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Increased production of cadaverine and anabasine in hairy root cultures of *Nicotiana tabacum* expressing a bacterial lysine decarboxylase gene

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Abstract

Several hairy root cultures of *Nicotiana tabacum* varieties, carrying two direct repeats of a bacterial lysine decarboxylase (*ldc*) gene controlled by the cauliflower mosaic virus (CaMV) 35S promoter expressed LDC activity up to 1 pkat/mg protein. Such activity was, for example, sufficient to increase cadaverine levels of the best line SR3/1-K1,2 from ca. 50 μg (control cultures) to about 700 μg/g dry mass. Some of the overproduced cadaverine of this line was used for the formation of anabasine, as shown by a 3-fold increase of this alkaloid. In transgenic lines with lower LDC activity the changes of cadaverine and anabasine levels were correspondingly lower and sometimes hardly distinguishable from controls. Feeding of lysine to root cultures, even to those with low LDC activity, greatly enhanced cadaverine and anabasine levels, while the amino acid had no or very little effect on controls and LDC-negative lines.

Introduction

We are interested in improving the formation of secondary metabolites by genetic transformation. As targets we have chosen enzymes linking primary and secondary metabolism. At the enzyme level, they are generally regarded as switches controlling the formation of secondary products. However, this conclusion does not mean that these enzymes are the only regulatory sites of secondary pathways or that the activities of these enzymes are indeed rate-limiting for a pathway. The exact knowledge whether a regulatory site is also the rate-limiting step of a biosynthetic sequence is needed if one wants to improve or alter product formation of secondary pathways by genetic transformation. Alterations at the rate-limiting site of a pathway, for example by enhancing the enzyme activity through constitutive expression of a corresponding heterologous gene, seem to be most promising for achieving distinct increases of target secondary products. The usefulness of this approach has recently been demonstrated for serotonin biosynthesis in *Peganum harmala* cell cultures where tryptophan decarboxylase activity is the only rate-limiting step [1]. The overexpression of the gene of a foreign plant tryptophan decarboxylase in *P. harmala* has resulted in 5- to 10-fold increases of serotonin levels in the transgenic cell cultures [3]. An important feature

of this system was that overproduced tryptamine did not accumulate but was immediately metabolized by the next and final step of serotonin biosynthesis.

Indeed we are especially interested in model systems in which the metabolite overproduced by the genetically engineered reaction is efficiently used for increased formation of derived secondary products. Such systems may eventually provide clues and identify factors (e.g. the impact of correct targeting) which have to be considered for altering product levels of economically important and complex pathways. A suitable model system where such studies may later be performed is anabasine biosynthesis (Fig. 1). It has been shown that the pathway from cadaverine to the alkaloid is not saturated [25, 26], as feeding of cadaverine led to high increases of anabasine. Enhancement of lysine decarboxylase (LDC) activity should stimulate cadaverine and thus anabasine formation. A hairy root culture of *Nicotiana glauca* expressing a *ldc* gene originating from the gram-negative bacterium *Hafnia alvei* [6] had indeed distinctly higher levels of cadaverine as well as twice as much anabasine as a control culture [8]. Anabasine is a major alkaloid in *N. glauca*, in contrast to many other tobacco species. As it is optically inactive [14], it has been proposed to be synthesized via a different pathway in *N. glauca* as in other tobacco species (probably via diamine oxidase (DAO); Fig. 1). Therefore we wished to check whether anabasine levels also would be enhanced in low or non-producing tobacco species by the overexpression of the bacterial *ldc* gene and where *N*-methyl putrescine oxidase (MPO) seems to be involved in its formation [27]. In addition, we had faced the problem that the *ldc* gene used for plant transformation was not well translated into an active enzyme when cloned behind the CaMV 35S promoter. In the previous report it was shown that the establishment of LDC-positive root cultures was unefficient when a binary vector was used containing only one expression cassette [8]. Only 2 out of 54 kanamycin-resistant hairy root cultures carrying one copy of the bacterial *ldc* gene had given rise to LDC-positive lines. We found it therefore reasonable to transform tobacco plants with a duplicated *ldc* expression cassette in order to see whether an increase of closely coupled transcription units might have a positive influence on the number of LDC-positive root cultures. Thus, we describe analyses of transgenic hairy root cultures of some varieties of *N. tabacum*, a tobacco species containing low anabasine levels (nicotine/anabasine ratio ca. 50:1), which were transformed with *Agrobacterium* strains carrying a double or a single *ldc* expression cassette under the control of the CaMV 35S promoter.

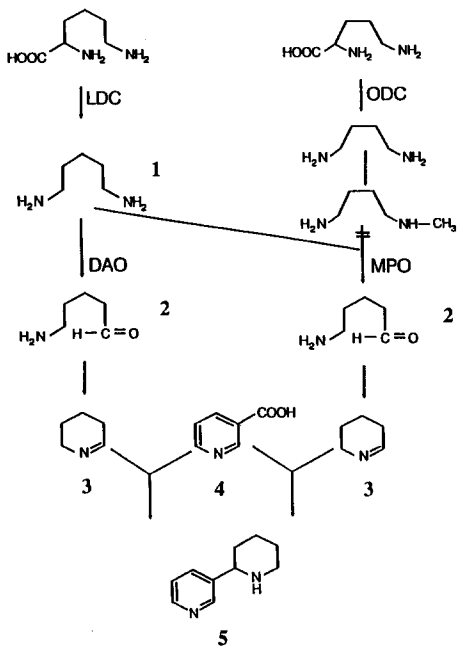

Fig. 1. Proposed biosynthetic pathways of anabasine. DAO, diamine oxidase; MPO, *N*-methylputrescine oxidase; ODC, ornithine decarboxylase; 1, cadaverine; 2, 5-aminopentanal; 3, Δ^1-piperideine; 4, nicotinic acid; 5, anabasine. The conversion of 3 to 4 is a non-enzymic reaction. Overproduced cadaverine evidently competes with *N*-methylputrescine for MPO.

Materials and methods

Biological materials

All plasmid constructions were propagated in *Escherichia coli* K12 strain DH5. Binary plant

transformation vectors were mobilized into *A. tumefaciens* strain C58C1 pRiA4b (12). Leaf disks of sterile grown plantlets of *N. tabacum* cv. Samsum (SS), cv. Petit Havana (SR1) and cv. Pirat (Pi) were then transformed with these *Agrobacterium* cultures as described [8, 10]. Binary vectors were isolated from the cultures used for leaf disc inoculations and analysed by restriction endonuclease cleavages. Root cultures, tolerant to 100 mg/l kanamycin, were cultivated in phytohormone-free B5 medium with 4% sucrose and 2.2 mM NaH_2PO_4 on a gyratory shaker [8].

Isolation and analyses of DNA and RNA

Plant DNA for Southern tests was isolated as previously described [2]. For preparation of total RNA for northern blot hybridizations 1–2 g of fresh root tissue, which had been previously blotted dry, were homogenized in a solution containing 4 M guanidinium thiocyanate, 0.5% (w/v) sodium lauroyl sarcosine, 25 mM sodium citrate (pH 7), 0.33% antifoam A (Sigma) and 100 mM 2-mercaptoethanol. Crude cell debris was removed by centrifugation at 2500 × *g* at 20 °C for 10 min. The supernatant was squeezed 10 times through a 20-gauge needle to sheer chromosomal DNA. The RNA was purified by ultracentrifugation [5]. Electrophoresis of total RNA (2.5–10 μg) after denaturation with glyoxal and transfer of the RNA to Pall nylon filters were performed as described previously [7].

For hybridizations the *Hin*d III fragment with the *ldc* expression cassette from pLX154.3 was labelled to a specific activity of 10^8–10^9 cpm/μg DNA with α-[^{32}P]-dCTP as described previously [2]. After hybridizations the filters were washed with 0.1% SSC, 0.2% SDS at 56 °C and exposed to Kodak XAR films at −70 °C.

Biochemical analyses

Neomycin phosphotransferase (NPTII) protein was extracted and quantified by an ELISA test as recommended by the supplier (5Prime-3Prime, West Chester, USA; ABCR Co., Karlsruhe, Germany). Lysine decarboxylase (LDC) activity was determined by measuring the amount of ^{14}C-labelled cadaverine extracted into pentan-l-ol [10] except that the specific activity of labelled lysine was enhanced 4-fold [8].

Cadaverine and anabasine were extracted from 25–50 mg dry mass with MeOH/$CHCl_3$/H_2O 12:5:3 [10] or 2.5 ml 0.1 μ H_2SO_4. The acid phase was extracted with ethyl acetate to remove lipids. Cadaverine levels were determined as benzoyl derivatives as described by Redmond and Tseng [20]. For alkaloid measurements, the acidic extract was made alkaline with NH_4OH and extracted with ethyl acetate. The ethyl acetate was evaporated and the residue taken up in 50 μl MeOH. Alkaloids were identified and quantified by GC [8] or by HPLC. Conditions: Licrosorb 100-RP18 column (5 μm, 125 mm × 4 mm); solvent: H_2O/AcOH/tetrahydrofuran 430:3:1, brought to pH 4 with triethylamine, flow rate 0.85 ml/min; nornicotine 3.3 min, nicotine 4.3 min, anabasine 6.0.

Results

Vector construction

The bacterial *ldc* coding region was available as *Bam* HI-*Bgl* II fragment 154 [10]. This fragment consisting of the *ldc* open reading frame of 2220 bp, a short stretch of 11 bp upstream of the start codon and a non-coding 630 bp region downstream of the stop codon was cloned into *Bam* HI site of pUC19. The resulting plasmid was linearized with *Bam* HI, the protruding ends were filled up with Klenow enzyme and the reaction product was cleaved with *Xba* I. The isolated fragment with the *ldc* gene and some adjacent bacterial DNA was ligated into pRT103 [23], which had previously been linearized with *Xho* I, filled up with Klenow enzyme and then cleaved with *Xba* I. In the resulting plasmid pRT103.154 the *ldc* gene was directly fused to the 35S promoter of pRT103. The *ldc* expression cassette from pRT103.154 was isolated as a *Hin*d III frag-

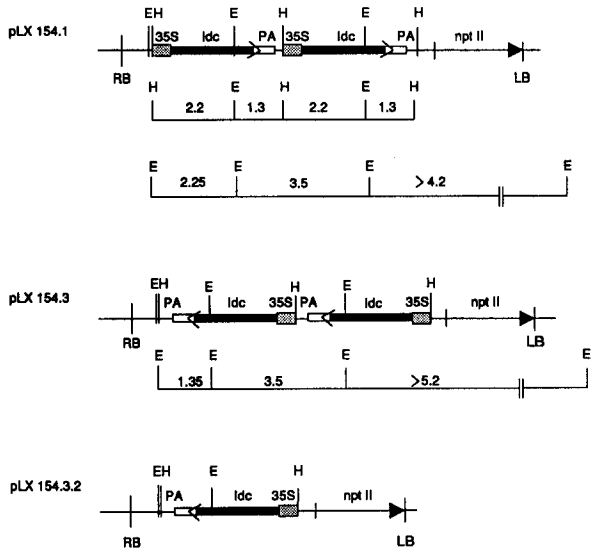

Fig. 2. Map between the right (RB) and left (LB) border of the plant transformation vectors pLX154.1 and pLX154.3 carrying the duplicated 35S-*ldc* gene and of pLX154.3.2. containing only one *ldc* expression cassette. The sizes of *Eco* RI and *Eco* RI/*Hin*d III fragments are indicated. 35S, 35S promoter of CaMV; *ldc*, bacterial lysine decarboxylase open reading frame (filled box) and adjacent 3′-non-coding region (open box); PA, CaMV-3′-polyadenylation signal of CaMV-strain Cabb B-D; *nptII*, neomycinphosphotransferase II in plant expression cassette of pLX222; H, *Hin*d III and E, *Eco* RI restriction sites.

ment and cloned into the *Hin*d III site of the binary vector pLX222 [13] to yield pLX154.1 and pLX154.3. The interesting features of these vectors were that each contained two 35S-*ldc* expression cassettes as direct repeats, transcribed either in the same (pLX154.1) or in the opposite direction (pLX154.3) as the *nptII* marker gene (Fig. 2). The plasmids were finally mobilized from *E. coli* DH5 into *A. tumefaciens* C58CIRiA4b [12]. Several transferred binary vectors were isolated from the selected *A. tumefaciens* pLX154.1 or pLX154.3 clones and analysed by restriction cleavages. One clone contained a recombination derivative of pLX154.3 with only one *ldc* expression cassette (pLX154.3.2). The *E. coli* and *A. tumefaciens* strains carrying a double *ldc* expression cassette showed a 20- to 40-fold increase of LDC activity which was in the same range as shown for bacterial strains carrying only one *ldc* gene [8].

Screening of hairy root cultures for LDC activity

Of 180 roots formed at infected leaf discs in the absence of kanamycin, 35 proved to be kanamycin-resistant. Twenty-four of the resistant lines had been transformed with the double *ldc* cassettes, 11 with pLX154.3.2. All of them expressed the *npt* gene and contained NPTII protein levels from 5 up to 250 ng/mg protein. Six of the resistant root cultures, containing quite different amounts of NPTII protein, had also low, but

Table 1. Neomycin phosphotransferase (NPTII) levels of some *lds*-transgenic, kanamycin-resistant hairy root cultures of *Nicotiana tabacum* with or without measurable LDC activity. The cultures had been grown for 2–3 growth cycles in liquid medium without the selective antibiotic. SS, cv. Samsun; SR, cv. Petit Havana SR1; Pi, cv. Pirat.

Subspecies/line	Binary vector used	NPTII protein (ng/mg protein)	LDC (pkat/mg)
SR 3/1-K1,2	pLX154.3	73.8	1.0
SR 3/1-K2	pLX154.3	35.0	n.d.
SS 1/1-K3	pLX154.1	7.9	0.4
SS 1/1-K6	pLX154.1	5.1	0.5
SS 3/2-G2	pLX154.3.2	83.7	n.d.
Pi 3/2-G4	pLX154.3.2	250.0	n.d.
Pi 3/1-k1	pLX154.3	111.2	0.4
Pi	untransformed	0	n.d.
SSgus	pLXGus	36	n.d.

n.d. = not detectable.

nevertheless distinctly enhanced LDC activity levels as compared to controls and to other lines transformed with the *ldc* gene constructs. The examples listed in Table 1 show that there was no correlation between NPTII activity and LDC activity. To verify the low activities in the range of 0.4–1 pkat/mg protein, the cadaverine formation was followed for up to 5 h using sometimes also two different protein concentrations. Interestingly the LDC-positive root cultures listed in Table 1 as well as two other positive lines had been transformed with bacteria carrying the double *ldc* cassettes, while all 11 lines transformed with pLX154.3.2 (Fig. 2) did not contain measurable LDC activity.

Molecular characterization of transgenic root cultures

The presence of the *ldc* expression cassettes in the transformed root cultures was proven by Southern blot hybridization of *Eco* RI/*Hin*d III digests. The expected two bands of 1.3 and 2.2 kb from the *ldc* expression cassette (Fig. 2) were detected in 16 from a total of 17 root lines analysed. Examples for the transformation of all three varieties of *N. tabacum* are given in Fig. 3. The signal of the 1.3 kb band was always distinctly lower than that of the 2.2 kb band. In some cases a DNA fragment of 3.5 kb was detected, probably due to partial digestion. No signals were obtained from the control cultures (6 and 9). The hybridization pattern confirmed that the *ldc* gene was integrated into the genome of all 6 LDC-positive lines (lanes 2, 7, 10–13) as well as into that of LDC-negative lines (lanes 3, 4, 8).

The hybridization pattern of Fig. 3 did not allow to recognize whether the double *ldc* cassette had completely been transferred to the plant genome. Line Pi3/1-k1 (lane 7, transformed with a double cassette) and line Pi3/2-G4 (lane 8, transformed with a single cassette) showed the same hybridization pattern. To analyse the structure of the DNA integrated into the plant genome more precisely, the genomic DNA was cleaved with *Eco* RI. In the lines transformed with the double *ldc* expression cassette pLX154.1 (Fig. 4, lanes 3, 9–11) two bands of 2.25 and 3.5 kb were seen. The detection of a 3.5 kb *Eco* RI fragment indi-

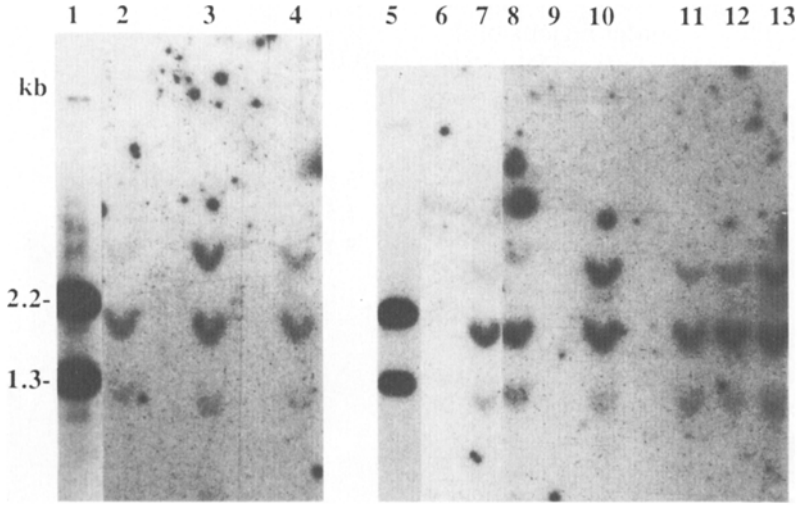

Fig. 3. Southern blot hybridization of some *ldc*-transgenic, kanamycin-resistant root cultures transformed with pLX154.1 or pLX154.3 and containing or lacking measurable LDC activity. The genomic DNA (7.5 μg) was cut with *Eco* RI and *Hin*d III, so that fragments of 1.3 and 2.2 kb (Fig. 1) were expected to be detected by the *ldc* probe. Lanes: 1, pLX154.3 (0.02 μg); 2, SR3/1-K1,2 (+); 3, SR3/1-K2 (−); 4, SR3/1-K3 (−); 5, pLX154.1; 6, Pi-untransformed roots; 7, Pi3/1-k1 (+); 8, Pi-3/2-G4 (−); 9, SSgus; 10, SS1/1-K3 (+); 11, SS1/1-K4 (+); 12, SS1/1-K6 (+); 13, SS1/1-K5 (+). (+) = LDC-positive, (−) = LDC-negative line. Exposure times after hybridizations to plasmid DNA were 3–5 h and to plant DNA 2 weeks.

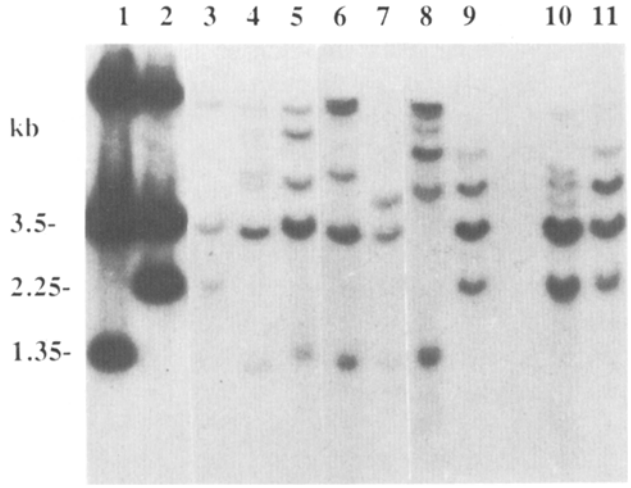

Fig. 4. Southern analyses of *Eco* RI fragments of some *ldc*-transgenic transformants. A fragment of 3.5 kb proved the intact integration of the double 35S-*ldc* gene constructs (Fig. 1). 8 μg of plant DNA and 3 ng of control plasmid DNA were digested with *Eco* RI. Exposure times see Fig. 2. 1, pLX154.3; 2, pLX154.1; 3, SR1/1-G5 (−); 4, SR3/1-K2 (−); 5, SR3/1-K1,2 (+); 6, Pi3/1-k1 (+); 7, Pi3/1-K4 (−); 8, SS3/2-G2 (−); 9, SS1/1-K6 (+); 10, SS1/1-K5 (+); 11, SS1/1-K4 (+).

cated the presence of the intact -ldc double cassette in the plant genome (see Fig. 2). Fragments larger than 3.5 kb contained 1.3 kb of one *ldc* expression cassette, the left border regions of the integrated DNA and variable of plant DNA. The conservation of the duplicated *ldc* gene was also seen in roots transformed with pLX154.3 (Fig. 4, lanes 4–7) with expected fragments of 3.5 kb and 1.35 kb. Line SS3/2-G2 (lane 8) was transformed with one *ldc* expression cassette and did thus show only a 1.35 kb band, but no signal of 3.5 kb. The detection of a number of larger border fragments in this *Eco* RI digest of the plant DNA may indicate that in most lines 2 or 3 of the duplicated expression cassettes have been integrated into the plant genome. Detailed analyses of copy numbers were not performed, as the low levels of LDC activity would not allow to establish a correlation between gene dosis and enzyme activity.

In summary, Southern analyses of all tested root lines, which were transformed with the *ldc* double cassette, showed that in all instances both *ldc* cassettes had been integrated into the plant genome and that rearrangements leading to an altered hybridization pattern between the homologous cassettes had not occurred.

As Southern hybridization showed that the bacterial *ldc* gene was not only present in LDC-positive, but also in LDC-negative transgenic root cultures, *ldc* mRNA levels of 14 lines were compared. In 13 lines having shown the correct hybridization pattern in Southern tests, *ldc* mRNA of 3.1 kb was detected, consisting of a short 5′-untranslated region, the *ldc* open reading frame and the long 3′-untranslated region. The lanes 5, 6 and 7 (Fig. 5) representing LDC-positive lines showed a stronger hybridization signal than the LDC-negative lines (lanes 1–4). Thus, the lines with LDC activity gave rise to the highest *ldc* mRNA signals. Of the LDC-negative lines, only lines SR3/1-K2 (lane 4) showed a signal in the northern test that might suggest the detection of at least some LDC activity. In all other cases (lanes 1 and 2) and 6 other LDC-negative lines (not shown) it was understandable that the low and often hardly visible mRNA levels were not sufficient for the synthesis of measurable amounts

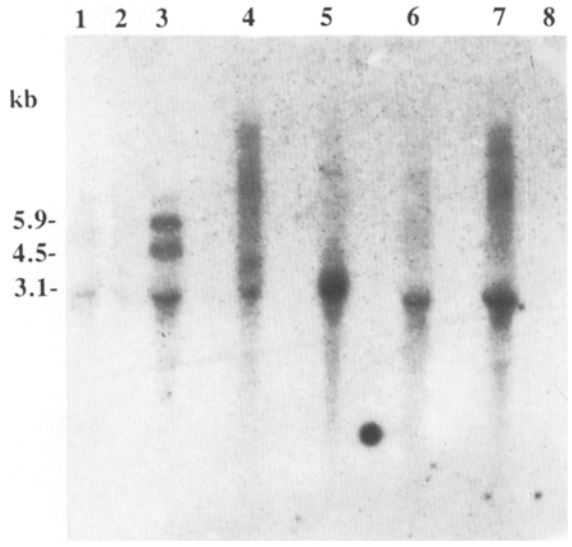

Fig. 5. Northern hybridization of some transgenic root cultures, carrying the bacterial *ldc* gene and expressing or lacking LDC activity. Total RNA/slot 10 μg; exposure time 16 days. 1, SR1/1-K3 (−); 2, SR1/1-G6 (−); 3, SR3/2-G3/1 (−); 4, SR3/1-K2 (−); 5, SR3/1-K1,2 (+); 6, SS1/1-K3 (+); 7, Pi3/1-k1 (+); 8, SSgus.

of LDC. The lack of LDC-activity of SR3/2-G3/1 (lane 3) and the occurrence of additional larger bands in the northern test is likely due to rearrangements, as also the Southern hybridization of this line (not shown) showed an unusual banding pattern. No *ldc* transcript could be detected in the control line SSgus (lane 8).

Biochemical analyses of the transgenic root cultures

All LDC-positive root cultures as well as some negative lines of the screening were repeatedly checked for LDC activity, cadaverine and anabasine levels. The line SR3/1-K1,2 (Table 1) always contained the highest LDC activity of all root cultures carrying the *ldc* double cassettes. The LDC activity and the accumulation pattern of cadaverine of this line was followed over a growth period of 3 weeks (Fig. 6). A similar pattern with LDC activities of up to 0.6 pkat/mg protein and cadaverine levels of about 0.5 mg/g dry mass was found for the transgenic line SS1/1-K5 (not shown). The control culture SSgus or SRgus hardly contained any measurable LDC activity (10–20-fold lower than the positive *ldc*-transgenic lines) and cadaverine levels were mostly below 50 μg/g.

In all cases the LDC-positive lines contained higher cadaverine levels than controls and lines

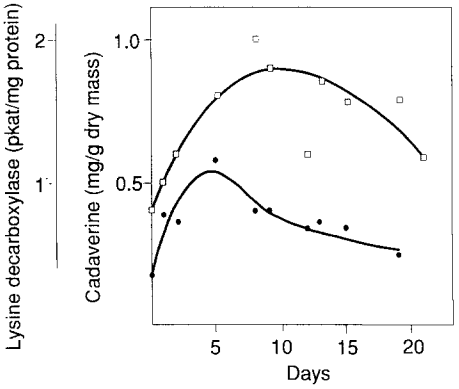

Fig. 6. Pattern of LDC activity (□) and cadaverine qqq(●) accumulation of the line *Nicotiana tabacum* SR3/1-K1,2 during a culture period of 3 weeks. Initial inoculum: 0.8 g (80 mg dry mass); final biomass: 6.1 g (604 mg dry mass).

without detectable LDC activity. Sometimes the differences between positive and negative lines were not that large to suggest an increased cadaverine level (see, for example, Pi3/2-G4 and Pi3/1-k1 in Table 2). During our screenings, it was noted that product levels of one line may vary. Whether such variations were based on different physiological states of the roots or introduced by the 'experimental handling' was not clear. Nevertheless, the root lines overproducing cadaverine were easily recognized as they contained, in contrast to the negative lines, more cadaverine than putrescine (Table 2). In all lines with good LDC activity the putrescine/cadaverine ratio fell from around 2 or more to 0.5 (Table 2).

Though the LDC-positive lines contained usually 3- to 8-fold higher levels of cadaverine than the controls, this was not a guarantee that specific levels of 300 to 800 μg cadaverine per g dry mass would be sufficient to affect anabasine levels. Indeed, except for the best line SR3/1-K1,2 where a 2- to 3-fold increase of the anabasine level was seen as compared to the other SR lines, anabasine levels were not clearly enhanced in the LDC-positive lines. The low overproduction of cadaverine and anabasine could not only be due to the low LDC activity but also to poor substrate supply. That the latter was a major cause of the low alterations in product levels was seen when lysine and cadaverine were fed to the root cultures (Fig. 7). The line SS3/2-G5 stands for negative and control lines. Due to the lack of LDC activity, lysine feeding had no effect on anabasine levels, though the line used exogenous cadaverine efficiently for anabasine formation. The low, but clearly measurable, LDC activity of line SS1/1-K6 (Table 1) was confirmed by a dramatic increase of the cadaverine pool after lysine feeding (Fig. 7). The line Pi3/1-k1 indicated that the subspecies Pirat might channel cadaverine most efficiently into anabasine biosynthesis. Again SR3/1-K1,2 was the best line. Lysine feeding enhanced the already increased anabasine content not only from 1.7 mg to 3.4 mg/g dry mass. The comparison of feeding of lysine or cadaverine indicated also that the pathway seemed to be

Table 2. Cadaverine and putrescine levels of some *ldc*-transgenic hairy root cultures of *N. tabacum* with and without measurable LDC activity. The roots were harvested after 3 weeks of cultivation. Line SSgus represents a *ldc*-minus rot culture.

Line	LDC activity	Putrescine (mg/g dry mass)	Cadaverine (μg/g dry mass)	Putrescine/ cadaverine ratio
Pi3/2-G4	n.d.	383	111	3.4
Pi3/1-kl	+ + +	111	260	0.4
Pi3/1-K4	n.d.	165	96	1.7
SSgus	n.d.	276	74	3.7
SS3/2-G5	n.d.	263	157	1.7
SS1/1-K5	+ + +	321	560	0.6
SS1/1-K6	+ +	459	459	1.0
SR3/1-K1,2	+ + +	240	643	0.4
SR3/1-K2	n.d.	280	157	1.8

n.d. = not detectable.

nearly saturated, as both resulted in very similar anabasine levels. The data of Fig. 7 clearly demonstrate that not only by feeding of cadaverine [25, 26], but also by an engineered overproduction of cadaverine, anabasine levels can be improved and that the nicotine/anabasine ratio, and thus the alkaloid pattern, was greatly affected by the genetic alteration.

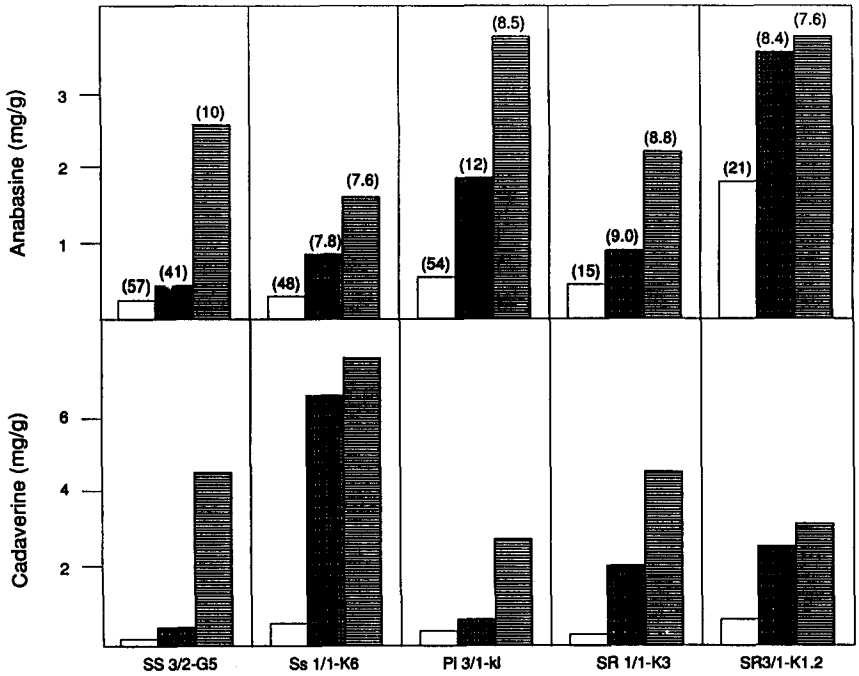

Fig. 7. Effect of feeding lysine (second column) or cadaverine (third column) on cadaverine and anabasine levels of hairy root cultures. The first column gives cadaverine and anabasine levels of the unfed lines. The line SS3/2-G5 had no LDC activity, SR1/1-K3 contained weak LDC-activity, SS1/1-K6, Pi3/1-k1 and SR3/1-K1,2 contained 0.3–0.6 pkat/mg protein, when the experiment was started. One gram of a 3-week-old root culture was transferred into 10 ml fresh medium for 5 days. Then 1 mM lysine or 1 mM cadaverine or water (1 ml) was added for 4 days. The number on the top of each column gives the nicotine/anabasine ratio. Note: in the presence of the high levels of lysine the nicotine content was sometimes greatly reduced without corresponding increases of anabasine. Alkaloid levels in the medium never exceeded 20% of the alkaloid stored in the cells.

Discussion

The biosynthesis of anabasine has not yet been fully clarified. The situation is additionally complicated by conflicting observations [25, 27]. On the one hand, cadaverine fed to hairy root cultures of tobacco was incorporated in anabasine without interfering with the incorporation of labelled lysine [25]. On the other hand, it was shown that there is no evidence that anabasine produced from cadaverine supplied arises from a mechanism different from that operating in intact plants [27]. Thus, it was not clear whether feeding of cadaverine and overproduction of cadaverine would both lead to increased formation of anabasine. In addition, if cadaverine synthesized and cadaverine supplied could be formed by different pathways, it was not clear whether low LDC activity is the rate-limiting step and main reason why many tobacco species contain little or no anabasine. The overproduction of cadaverine in a *ldc*-transgenic line of *N. glauca* [8] could not give a clear answer to this, as it has been proposed that anabasine is formed in this species via a different pathway than in other tobacco species [14]. The results obtained with the root cultures of *N. tabacum* provide now evidence that LDC activity is indeed the rate-limiting step of anabasine biosynthesis. Overproduction of cadaverine by the expression of the bacterial gene in *N. tabacum* led not only to increased anabasine production but also to reduced accumulation of nicotine. The finding of Watson *et al.* [27] that cadaverine may compete with *N*-methylputrescine for *N*-methylputrescine oxidase (Fig. 1) could explain the change of the alkaloid levels. However, competition of the cadaverine derived precursor with the putrescine derived precursor for nicotinic acid (Fig. 1) would also lead to reduced nicotine formation.

In previous transformation experiments using vectors in which the *ldc* gene was present as single expression cassette and controlled by either the bidirectional Tr promoter of *A. tumefaciens* [10] or the CaMV 35S promoter [8], we had experienced that none [10] or extremely few [8] of the transformed lines contained measurable LDC activity. Thus, it was justified to test a vector containing a double *ldc* expression cassette. The fact that 25% of the lines transformed with the double cassettes contained measurable LDC activity, while all lines transformed with the vector containing only one expression cassette were negative, is a good approval of our approach. These direct repeats were preserved when integrated into the plant genome. We assume that the presence of these duplicated genes had a positive effect on the *ldc* mRNA formation which resulted in the necessary amount of transcripts for obtaining measurable LDC activity. As we had also selected several root cultures with duplicated *ldc* expression cassettes but lacking LDC activity, the positive effect of the duplicated *ldc* gene was also dependent on its position in the plant genome.

In contrast to rearrangements of the duplicated expression cassette observed in *A. tumefaciens* pLX154.3 (for example, the deletion of one cassette), no rearrangements were detected in the transgenic root lines. This result is in good agreement with the observation of low somatic recombination frequencies (10^{-5}) for short, closely linked homologous repeats, as determined in recombination studies with transformed *N. tabacum* [19, 24]. However, we cannot exclude that in small subpopulations of root cells intrachromosomal recombinations between the direct *ldc* repeats may have resulted in loss of one expression cassette or in other limited DNA rearrangements which would not be detected by our Southern analyses.

We have no evidence that a poor transcription is the main reason for the low level of the LDC. When the steady-state levels of mRNAs of the co-transferred *nptII* and *ldc* gene of some kanamycin-tolerant, LDC-positive root cultures (not shown) were compared, we did not see significant differences in the intensity of the northern signals. While similar levels of mRNA resulted in one case in the accumulation of substantial amounts of NPTII (Table 1), LDC activity was very low. As LDC activities of 100 pkat/mg protein in transgenic tobacco leaves [10] allowed immunological detection of the LDC protein in western blots only after a two-step en-

richment by FPLC [22], we assume that the reason for the low LDC levels must mainly be due to poor translation of the mRNA of the bacterial *ldc*. Nevertheless, it seems useful to further improve transcription of the bacterial gene, as *ldc* mRNA levels and LDC activity clearly showed a positive correlation. If one compares the LDC activities of leaves of the best transgenic tobacco plants (140 pkat/mg protein) in which the gene had been fused to the *rbcS* transit peptide and the *rbcS* promoter [10] with the data obtained with the 35S promoter, one could find at least 3 explanations for the different outcome. Firstly the *rbcS* promoter may provide more transcript; secondly fusion of the *ldc* gene to the transit peptide may improve the translational efficiency; and thirdly without a targeting signal the LDC might be more rapidly degraded. Weak transcription and translation of bacterial genes in transgenic plants is not uncommon [15] and was in some cases overcome by changing amino acid codons to those commonly used by plants [17, 18]. In the case of the *ldc* gene the coding region contains in addition some sequences which mimic putative plant signal sequences, for example two signals for polyadenylation. Thus, for highest expression of the bacterial *ldc* gene under the control of a root-active promoter such as the 35S promoter, adaption of the sequence to the codon usage of plants might be necessary. Whether fusion of translational enhancing elements, such as the TMV Ω or the AMV leader [9, 11], to the *ldc* gene can improve its translation efficiency has to be tested, as the effects of such leaders may differ [4].

However, it is questionable whether improvement of the translational efficiency alone would be sufficient to affect cadaverine and anabasine formation to the desired extent. The large effect of feeding lysine on cadaverine levels indicates that even the low LDC activities were not saturated with substrate. The high accumulation of cadaverine in the transgenic leaves of tobacco was also probably due to the fact that the protein was targeted to the chloroplast, the compartment of lysine biosynthesis [10]. The importance of targeting the engineered enzyme to the correct cellular compartment has also been shown for lysine biosynthesis. No lysine overproduction was obtained when the gene of a bacterial dihydrodipicolinate synthase was expressed in the cytoplasm, while the protein imported into chloroplasts resulted in large increases of lysine biosynthesis [16, 21]. These studies also provided good evidence that the biosynthesis of lysine in root cells also operates in the plastids of non-green tissues [16]. Vector constructs allowing the transport of the LDC in this compartment may thus be important for achieving the best substrate supply.

In conclusion, overproduction of cadaverine in transgenic root cultures can enhance anabasine levels and thus alter the alkaloid pattern of tobacco species. It is suggested that even larger effects, as seen with the double 35S-*ldc* expression cassette, will be obtained when the translational efficiency of the *ldc* mRNA is improved and the LDC protein is targeted to the compartment of lysine biosynthesis.

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