Plant Molecular Biology **17**: 475–486, 1991.

Expression of a bacterial lysine decarboxylase gene and transport of the protein into chloroplasts of transgenic tobacco

S. Herminghaus [1], P.H. Schreier [2], J.E.G. McCarthy [3], J. Landsmann [1], J. Botterman [4] and J. Berlin [1,3]*

[1] *BBA – Institut für Biochemie, Biologische Bundesanstalt für Land- und Forstwirtschaft, Messeweg 11/12, D-3300 Braunschweig, Germany (*author for correspondence);* [2] *Institut für Biotechnologie PF-E, Bayer-AG, D-5090 Leverkusen, Germany;* [3] *GBF – Gesellschaft für Biotechnologische Forschung m.b.H., D-3300 Braunschweig, Germany;* [4] *Plant Genetic Systems NV, Gent, Belgium*

Received 28 February 1991; accepted in revised form 24 May 1991

Key words: Nicotiana tabacum, plant transformation, gene expression, bacterial lysine decarboxylase, protein transport, chloroplasts, cadaverine

Abstract

A possible approach for altering alkaloid biosynthesis in plants is the expression of genes encoding key enzymes of a pathway such as lysine decarboxylase (*ldc*) in transgenic plants. Two strategies were followed here: one focused on expression of the gene in the cytoplasm, the other on subsequent targeting of the protein to the chloroplasts. The *ldc*gene from *Hafnia alvei* was therefore (a) placed under the control of the 1′ promoter of the bidirectional Tr promoter from *Agrobacterium tumefaciens* Ti- plasmid, and (b) cloned behind the *rbcS* promoter from potato fused to the coding region of the *rbcS* transit peptide. Both *ldc* constructs, introduced into *Nicotiana tabacum* with the aid of *A. tumefaciens*, were integrated into the plant genome and transcribed as shown by Southern and northern hybridization. However, LDC activity was only detectable in plants expressing mRNA under the control of the *rbcS* promoter directing the LDC fusion protein into chloroplasts with the aid of the transit peptide domain. In plants expressing the processed bacterial enzyme cadaverine levels increased from nearly zero to 0.3–1% of dry mass.

Introduction

One approach towards optimizing plant secondary metabolite pathways is to introduce genes encoding enzyme activities that are rate-controlling or could have a regulatory function in the biosynthesis of a pathway. Given our presently limited knowledge of these pathways, an attractive strategy is to examine short and simple, rather than long and branched, pathways. We accordingly became interested in the quinolizidine alkaloid pathway of Fabaceae, in which the tetracyclic structure is probably formed via two enzymatic steps [43]. Lysine is decarboxylated to cadaverine which is then incorporated in a not fully understood way into the alkaloids [14]. In general, lysine decarboxylase (LDC) activity is rather low in higher plants. Indeed, there has been no report of the purification and characterization of any plant LDC. LDC activity could thus be regarded as one limiting factor in the synthesis of cadaverine-derived secondary metabolites. The

integration and expression of a bacterial *ldc* gene in higher plants represents one possible way of increasing their cadaverine pools.

A bacterial *ldc* gene of *Hafnia alvei* has recently been cloned [9]. In the present communication we describe the construction of two different *ldc* gene constructs one of which should lead to expression in the cytoplasm, and the other should facilitate transport of the LDC protein into chloroplasts, its natural compartment in *Lupinus* [14, 44]. Despite the fact that transformation of *Lupinus* has previously been achieved using wild-type strains of *Agrobacterium tumefaciens* and *A. rhizogenes* [3], *Agrobacterium* strains carrying the *ldc* gene were first used to transform *Nicotiana tabacum*.

Materials and methods

Biological materials

Sterile-grown plantlets of *N. tabacum* cv. Petit havana SR1 and cv. Samsum were used in transformation experiments. The *Escherichia coli* strains used are commonly applied [22] or have been described in the corresponding references. *E. coli* strains were transformed according to Hanahan [12], and transfers of chimaric gene constructs were performed by donation from *E. coli* to *A. tumefaciens* [8] using the helper strains *E. coli* GJ23 or RK2013. *A. tumefaciens* C58CIRif pGV3850 [46] and LBA4404 [15] were used.

Biochemicals, kits and vectors

Chemicals, labelled compounds and enzymes were purchased from well known suppliers. The following kits were used: random-primed DNA labelling kit (Boehringer); Sequenase version 2.0 kit (USB); rabbit reticulocyte lysate N.90Y (Amersham); *in vitro* transcription kit, Riboprobe Gemini II core system P1270 (Promega). The primary vectors used for cloning of the *ldc* gene and the new constructs are listed in Table 1.

Cloning, isolation and sequencing of bacterial DNA

Unless mentioned otherwise, standard procedures were used as described by Maniatis *et al.* [22] or Sambrook *et al.* [33], or as recommended in the protocols of the commercial suppliers. Sequence analyses were performed using M13mp18.

Plant transformation

Leaf protoplasts of sterile-grown plantlets (MSO medium [25]) of *N. tabacum* cv. Samsum and cv. SR1 were essentially isolated as described by Medgyesy *et al.* [24] using for Samsum 0.25% Driselase, and for SR1 1% Cellulase R10 Onozuka, 0.25% Macerozyme R10 in K3 medium with 0.4 M sucrose. Transformation by co-cultivation [11, 23] and imbedding of the protoplasts in 0.8% sea-plaque-LMT-agarose [36]

Table 1. Plasmid-constructs bearing the *ldc* gene.

Primary plasmid vector	Ref.	Name of *ldc* construct	Procaryotic antibiotic resistances of *ldc* plasmids
pBR322	[4]	pLD1-101 (9)	amp
		pLD2-19 (9)	amp
		pLD2-101	amp
		p322.154	amp
pJLA505	[34]	p5.154	amp
pAP2036	[39]	p6.154	spec/strep/carb
pGV3850	[46]	p50.154	rif/spec/strep/carb
pSP72	[18]	p72.154	amp
pLS1	(PGS, Ghent)	p1.154	amp
pLX222	[21]	p22.154	amp/tet

were performed as described. Microcalli growing under selective conditions (100–1000 mg kanamycin per litre K3 medium with 500 mg Claforan) were stepwise adapted to growth on MSO agar with 1 mg/l NAA and 0.2 mg/l kinetin.

Transformation by the leaf disc method was performed according to Horsch *et al.* [16]. The shoots obtained on MS9 medium (0.5 mg/l IAA, 1 mg/l BAP) were rooted on MSO medium in the presence of 100–300 mg/l kanamycin. All analyses of transgenic plants were performed with first-generation plants.

Nucleic acid isolation from plant material

Isolation of DNA was performed according to Dellaporta *et al.* [6], but additionally purified by RNAse and proteinase-K digestion. RNA isolation has been described by Verwoerd *et al.* [40].

Hybridization

Southern hybridizations were performed as described by Maniatis *et al.* [22]. In the case of bacterial DNA the probe was labelled by nick translation [32]. Hybridization conditions were: 4 × SSC, 5 × Denhardt's solution, 0.1% SDS, 100 μg/ml herring sperm DNA at 65 °C overnight. The filters were washed with 2 × SSC and 0.1 × SSC, 0.1% SDS (2–4 times).

Plant DNA was applied at 20 μg/slot. Probes were labelled by the random-primed DNA method (Boehringer). Hybridization conditions were: 3 × SSC, 10 × Denhardt's solution, 50 mM phosphate buffer, 50% formamide, 100 μg/ml herring sperm DNA; preincubation at 42 °C for 2–4 h, hybridization for 40–48 h. Washings were done as described above at 42 °C.

RNA was separated on 1% agarose gels in formaldehyde running buffer (MEN) pH 7.0 [33]. For denaturation, RNA (20 μg/20 μl) was incubated with 5 μl 10 × MEN buffer, 6 μl formaldehyde and 20 μl formamide for 15 min at 60 °C. The blotting and hybridization conditions were as for Southern blotting of plant DNA.

In vitro *transcription and translation*

The DNA was cloned in vector pSP72 [18], linearized and transcribed with 5′-capping of RNA according to the protocol of the transcription kit. The RNA (1 μg/μl) was kept at 67 °C for 10 min and used in the rabbit reticulocyte translation system with ^{35}S-methionine as recommended. 1 μl was fixed on a filter paper by putting it in 10% TCA at 65 °C and allowing it to incubate for 5 min (2 ×). The filter was washed several times with EtOH before determination of the radioactivity. The translation products were analysed after SDS-PAA electrophoresis by fluorography.

Immunodetection

Transfer of protein in western blots from the PAA gel to nitrocellulose was performed according to the semi-dry method of Kyhse-Andersen [19]. The membrane was washed with 0.85% Na Cl (3 ×) for 5 min and then saturated overnight with non-fat dry milk powder buffer (0.01 M Tris, 0.85% NaCl, 2% Triton X-100, 5% milk powder) [17]. The membrane was then incubated for 2 h in the same buffer with the LDC-specific antibodies from rabbit [2], washed with 0.85% NaCl (7 ×), and again incubated in the buffer for 30 min before being incubated with an anti-rabbit IgG alkaline phosphatase conjugate for 2 h. After washing with 0.85% NaCl (2 ×) the nitrotetrazolium blue reaction was performed as described by Sambrook *et al.* [33].

Enzyme preparations and tests

For determination of NPTII activity the radioactive assay [30] was used as modified by Schreier *et al.* [35]. For measurement of LDC activity, 4 g plant cells were homogenized in 4 ml extraction buffer (0.1 M Tris-HCl pH 6.0, 0.1% MSH, 10 μM PLP) with quartz sand and buffered Polyclar AT. After centrifugation, 80% $(NH_4)_2SO_4$ precipitation and desalting on a PD10 column (Pharmacia), the extract was used in a radioactive

assay (2×10^5 cpm lysine per test) based on the photometric assay described for ornithine decarboxylase by Ngo *et al.* [26]. Labelled cadaverine was extracted with 2 ml pentanol using repeated vortexing. Protein was determined by a modified Bradford assay [28].

Isolation of chloroplasts

Intact chloroplasts were isolated in a Percoll gradient as described by Cline *et al.* [5] using an Ultraturrax (1 s, medium speed). The chloroplast pellet was finally suspended in 50 mM Tris-acetate buffer pH 6.0 and used for the LDC measurements. Chlorophyll was determined according to Arnon [1].

Determination of cadaverine

100 mg freeze-dried plant material was extracted twice with 5 ml MeOH $CHCl_3$ H_2O 12:5:3 at 70 °C. For removing lipids, $CHCl_3$ and H_2O were added to the combined extracts. The $CHCl_3$ phase was discarded or analysed for alkaloids. The MeOH H_2O phase was evaporated and taken up in 1 ml H_2O. An aliquot was benzoylated, extracted with ether and chromatographed on a Licrosorb RP18 column as described by Redmond and Tseng [29]. The identity of cadaverine isolated from the transformed plants was established by means of GC/MS analysis of the benzoylated compound.

Results

Construction of A. tumefaciens *strain 50.154 carrying a bacterial* ldc *gene under the control of the Tr promotor*

Cloning of *ldc* from *H. alvei* in pBR322 has provided previously two full-length clones: pLD1-101 and pLD2-19 [9]. After cleavage of both plasmids with *Hind* III (restriction site in pBR322) and *Eco* RI (site in *ldc*) and religation, a new clone pLD2-101 (Fecker, unpublished) was obtained carrying as unique restriction sites the HpaI-site of pLD1-101 before and the BglII-site of pLD2-19 behind the *ldc*-coding region (L.F. Fecker, unpublished). As an ATG in a different reading frame 56 bp before the known coding region might have been recognized as a translational start codon in plant cells, it was removed by *Bal* 31 digestion after cleavage with *Hpa*I. A *Bam* HI linker was ligated onto the 'filled-in' ends. The insert of clone 154 starting 2 bp before the *ldc* open reading frame was chosen for further subcloning and expression work. The *ldc* (*Bam* HI-*Bgl* II fragment) of this clone (p322.154) was integrated into the *Bam* HI site of the *E. coli* expression vector pJLA505 [34] carrying the *atpE* TIR of the *E. coli* ATPase operon, the P_R/P_L promoters and the temperature-sensitive CIts857 repressor of lambda. The identity of the induced protein bands of 78 and 65 kD was verified by PAGE and immunodetection (Fig. 1). Both bands are typical for LDC of *H. Alvei* [2]. The

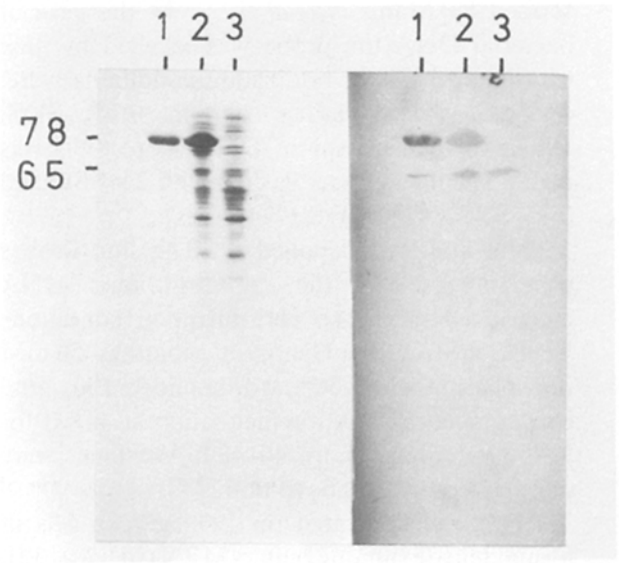

Fig. 1. Detection of LDC induced in *E. coli* p5.154 with the expression vector pLJA505. Left: 15% PAA gel with Coomassie detection; right: immunodetection on nitrocellulose. 1, purified LDC from *H. alvei*; 2, protein extracts of *E. coli* p5.154 cells after 240 min at 42 °C; 3, *E. coli* extracts without the plasmid. Molecular weight: $M_r \times 10^{-3}$.

LDC activity in *E. coli* extracts increased from zero to 70 nkat/mg protein within 4 h.

Having shown that the shortened fragment coded for an active LDC, the *ldc* insert of p322.154 was cloned into the *Bam* HI site in the polylinker of pAP2036, a derivative of pAP2022 [39]. The *ldc* was thus under the control of the 1′ promoter of the bidirectional Tr-promoter while the marker gene *nptII* was under the control of the 2′ promoter. Sequence and restriction analyses of the resulting plasmid p6.154 demonstrated that the *ldc* gene was correctly fused to the promoter and integrated into the vector (data not shown). The *ldc* gene of p6.154 was integrated into *A./ tumefaciens* C58CIRif pGV3850 [46] by conjugation and yielded *A. tumefaciens* strain 50.154 for plant transformation. The complete and correct integration of the *ldc* gene in the co-integrative vector was proven by Southern tests using 5 probes covering all decisive areas of the construct (data not shown).

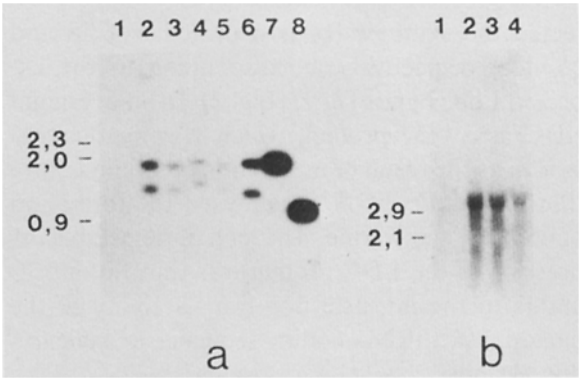

Fig. 2. a. Southern hybridizations of *Bam* HI/*Eco* RI restricted DNA of untransformed callus (1) and calli of *N. tabacum* cv. Samsum 50.154 (2–5) transformed with *A. tumefaciens* 50.154 (6). The hybridization probes S3 (7) and S4 (8) are *ldc* fragments of 1.8 and 1.1 kb respectively covering the whole coding region. b. Northern hybridization of total RNA from the roots of the transformed *N. tabacum* plants using S3 as LDC probe. 1, Samsum untransformed; 2–4, Samsum 50.154 transformants. Fragment lengths in kb.

Transformation of tobacco cells with A. tumefaciens *strain 50.154*

Co-cultivation of tobacco protoplasts and bacteria yielded 15 kanamycin-tolerant transformants with NPTII activity (data not shown). Nine of the transformed calli were regenerated to intact plants. While all calli grew on 1 mg/ml kanamycin, growth and root formation of the plantlets remained unaffected up to 0.3 mg/ml.

Southern hybridization clearly demonstrated the presence of the complete *ldc* gene in 14 of the transformants obtained by co-cultivation (Fig. 2a). By using the H23 fragment [7] as probe it was shown that the T-DNA containing the *ldc* gene was integrated 1–3 times into the genome of the various calli (data not shown). As the Tr promoter should be especially effective in root cells [38], mRNA was isolated from roots. All of the 14 50.154 transformants expressed *ldc* mRNA with a size of above 3000 bases (Fig. 2b). This correlated well with an expected minimal size of 3100 bases, the size of the *ldc* insert including the polyadenylation region in pAP2036 [39]. Despite the co-transcriptional activity of the Tr promoter for the *nptII* and *ldc* genes, LDC activity was not detected in any protein extract of the NPTII-positive transformants. In spite of a detection limit for LDC of 0.004% of total protein, LDC could also not be detected in western blot experiments.

In vitro *transcription and translation of the* ldc *gene in a eucaryotic vector system*

Given the absence of detectable LDC in the transformants we checked whether the bacterial *ldc* construct was generally translatable in a eucaryotic system. For this purpose, the *ldc* construct including the polyadenylation region of p6.154, was isolated by *Eco* RI (partial)/*Bam* HI digestion and cloned into the corresponding sites of the transcription vector pSP72. After linearization with *Bgl* II, the resulting plasmid (p.72.154) yielded 'run-off' transcripts of about 3300 bases as estimated by comparison with BMV (brome mosaic virus) markers (not shown). In the *in vitro* translation system this mRNA di-

rected the synthesis of two proteins of 78 and 65 kDa, respectively, corresponding to the expected LDC bands [2, 9] (Fig. 3). In an overnight LDC assay the protein, which was synthesized *in vitro* as the result of translation of 1.5 μg *in vitro* transcribed *ldc* mRNA, catalysed the formation of 46 nmol cadaverine. The lack of detectable expression of the LDC protein was thus not attributable to the intrinsic non-translatability of the bacterial *ldc* mRNA coding sequence in a eucaryotic system.

Cloning of the ldc *gene into vector pLS1*

There are several possible reasons for the lack of LDC protein accumulation in the transformants obtained from *A. tumefaciens* 50.154. A low translational activity of the mRNA and/or rapid degradation of the protein in the cytoplasm, for example, would easily explain the absence of the LDC protein. As the quinolizidine alkaloid biosynthesis pathway of Fabaceae, the original target for the *ldc* gene transformation experiments, seems to be localized in chloroplasts, we chose a new vector which would allow not only a very high expression but would also facilitate transport of the LDC protein into these organelles. A suitable vector for this purpose was the plasmid pLS1, which carries the promoter of the small subunit of Rubisco from *rbcS1* from potato together with the coding sequence of the transit peptide [10, 45].

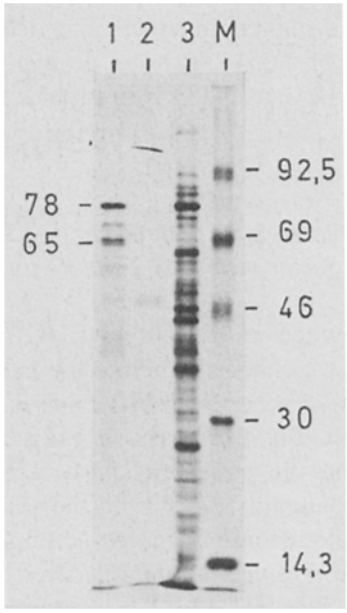

Fig. 3. Fluorogram of the *in vitro* translation products of p72.154 separated on a 12.5% PAA gel. 1, proteins of the *in vitro* RNA transcript of p72.154; 2, control without the RNA-transcript; 3, proteins of *E. coli* DH5 p5.154 labelled with ^{35}S-methionine. Molecular weight: $M_r \times 10^{-3}$.

The *ldc* gene together with the polyadenylation and the transcription termination site was isolated from p6.154 by *Eco* RI (partial)/*Bam* HI digestion. The ends were filled and the fragment was cloned into the filled *Bam* HI site of pLS1 directly behind the transit peptide. In pLS1 the sequence of the *rbcS1* gene at the coding sequence of the transit peptide processing site TGC, ATG, CAG was changed to TGC, ATG, GAT, CC forming a *Bam* HI site. The fill-in reactions at the *Bam* HI sites of pLS1 and the cloned *ldc* fragment created a translational gene fusion of the sequence TGC, ATG, GAT, CGA, TCC, CGC, ATG, thus adding 5 additional amino acids to the mature LDC protein, if processing would occur at the site as in the genuine small subunit protein precursor, i.e. between Cys and Met encoded by codons 1 and 2 of the sequence mentioned above. This cloning strategy was verified by sequence analysis of a 360 bp *Pst* I-*Sst*I fragment containing the transition of the transit peptide and the *ldc* gene. The resulting plasmid p1.154 was cleaved with *Hin*d III and cloned into the corresponding site of the binary plant transformation vector pLX222 [21]. *E. coli* DH5 cells carrying the plasmid with the insert in either of the two orientations were selected according to the restriction patterns. The new plasmids, named p22.154 B1 and B2, were introduced into *A. tumefaciens* LBA4404 via triparental mating. Southern and restriction pattern analyses confirmed the presence of p22.154 B1 and B2 in the transformed *Agrobacterium* strains termed 04.154 B1 and 04.154 B2.

Characterization of tobacco cells transformed with A. tumefaciens *04.154 B1 and B2*

Transformation of tobacco was performed by the leaf disc method. A total of 742 shoots was obtained from 240 infected leaf discs (50% from cv. SR1, 50% from cv. Samsum) on MS9 medium containing kanamycin and claforan. A total of 103 shoots (mostly variety Samsum) showed good root formation in the presence of 100–300 mg/l kanamycin. All of them were analysed for NPTII activity. Twenty-two of the seemingly kanamycin-tolerant plants showed no detectable NPTII activity and most of the 22 NPTII-negative plants died within the next 2 weeks.

The DNA of the leaves of 51 of the 81 NPTII-positive plants were analysed by Southern hybridization. Twenty-seven contained the expected DNA fragments of 3.6 and 4.8 kb (Fig. 4a), which indicated that promoter, transit peptide, the *ldc* gene, and the polyadenylation site were correctly integrated into the plant DNA and not rearranged.

The total RNA of the leaves of 20 of the 27 plants was analysed using the northern blotting technique. The mRNA of 15 of the plants hybridized with LDC probes, and their size (Fig. 4b) was in good agreement with the mRNA detected in plants transformed with the plasmid p50.154 (Fig. 2b).

The latter plants were chosen for the determination of LDC-activity (Fig. 5). Three of the transformed plants, all derivatives of cv. SR1, had in two independent experiments distinctly higher LDC activity than all of the others (Table 2). In several plants the activity was 3–5 fold above the background level, whereas in other

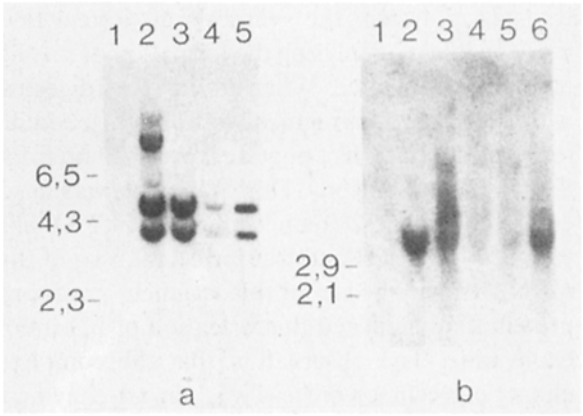

Fig. 4. a. Southern hybridization of *Hind* III/*Sal* I-restricted DNA of untransformed leaves (1) and leaves of *N. tabacum* SR1-plants (2–4) transformed with *A. tumefaciens* 04.154 B1/B2 (5). Hybridization with probes S3 and S4 (Fig. 2). b. Northern hybridization of leaf RNA of transformed *N. tabacum* cv. Samsum plants. Untransformed control (1), Samsum transformed with *A. tumefaciens* 50.154 (root RNA) (2) and with *A. tumefaciens* 04.154 B1/B2 3–6). Fragment lengths in kb.

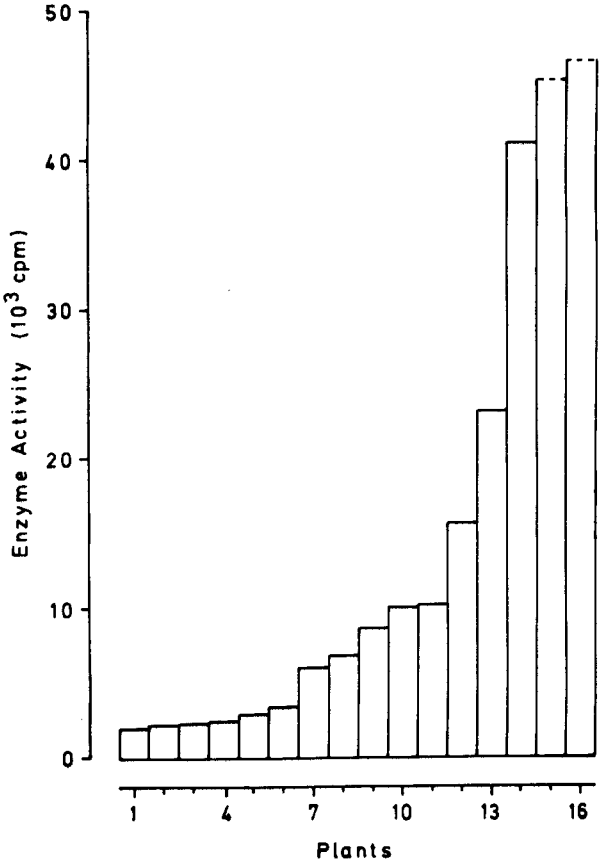

Fig. 5. Measurement of LDC activity in *N. tabacum* 04.154 B1 and 04.154 B2 transformants. The enzyme assays with 2×10^5 cpm (U-^{14}C)-lysine were run overnight. As only 1/4 of the pentanol phase was counted, the plant 14–16 had converted all lysine to cadaverine. 1: control Samsum and SR1. Extracted radioactivity of the controls corresponded to the background of the test. Transformed SR1 plants were plants 8, 12, 14–16, while the other ones were transformed Samsum plants. Plants 14 and 16 contain the LDC in B2, plant 15 in B1 orientation.

Table 2. Measurement of specific LDC activity and cadaverine in *N. tabacum* 04.154 transformants. Enzyme activity was determined in two experiments at 8-week intervals. L means growth of plantlets in the presence of 0.2 mM lysine. Under the conditions used here, neither LDC activity nor cadaverine was detected in untransformed Samsum (Ss) and SR1 plants.

Transformant		SR1 B1b	Sr1 B2g	Sr1 B2c	Ss B2hL	Sr1 B2f	Ss B2e	Ss B2jL
LDC activity	a)	144	126	83	23	18	11	8
pkat/mg protein	b)	143	68	124	28	29	21	27
Cadaverine mg/g dry mass		5.7	7.5	10.4	3.3	4.9	7.9	3.5

cases LDC activity was hardly distinguishable from the background level (Fig. 5). The LDC activity of untransformed plants never exceeded the background activity. Up to now, the highest specific LDC activity measured in the transformed plants was 144 pkat/mg protein (Table 2). Even in plants containing high LDC activity, the LDC protein was not detectable in western blots. No LDC activity was detected in enzyme extracts prepared from root and callus of transformed plants, since the *ldc* gene was under the control of the leaf-specific *rbcS1* promoter. Accordingly, northern hybridizations performed with RNA extracted from roots and callus tissue were also negative.

In order to test whether the LDC protein was transported into chloroplasts, intact chloroplasts were separated in a Percoll gradient from soluble proteins and broken chloroplasts. The sharp band of intact chloroplasts contained a 9-fold higher LDC activity than the broad, diffuse band of broken chloroplasts (Table 3). Since during chloroplast preparation large portions of the chloroplasts were destroyed and their contents were mixed with the soluble enzyme fractions, it was not possible to exclude that some LDC activity was present in the cytoplasm. However, further indirect evidence suggests that the chimeric LDC protein has to be processed to yield an active enzyme. In order to examine this problem, two different fragments were excised from p1.154 for cloning into the *in vitro* transcription vector pSP72. When a *Eco* RI (partial digestion)/*Sma* I fragment of p1.154 was cloned into the corresponding sites of pSP72, the resulting plasmid p72.154 ES carried the complete sequence of the transit peptide fused to the *ldc* gene. After linearization of p72.154 ES with *Hin*d III and 'run-off' experiments, 1.5 μg mRNA directed in the *in vitro* translation system the synthesis of protein that was capable of catalysing the formation of merely 3.6 nmol cadaverine. When p1.154 was digested with *Pst* I/*Sma* I, a fragment was obtained coding for a truncated transit peptide lacking 20 bp from the 5'-coding region. This fragment was also cloned into pSP72 (named pSP72.154 PS), linearized with *Bgl* II and transcribed. 1.5 μg of the mRNA transcribed from this fragment produced protein that catalyzed the formation of 221 nmol cadaverine. The observation that the complete chimeric precursor protein was almost enzymatically inactive, whereas the truncated version yielded a 50-fold higher LDC activity, is consistent with the hypothesis that processing during or after transport of the chimeric protein into the chloroplasts has occurred. It is less likely that unspecific processing in the cytoplasm prior to protein transport is responsible for the observed LDC activity since removal of the first 3 codons

Table 3. LDC activity in isolated and broken chloroplasts of *N. tabacum* 04.154 transformants. Leaves of SR1 B1b/B2c/B2f and B2g (Table 2) were pooled and used for chloroplast preparation. The background radioactivity was 5000 ± 200 cpm.

	Transformant chloroplasts		Samsum control chloroplasts		Buffer
	intact	broken	intact	broken	
LDC activity (cpm extracted)	76000	8600	0	0	0

of the transit peptide coding sequence severely impaired binding of the precursor and transport *in vitro* [31].

The expression and transport of the bacterial LDC into the chloroplasts of the transformed tobacco plants resulted in a dramatic increase in the amount of cadaverine in the leaves (Fig. 6). While in untransformed or transformed plants lacking the LDC protein cadaverine was hardly detectable the LDC-active plants contained an amount between 0.3 and 1% of dry mass (Table 2). A close correlation between the LDC activity and the cadaverine levels has not yet been established, as this requires detailed physiological and metabolic studies of the factors controlling LDC expression and the cadaverine accumulation pattern. As expected from the lack of LDC activity in roots and calli, cadaverine pools were not enhanced in these tissues. This fact indicates that transport of cadaverine from the leaves into the roots is low. Preliminary experiments have so far provided no indication that the enlarged cadaverine pool in the leaves affects alkaloid synthesis in the roots.

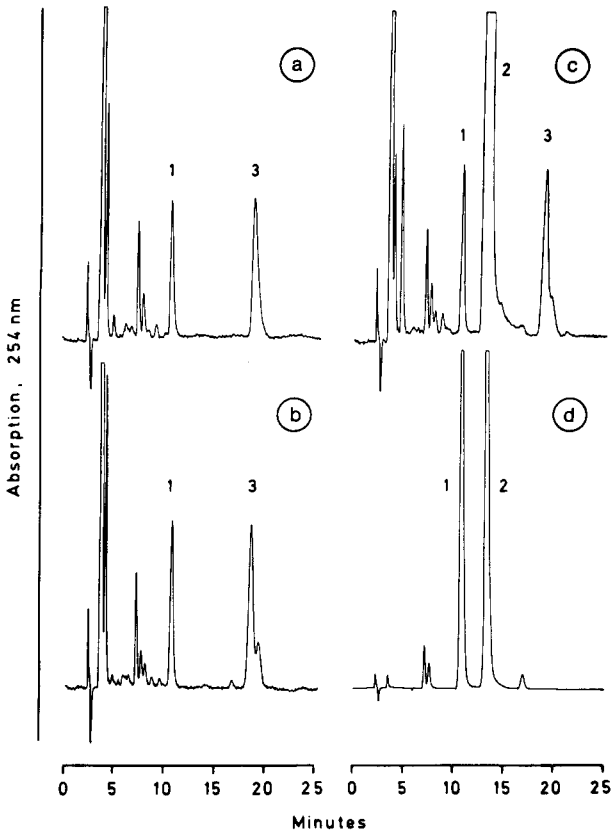

Fig. 6. Polyamine determination in leaf extracts of *N. tabacum* by reversed-phase chromatography. a, SR1 control; b, Samsum 50.154W; c, SR1 04.154 B2c; d, putrescine (1), cadaverine (2). As internal standard 1,6-diaminohexane (3) was added to plant extracts.

Discussion

An important first step towards altering biosynthetic pathways in higher plants by means of genetic transformation is to demonstrate not only that an appropriate gene is expressed, but also that the corresponding product is enzymatically active. If the compartment where the synthesis of the substrate is localized is known, one should direct the foreign protein to that site, or one should transport the foreign protein to compartments to which the substrate is transported for further metabolic reactions. Evidence is available that lysine biosynthesis is located in the chloroplasts [41]. The large accumulation of cadaverine in the transgenic plants in which LDC was directed into the chloroplasts is a clear demonstration of the importance of this strategy. Indeed, to the best of our knowledge, synthesis of such high levels of cadaverine have not previously been reported for any higher plant. The high LDC activity found in these transgenic plants might be related both to the high transcriptional activity of the *rbcS1* promoter [10, 45] and the import of the protein into the chloroplasts. However, it was unexpected that the LDC protein levels remained too low to be detectable in western blots. In the light of this observation, the failure to detect the LDC protein by western blotting in transgenic plants or calli bearing the *ldc* gene under the control of the weaker Tr promoter, is not surprising. Since in the case of the latter plants neither LDC activity nor enhanced cadaverine levels were measured, one has to assume that any LDC protein formed was immediately degraded or inactivated in the cytoplasm, while the chimaeric protein due to its

routing into chloroplasts might be less vulnerable after successful transport.

Presently, one can only speculate about the reasons why LDC protein levels driven by the *rbcS1* or the Tr promoter were so low, or even lacking, in the transgenic plants despite the fact that seemingly good levels of the corresponding mRNA were formed. The primary or secondary structure of the resulting mRNA could inhibit translation or impair its efficiency. A correlation between the levels of the mRNA and the translation product has often not been apparent in transgenic plants, especially when protein transport and processing is involved. For example, overexpression of an acetolactate synthase in *Arabidopsis* led to a 25-fold increase in the specified mRNA level while the enzyme activity increased only 2-fold [27]. One assumption was that the untransported unprocessed protein was rapidly turned over in the cytoplasm [27]. Given that protein import into chloroplasts depends not only on a functional transit peptide, but also on features of the fusion protein [42], it is possible that the chimaeric transit peptide/LDC protein was not efficiently transported and/or processed. Most of the chimaeric protein might therefore have been degraded in the cytoplasm while only reduced amounts of LDC entered the chloroplasts. However, until we have data relating to protein turnover in this system, it will remain unclear to what extent poor translation and/or protein instability contribute to the failure to detect the LDC in western blots. Another possibility is that post-translational modifications of the LDC protein caused the loss of antigenic determinants recognized by the specific antibodies raised against the bacterial LDC. This possibility will be tested as soon as sufficient LDC protein can be purified from LDC-expressing plants.

Despite the fact that LDC activity was only detected when the protein was transported into chloroplasts, it would be premature to conclude that the lack of activity of this bacterial enzyme in the cytoplasm is attributable to specific degradative processes located in this cellular compartment. Two other amino acid decarboxylases have recently been expressed in the cytoplasm of tobacco. The gene of the tryptophan decarboxylase of *Catharanthus roseus* under the control of the CaMV 35S promoter was expressed in tobacco plants [37]. The enzyme activity of TDC was increased up to 40-fold, and tryptamine levels reached up to 1% of dry mass in young leaves [37]. Whether the overexpression of the *tdc* gene led to dramatic changes of TDC activity and tryptamine levels in roots was not mentioned. The metabolic effects reported for the expression of an ornithine decarboxylase (ODC) of yeast, driven by the CaMV 35S promoter, in tobacco root cultures were not as dramatic [13]. The authors found the levels of putrescine and compounds derived therefrom increased in the order of 2-fold, despite distinctly enhanced ODC activity (2–20-fold depending upon the culture period), which indicates that overexpression of a biosynthetic gene might not be sufficient to increase the metabolic flux in the desired magnitude. The enzyme activities of the overexpressed foreign proteins (ODC in roots 40 pkat/mg protein, TDC and LDC in leaves 20 and 30–140 pkat/mg, respectively) were not sufficiently different to explain the much larger differences in product accumulation between roots (up to 0.15% putrescine and derived metabolites) and leaves (up to 1% tryptamine and cadaverine). In order to get more insight into this problem, we are presently studying the metabolic effects of *ldc* gene expression under the control of other promoters and signal peptides. The results should provide information about the importance of the site of expression and the final destination of the synthesized protein in relation to the metabolic consequences. Direction of the protein to the natural compartment of the targeted biosynthetic chain may be of particular importance, since the overproduction of one pathway enzyme is likely only to have significant effects when the product of the corresponding reaction is immediately available to the next enzyme of a pathway.

Acknowledgements

We would like to thank R. Hain (Bayer, Leverkusen) for the H23 probe and L. Witte (Tech-

nische Universität, Braunschweig) for the GC/MS analysis. We are especially grateful to C. Rügenhagen for her expert assistance. We thank U. Commandeur for many helpful suggestions and comments. The financial support of the GBF, Braunschweig, is gratefully acknowledged.

References

1. Arnon DJ: Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1–15 (1949).
2. Beier H, Fecker LF, Berlin J: Lysine decarboxylase from *Hafnia alvei*: Purification, molecular data and preparation of polyclonal antibodies. Z Naturforsch 42c: 1307–1312 (1987).
3. Berlin J, Fecker LF, Mollenschott C, Sator C, Strack D, Witte L, Wray V: Isoflavonoid formation in transformed and non-transformed suspension and hairy root cultures of *Lupinus polyphyllus* and *L. hartwegii*. Z Naturforsch 46c (in press).
4. Bolivar F, Rodriguez RL, Greene PJ, Betlach ML, Heyneker HL, Boyer HW, Crosa JH, Falkow S: Construction and characterization of new cloning vehicles II. a multipurpose cloning system. Gene 2: 95–113 (1977).
5. Cline K, Andrews J, Mersey B, Newcomb EH, Keegstra K: Separation and characterization of inner and outer envelope membranes of pea chloroplasts. Proc Natl Acad Sci USA 78: 3595–3599 (1981).
6. Dellaporta SL, Wood J, Hicks JB: A plant DNA minipreparation: Version II. Plant Mol Biol Rep 1: 19–21 (1983).
7. Depicker A, De Wilde M, De Vos G, De Vos R, Van Montagu M, Schell J: Molecular cloning of overlapping segments of the nopaline Ti-plasmid pTiC58 as a means to restriction endonuclease mapping. Plasmid 3: 193–211 (1980).
8. Ditta G, Stanfield S, Corbin D, Helinski DR: Broad host range DNA cloning system from gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc Natl Acad Sci USA 77: 7347–7351 (1980).
9. Fecker LF, Beier H, Berlin J: Cloning and characterization of a lysine decarboxylase gene from *Hafnia alvei*. Mol Gen Genet 203: 177–184 (1986).
10. Fritz CC, Herget T, Wolter FP, Schell J, Schreier PH: Reduced steady state of *rbc*S mRNA in plants kept in the dark are due to differential degradation. Proc Natl Acad Sci USA (in press).
11. Hain R, Stabel P, Czernilofsky AP, Steinbiss HH, Herrera-Estrella L, Schell J: Uptake, integration, expression and genetic transmission of a selectable chimeric gene by plant protoplasts. Mol Gen Genet 199: 161–168 (1985).
12. Hanahan D: Study on the transformation of *Escherichia coli* with plasmids. J Mol Biol 166: 557–580 (1983).
13. Hamill JD, Robins RJ, Parr AJ, Evans DM, Furze JM, Rhodes MJC: Overexpressing a yeast ornithine decarboxylase gene in transgenic roots of *Nicotiana rustica* can lead to enhanced nicotine accumulation. Plant Mol Biol 15: 27–38 (1990).
14. Hartmann T: Quinolizidines and pyrrolizidines. In: Constabel F, Vasil IK (eds) Cell Culture and Somatic Cell Genetics of Plants, vol. 5; Phytochemicals in Plant Cell Cultures pp. 277–288. Academic Press, San Diego (1988).
15. Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA: A linary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303: 179–180 (1983).
16. Horsch RB, Fry J, Hoffmann N, Niedermeyer J, Rogers SG, Fraley RT: Leaf disc transformation. In: Gelvin SB, Schilperoort RA (eds) Plant Molecular Biology Manual, A5: 1–9 Kluwer Academic Publishers, Dordrecht (1988).
17. Johnson DA, Gautsch JW, Sportsman JR, Elder JH: Improved technique utilizing non fat dry milk for proteins and nucleic acids transferred to nitrocellulose. Gene Anal Techn 3: 1–8 (1984).
18. Krieg PA, Melton DA: *In vitro* RNA-synthesis with SP6 RNA-polymerase. Meth Enzymol 155: 397–415 (1987).
19. Kyhse-Andersen J: Electrolabelling of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J Biochem Biophys Meth 10: 203–209 (1984).
20. Laemmli UK: Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227: 680–685 (1970).
21. Landsmann J, Llewellyn D, Dennis ES, Peacock WJ: Organ regulated expression of the *Parasponia andersonii* hemoglobine gene in transgenic tobacco plants. Mol Gen Genet 214: 68–73 (1988).
22. Maniatis T, Fritsch EF, Sambrook J: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).
23. Marton L, Wullems GJ, Molendijk L, Schilperoort RA: *In vitro* transformation of cultured cells from *Nicotiana tabacum* by *Agrobacterium tumefaciens*. Nature 277: 129–131 (1979).
24. Medgyesy P, Menzel L, Maliga P: The use of cytoplasmic streptomycin resistance: chloroplast transfer from *Nicotiana tabacum* into *Nicotiana sylvestris*, and isolation of their somatic hybrids. Mol Gen Genet 179: 693–698 (1980).
25. Murashige T, Skoog F: A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15: 473–497 (1962).
26. Ngo TT, Brillhart KL, Davis RH, Wong RC, Bovaird JH, Digangi JJ, Ristow JL, Marsh JL, Phan APH, Lehnhoff HM: Spectrophotometric assay for ornithine decarboxylase. Anal Biochem 160: 290–293 (1987).
27. Odell JT, Caimi PG, Yadav NS, Mauvais CJ: Compari-

son of increased expression of wild-type and herbicide-resistant acetolactate synthase genes in transgenic plants, and indication of posttranscriptional limitation on enzyme activity. Plant Physiol 94: 1647–1654 (1990).
28. Read SM, Northcote DH: Minimizing of variation in the response to different proteins of the Coomassie blue G dye-binding assay for protein. Anal Biochem 116: 53–64 (1981).
29. Redmond JW, Tseng A: High pressure liquid chromatographic determination of putrescine, cadaverine, spermidine and spermine. J Chromatogr 170: 479–481 (1979).
30. Reiss B, Sprengel R, Will H, Schaller H: A new sensitive method for qualitative assay of neomycin phosphotransferase in crude cell extracts. Gene 30: 211–218 (1984).
31. Reiss B, Wasmann CC, Schell J, Bohnert HJ: Effect of mutations on the binding and translocation functions of a chloroplast transit peptide. Proc Natl Acad Sci USA 86: 886–890 (1989).
32. Rigby PWJ, Dieckmann M, Rhodes C, Berg P: Labelling deoxyribonuleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J Mol Biol 113: 237–251 (1977).
33. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY (1989).
34. Schauder B, Blöcker H, Frank R, McCarthy JEG: Inducible expression vectors incorporating the *Escherichia coli* atpE translation initiation region. Gene 52: 279–283 (1987).
35. Schreier PH, Seftor EA, Schell J, Bohnert HJ: The use of nuclear encoded sequences to direct the light-regulated synthesis and transport of a foreign protein into plant chloroplasts. EMBO J 4: 25–32 (1985).
36. Shillito RD, Paszkowski J, Potrykus I: Agarose plating and a bead type culture technique enable and stimulate development of protoplast-derived colonies in a number of plant species. Plant Cell Rep 2: 244–247 (1983).
37. Songstad DD, De Luca V, Brisson N, Kurz WGW, Nessler CL: High levels of tryptamine accumulation in transgenic tobacco expressing tryptophan decarboxylase. Plant Physiol 94: 1410–1413 (1990).
38. Teeri TH, Lehväslaiho H, Franck M, Uotila J, Heino P, Palva ET, Van Montagu M, Herrera-Estrella L: Gene fusions to lacZ reveal new expression patterns of chimeric genes in transgenic plants. EMBO J 8: 343–350 (1989).
39. Velten J, Schell J: Selection-expression plasmid vectors for use in genetic transformation of higher plants. Nucl Acids Res 13: 6981–6998 (1985).
40. Verwoerd TC, Dekker BMM, Hoekema A: A small scale procedure for the rapid isolation of plant RNAs. Nucl Acids Res 17: 2362 (1989).
41. Wallsgrove RM, Mazelis M: The enzymology of lysine biosynthesis in higher plants. FEBS Lett 116: 189–192 (1980).
42. Wasmann CC, Reiss B, Bartlett SG, Bohnert HJ: The importance of the transit peptide and the transported protein for protein import into chloroplasts. Mol Gen Genet 205: 446–453 (1986).
43. Wink M, Hartmann T: Cadaverine-pyruvate transamination: The principal step of enzymatic quinolizidine alkaloid biosynthesis in *Lupinus polyphyllus* cell suspension cultures. FEBS Lett 101: 343–346 (1979).
44. Wink M, Hartmann T: Sites of enzymatic synthesis of quinolizidine alkaloids and their accumulation in *Lupinus polyphyllus*. Z Pflanzenphysiol 102: 337–344 (1981).
45. Wolter FP, Fritz CC, Willmitzer L, Schell J, Schreier PH: rbcS genes in *Solanum tuberosum*: Conservation of transit peptide and exon shuffling during evolution. Proc Natl Acad Sci USA 85: 846–850 (1988).
46. Zambryski P, Joos H, Genetello C, Leemans J, Van Montagu M, Schell J: Ti-plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO J 2: 2143–2150 (1983).