420:3:1, pH 4.0 with $N(Et)_3$; Flow rate: 1 ml/min; Detection: 260 nm). Calibration was performed with anabasine Rotichrom (Roth, Karlsruhe). For polyamine determination 250 μl extract were benzoylated and analysed on the above column as described in ref. [12].

Feeding experiments with labelled and unlabelled precursors

[^{14}C]-Cadaverine was prepared from L-[U-^{14}C]-lysine [Dupont-NEN] by incubation with a crude lysine decarboxylase extract isolated from *Hafnia alvei* [13]. The reaction was stopped when 95% of the radioactivity was extractable with pentan-1-ol. Labelled cadaverine and lysine were added to the cultures after sterile filtration. The distribution of radioactivity in the extracts was determined either by counting the radioactivity in the HPLC separated peaks or by fractionation. The extract was made alkaline and was first extracted twice with EtOAc (anabasine), then twice with pentan-1-ol (cadaverine). The extract was then

brought to pH 3–4, passed through a Dowex-50(H^+)-column to determine radioactivity in the amino acid fraction (lysine). Total radioactivity minus anabasine, cadaverine and lysine gave a rough indication about the percent label of HCCs. The correlation of the extract radioactivity with incorporation into anabasine, cadaverine and HCCs was confirmed by HPLC.

Acknowledgement—We would like to thank Dr J. Negrel, Dijon, for a gift of feruloylcadaverine. Most of this work was financially supported by the GBF while the group was working at the BBA during a cooperative project of both institutions.

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