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Increased lysine synthesis in tobacco plants that express high levels of bacterial dihydrodipicolinate synthase in their chloroplasts

Orit Shaul and Gad Galili*
Department of Plant Genetics, The Weizmann Institute of Science, Rehovot 76100, Israel

Summary

A major nutritional drawback of many crop plants is their low content of several essential amino acids, particularly lysine. The biosynthesis of lysine in plants is regulated by several feedback loops. Dihydrodipicolinate synthase (DHPS) from *Escherichia coli*, a key enzyme in lysine biosynthesis, which is considerably less sensitive to lysine accumulation than the endogenous plant enzyme has been expressed in chloroplasts of tobacco leaves. Expression of the bacterial enzyme was accompanied by a significant increase in the level of free lysine. No increase in protein-bound lysine was evident. Free lysine accumulation was positively correlated with the level of DHPS activity in various transgenic plants. Compartmentalization of DHPS in the chloroplast was essential for its participation in lysine biosynthesis as no lysine overproduction was obtained in transgenic plants that expressed the bacterial enzyme in the cytoplasm. The elevated level of free lysine in the transgenic plants was sufficient to inhibit, *in vivo*, a second key enzyme in lysine biosynthesis, namely, aspartate kinase, with no apparent influence on lysine accumulation. The present report not only provides a better understanding of the regulation of lysine biosynthesis in higher plants but also offers a new strategy to improve the production of this essential amino acid.

Introduction

Many crop plants have poor nutritional quality because of their limited synthesis and accumulation of several essential amino acids, particularly lysine. In higher plants, lysine is synthesized from aspartate via the aspartate-family biosynthetic pathway (Bryan, 1980), which also leads to the synthesis of threonine, methionine and isoleucine (Figure 1). A similar pathway also operates in many bacterial species (Umbarger, 1978). Both in plants and bacteria, lysine biosynthesis is controlled by two key enzymes. The first one, aspartate kinase (AK), generally appears in at least two isozymic forms whose activities are subject to feedback inhibition either by lysine or threonine. The second key enzyme, dihydrodipicolinate synthase (DHPS), is subject to feedback inhibition only by lysine (Bryan, 1980; Umbarger, 1978).

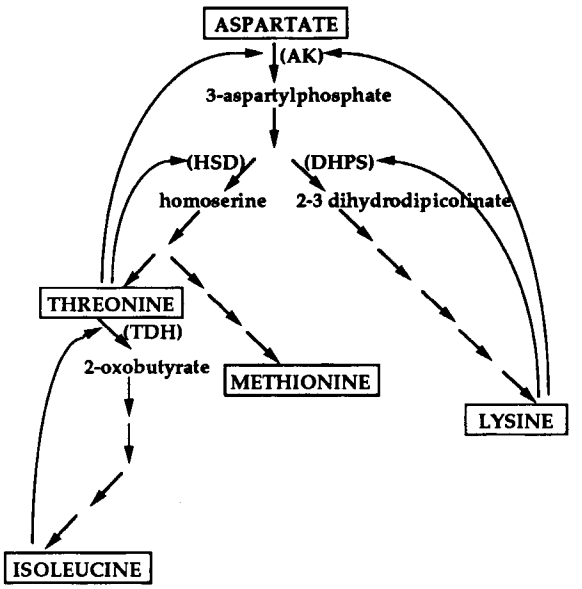

Figure 1. A diagram of the aspartate-family biosynthetic pathway.
Only the major key enzymes and their products are indicated. Curved arrows represent feedback inhibition by the end-product amino acids. AK: aspartate kinase; DHPS: dihydrodipicolinate synthase; HSD: homoserine dehydrogenase; TDH: threonine dehydratase.

In bacteria, AK was shown to be the major rate-limiting enzyme in lysine biosynthesis (Cohen and Saint-Girons, 1987). Little is known, however, about the relative roles of AK and DHPS in regulating lysine biosynthesis in higher plants. Despite many attempts to obtain lysine overproducing plants, employing mutagenesis followed by selection for resistance to the lysine analogue S-aminoethyl L-cysteine, only a few cases using plant tissue culture were successful (Boyes and Vasil, 1987; Negrutiu *et al.*, 1984; Schaeffer and Sharpe, 1981; Widholm, 1976). In one case, using tobacco protoplasts, the biochemical basis for lysine overproduction was characterized and identified as a mutation in DHPS, rendering it less sensitive to lysine inhibition (Negrutiu *et al.*, 1984). These results indicated that, unlike bacteria, DHPS may play a major role in the regulation of lysine biosynthesis in higher plants. Indeed, whereas the I_{50} of DHPS in plants ranges between 10 and 50 μM lysine (Bryan, 1980), in *E. coli* it is 1 mM

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*For correspondence (fax +972 8 469124).

(Yugari and Gilvarg, 1962). Support for the major role of DHPS in lysine biosynthesis in higher plants also came from studies of mutant plants possessing desensitized AK isozymes. Such plants, selected by resistance to lysine plus threonine (Bright *et al.*, 1982; Cattoir-Reynaerts *et al.*, 1983; Hibberd *et al.*, 1980), generally overproduce threonine but not lysine.

Although DHPS and other enzymes of the aspartate-family pathway in plants were located to the chloroplast (Bryan *et al.*, 1977; Ghislain *et al.*, 1990; Mills *et al.*, 1980), it is not yet known whether this pathway is confined to the chloroplast or whether it also operates in the cytoplasm.

In order to study the role of DHPS in the biosynthesis of lysine in higher plants, we expressed the *E. coli dapA* gene coding for DHPS (Richaud *et al.*, 1986) in transgenic tobacco plants. This bacterial enzyme is much less sensitive to lysine inhibition than its plant counterpart. Our results showed that not only the sensitivity of DHPS to lysine inhibition but also the actual level of this enzyme greatly influenced the accumulation of lysine in tobacco leaves. We also showed that the compartmentalization of DHPS within the chloroplast is essential for lysine biosynthesis.

Results

Expression and subcellular localization of the E. coli *DHPS in leaves of the transgenic tobacco plants*

In order to study the role of DHPS and the significance of its chloroplast compartmentalization in the biosynthesis of lysine in higher plants, we have expressed the *E. coli dapA* gene coding for DHPS (Richaud *et al.*, 1986) in transgenic tobacco plants. Two types of chimeric genes, expected to express the *E. coli* DHPS in the cytoplasm or within the chloroplast, were constructed (Figure 2). These chimeric genes were subcloned in the binary Ti vector pGA492 of *Agrobacterium tumefaciens* (An, 1986) and introduced into *Nicotiana tabacum* cv. Samsun NN by the leaf disc protocol (Horsch *et al.*, 1985). To test for the expression of

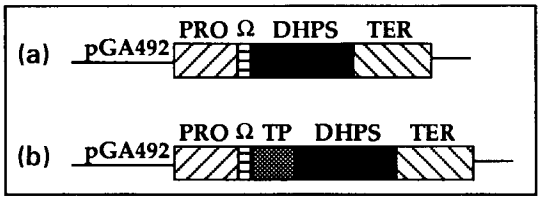


Figure 2. A schematic diagram of the chimeric genes utilized in the present study.
(a) Cytoplasmic-type gene; (b) chloroplastic-type gene. PRO, the 35S promoter; Ω, the DNA coding for the Ω mRNA leader sequence; TP, the DNA coding for the pea *rbcS-3A* chloroplast transit peptide; DHPS, the coding DNA sequence of the *E. coli dapA* gene; TER, the octopine synthase 3′ terminator DNA sequence.

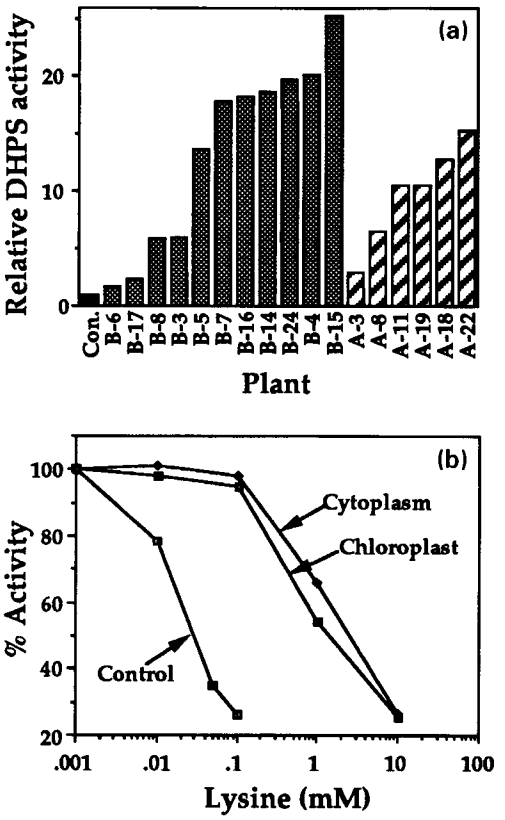
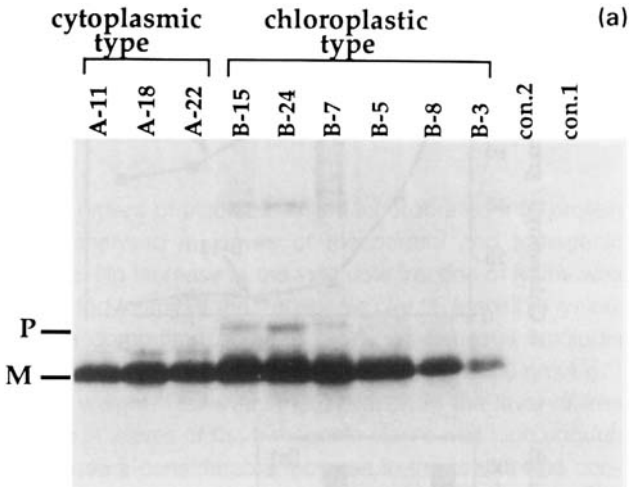


Figure 3. Relative DHPS activity (a) and sensitivity to lysine inhibition (b) in the transgenic plants.
(a) DHPS activity in each transgenic genotype (average of two measurements for each transgenic genotype) is plotted relative to the average DHPS activity of four control (Con.) plants which was given the value of 1. B-3 to B-24, transgenic genotypes expressing the chloroplastic-type DHPS; A-3 to A-22, transgenic genotypes expressing the cytoplasmic-type DHPS.
(b) DHPS activity was assayed as in (a) in the presence of increasing lysine concentrations. At each lysine concentration DHPS activity is presented as a percentage of the activity in extracts with no lysine added. Control, nontransformed plants; Cytoplasm, average DHPS activity of plants A-11 and A-22 containing the cytoplasmic-type *E. coli* DHPS; Chloroplast, average DHPS activity of plants B-7 and B-15 containing the chloroplastic-type *E. coli* DHPS.

the chimeric genes, DHPS activity was assayed in leaves harvested from transgenic plants grown for about 1 month on Nitsch minimal medium in Magenta boxes. Both types of transgenic plants, expressing the cytoplasmic-type or the chloroplastic-type *E. coli* DHPS, exhibited up to 25-fold increases in the level of leaf DHPS activity compared to nontransformed control plants (Figure 3a). DHPS activity in both types of transgenic plants was less sensitive to lysine inhibition compared to control plants, having an I_{50} of about 1 mM lysine, as expected for the *E. coli* enzyme (Figure 3b).

Transgenic plants exhibiting different levels of DHPS activity were selected for immunoblotting with anti-*E. coli* DHPS serum (Figure 4a). A major band with the expected



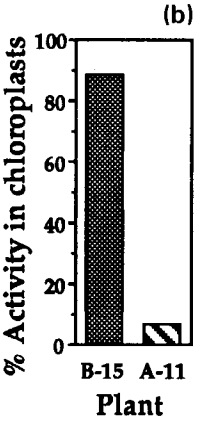


Figure 4. Western blot analysis and chloroplast compartmentalization of the *E. coli* DHPS in transgenic plants.
(a) Proteins extracted from leaves of control and transgenic plants containing the cytoplasmic- and chloroplastic-type enzymes were reacted in Western blots against antibodies specific to *E. coli* DHPS. M, band corresponding in molecular weight to the natural *E. coli* DHPS; P, band corresponding to the expected size of the precursor *E. coli* DHPS containing the chloroplast transit peptide.
(b) DHPS activity measured in isolated intact chloroplasts from transgenic plants carrying the chloroplastic-type (B-15) and the cytoplasmic-type (A-11) gene. Data are given as a percentage of the activity measured in a total extract of the same plant, using equal amount of chlorophyll for each assay.

size of the *E. coli* DHPS appeared in all plants. The comigration of the *E. coli* DHPSs from both types of transgenic plants indicated that in the chloroplastic-type the transit peptide had been removed and the enzyme was apparently translocated into the chloroplast. In plants expressing high levels of DHPS in their chloroplasts, a faint band of higher molecular weight, apparently representing a small proportion of unprocessed precursor protein, was also detected (Figure 4a). The intensities of the anti-DHPS bands (Figure 4a) were positively correlated with the levels of DHPS activity (Figure 3a). To confirm the compartmentalization of the chloroplastic-type protein within this organelle, intact chloroplasts were isolated. In plants expressing the chloroplastic-type enzyme, about 90% of the DHPS activity measured in a total extract was co-purified with the intact chloroplast fraction (equal amounts of chlorophyll were used for the enzymatic assay). In contrast, in plants expressing the cytoplasmic-type enzyme, only 7% of the total DHPS activity was co-purified with the chloroplast fraction (Figure 4b). The purity of the chloroplast preparation was verified by determining the relative proportions of the chloroplastic and cytoplasmic isozymes of superoxide dismutase (SOD). While in the total extracts all SOD isozymes were observed, in the intact chloroplast fraction only the chloroplastic isozyme was detected (data not shown).

Lysine accumulation in leaves of the transgenic plants

The relationship between DHPS activity and the content of free lysine was studied in young leaves of transgenic plants having different levels of DHPS activity. Plants with high levels of the *E. coli* enzyme in the chloroplast exhibited a significant increase in the level of free lysine, amounting up to 15-fold over the lysine level in control plants (Figure 5). The level of free lysine in these plants was positively correlated with their level of DHPS activity (Figures 3a and 5). No significant change was observed in the amount of other free amino acids, including aspartate, the precursor of lysine, and threonine, which is synthesized through another branch of the same pathway. Methionine was below the detection level both in the control and in the transgenic plants. Plants containing the cytoplasmic-type *E. coli* DHPS activity showed no increase in free lysine (Figure 5).

Inheritance and dosage effect of the chimeric DHPS gene

The number and stability of the integrated, chloroplastic-type chimeric genes were studied by analysing DHPS

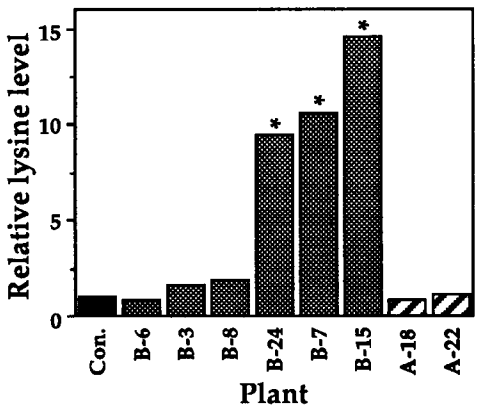


Figure 5. Relative levels of free lysine in leaves of transgenic plants.
The level of free lysine in each genotype (average of at least three measurements of different plants for each transgenic genotype) is plotted relative to the level of free lysine in the control plants (average of 14 measurements of different plants). Con., control nontransformed plants; B-3 to B-24, transgenic plants expressing the chloroplastic-type DHPS; A-18 and A-22, transgenic plants expressing the cytoplasmic-type DHPS. An asterisk on top of the bar indicates a significant difference from the control at the 1% level, as determined by the Dunnett treatment versus control test (Dunnett, 1955). Analysis was carried out on a log. transformation to stabilize variances within different transgenic genotypes.

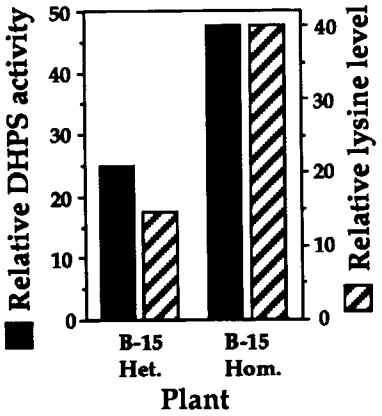


Figure 6. Relative DHPS activity and lysine accumulation in young leaves from heterozygous and homozygous seedlings derived from plant B-15. The levels of DHPS activity and lysine accumulation (average of 10 measurements of heterozygous and 14 measurements of homozygous plants) are plotted relative to the levels of DHPS activity and lysine accumulation in the control plants, determined as described in Figures 3a and 5, respectively.

activity in seedlings of progeny derived from various transgenic plants. In all cases, approximately 75% of the seedlings exhibited the *E. coli* DHPS activity, showing that all the transgenic plants tested contained a single, stably inherited chimeric gene. To test for lysine accumulation as a function of the dosage of the chimeric gene, seedlings derived from plant B-15 were divided into heterozygotes and homozygotes, the latter having twice the level of DHPS activity. The average level of lysine in the homozygous plants was about 2.7-fold higher than in the heterozygotes (Figure 6). Lysine accumulation in the homozygous progeny of the plant B-15 was elevated about 40-fold compared to control plants.

The effect of lysine accumulation on AK activity

The positive correlation between the level of DHPS activity and content of free lysine demonstrated that DHPS represents a major rate-limiting step for lysine biosynthesis in tobacco leaves. Regarding AK, however, since at least one of its isozymes is sensitive to lysine accumulation, it was of interest to evaluate whether this isozyme was inhibited *in vivo* to a greater extent in the lysine overproducing plants than in control plants. We have taken advantage of the known sensitivity of plants to the exogenous addition of lysine plus threonine. Plants are relatively tolerant to supplementation of threonine into their growth medium, but administration of lysine in addition to threonine arrests their growth, apparently because of methionine starvation caused by complete inhibition of AK activity (Green and Phillips, 1974). Growth of shoots regenerated from plant B-15, the highest lysine overproducing transgenic plant, was much more sensitive to

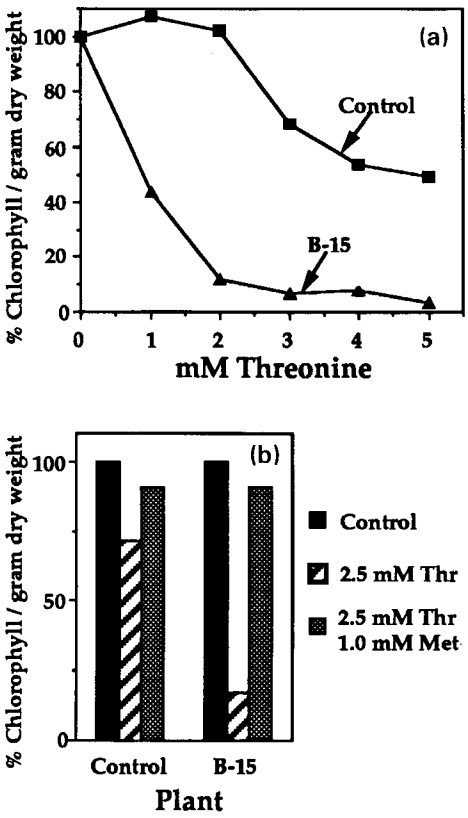


Figure 7. The sensitivity of tobacco shoot growth to exogenously added threonine.
Shoots of B-15 and control plants, regenerated from leaf discs, were grown on Nitsch minimal medium supplemented with increasing threonine concentrations (a) or threonine plus methionine (b). The chlorophyll content per gram dry weight of the shoots is represented as a percentage of the chlorophyll content in shoots with no added amino acids.

the presence of threonine in the growth medium compared to control plants (Figure 7a). This sensitivity could be completely overcome by the addition of methionine (Figure 7b). These results indicated that at least in plant B-15 the lysine level was sufficiently high to inhibit the activity of the lysine-sensitive AK *in vivo*.

Influence of high lysine accumulation on plant phenotype

The highest lysine overproducing plants, B-15, B-7 and B-24, exhibited some abnormal phenotypic characteristics, that were most noticeable during later stages of plant development. Newly developed leaves at the tip apex showed a mosaic green colour, and a partial loss of apical dominance was noticed. Some flowers on the primary stem of these plants were sterile, but those that developed on secondary stems were fully fertile and produced seeds. Senescence was delayed and new buds continued to develop for a relatively long period of time. Homozygous plants showed more severe symptoms which also appeared at younger stages of growth. Thus

there was a positive correlation between lysine overproduction and abnormal plant phenotype.

Effect of free lysine accumulation on the level of insoluble lysine

The content of insoluble lysine incorporated into protein was analysed in leaves of the control and transgenic plants. No increase in the insoluble fraction of lysine was detected in any of the transgenic plants. Insoluble lysine, which comprised about 6 mol% of the total insoluble amino acids, amounted to approximately 8000 nmol g^{-1} fresh weight. However, the elevation in the level of free lysine in leaves of the transgenic plants was high enough to cause a considerable increase in the total lysine content. For example, the increased level of free lysine in the homozygous plant B-15 (4680 nmol g^{-1} fresh weight) compared to control plants (117 nmol g^{-1} fresh weight) brought about an elevation of 56% in the total lysine content.

Discussion

The results presented in this paper support a previous report (Negrutiu *et al.*, 1984) showing that the activity of DHPS in tobacco leaves represents a major rate-limiting step in lysine biosynthesis. However, several new principles emerge from this study concerning the regulation of lysine biosynthesis.

(i) The major rate-limiting factor(s) controlling lysine biosynthesis is not only the sensitivity of DHPS activity to lysine inhibition but also the actual level of this enzyme. This can be deduced from the positive correlation between the activity of the *E. coli* DHPS and the content of free lysine in the leaves (Figures 3a, 5 and 6). In fact, the level of DHPS may be even more important than its sensitivity to lysine inhibition. For example, the activity of the *E. coli* DHPS in plant B-6 was similar to the activity of the endogenous enzyme, yet this plant failed to produce significantly more lysine (Figure 5).

(ii) The level of aspartate, the precursor of lysine, is not a rate-limiting factor in lysine biosynthesis, at least in tobacco leaves. This is inferred from the fact that the level of aspartate was not reduced even in leaves that accumulated the highest levels of free lysine.

(iii) Compartmentalization of DHPS within the chloroplast is essential for lysine biosynthesis. Evidently, expression of high levels of this enzyme outside this organelle was not accompanied by increased lysine production. This indicates that the lysine pathway operates largely, if not entirely, within the chloroplast and that transport of intermediate compounds of the aspartate pathway through the chloroplast membrane may be limited.

Although DHPS activity plays a major role in lysine biosynthesis in tobacco leaves, lysine accumulation may also be affected by the activity of the lysine-sensitive AK isozyme. We have shown that the activity of this isozyme is inhibited *in vivo* to a greater extent in the heterozygous transgenic plant B-15 than in the control plant. However, the positive correlation between DHPS activity and lysine accumulation, which was not saturated even in the homozygous plant B-15, indicates that at least in young tobacco leaves the effect of the lysine-sensitive AK on lysine accumulation is limited. This may be explained by the presence of relatively high levels of lysine-insensitive AK activity in tobacco leaves.

The approach presented here not only provides a new tool for studying the regulation of lysine biosynthesis in plants, but it may also open a new avenue towards breeding of crop plants with a more balanced level of this essential amino acid. We show that a substantial increase in free lysine may be sufficient by itself to elevate considerably the total lysine content in the plant. However, our results imply that in order to obtain a significant overproduction of free lysine, at least two types of modifications should be considered: an alteration of DHPS to a form which is less sensitive to lysine inhibition, and an elevation in DHPS expression. The main advantage of the present approach over previously used genetic methodologies is the potential to modulate both traits of DHPS in the same plant simultaneously. Plants that express higher levels of DHPS and consequently accumulate more free lysine may also be obtained by crossing different transgenic plants in which the chimeric genes are inserted in different locations in the genome. In addition, using tissue-specific promoters, our approach enables the targeting of DHPS to specific tissues where increased lysine synthesis is desirable. Such targeting may eliminate the phenotypic modifications observed in the highest lysine overproducing plants, namely a partial loss of apical dominance, delayed flowering and senescence, partial sterility and abnormal leaf morphology.

Another approach to increase the level of lysine in plants is the introduction of new genes coding for lysine-rich proteins (Wallace *et al.*, 1988; Yang *et al.*, 1989). The fact that elevation in free lysine did not alter the level of insoluble lysine in tobacco leaves suggests that the normal level of free lysine, at least in this tissue, was not a limiting factor for the production of endogenous proteins which are relatively rich in lysine. However, it is not known whether the normal level of free lysine is sufficient to promote a significant production of lysine-rich proteins derived from newly introduced genes. Yet, as it is now feasible both to introduce genes for lysine-rich proteins and to bring about an elevation in the level of free lysine, crossing these two types of transgenic plants may combine both traits in the same plant.

Experimental procedures

Construction of chimeric genes and plant transformation

The various DNA fragments were joined using general cloning protocols (Maniatis *et al.*, 1982). Two types of chimeric genes were constructed: one designed to express the *E.coli* DHPS in the cytoplasm and the other within the chloroplast. In the first (Figure 2a), the *Nru*I to *Hin*dIII DNA fragment of pDA3 (Richaud *et al.*, 1986), containing the entire coding sequence of the *E. coli dapA* gene, except for the first 22 bp, was ligated with a synthetic linker that restored these 22 bp and had *Sph*I and *Nru*I sites. This fragment was introduced between the *Hin*dIII and *Sph*I sites of pUC18 (Pharmacia). We have inserted between the *Bam*HI and *Sal*I sites of this plasmid, upstream of the *dapA* coding region, the *Bam*HI to *Sal*I fragment of pJD330, kindly provided by Dr D.R. Gallie. This *Bam*HI to *Sal*I fragment of pJD330 contained the 35S promoter of the CaMV (Guilley *et al.*, 1982; Odell *et al.*, 1985), and an Ω DNA sequence from the coat protein gene of tobacco mosaic virus, previously shown to code for an mRNA leader sequence that enhances translation of eukaryotic mRNAs (Gallie *et al.*, 1989). A 0.7-kb DNA fragment containing the transcription termination and polyadenylation signal of the *A. tumefaciens* octopine synthase gene (Greve *et al.*, 1983) was cloned into the *Sma*I site of pBluescript SK+ (Stratagene). The *Bam*HI site in the polylinker was then eliminated by *Bam*HI digestion, fill-in reaction with Klenow and ligation. We have inserted between the *Kpn*I and *Hin*dIII sites of this plasmid, upstream of the terminator, the *Kpn*I to *Hin*dIII fragment of the previous plasmid that contained the *dapA* coding sequence as well as the 5′ flanking regions. Thus we have obtained the cytoplasmic-type chimeric gene shown in Figure 2a. In the second chimeric gene (Figure 2b), the pea *rbcS-3A* transit peptide coding sequence (Fluhr *et al.*, 1986), kindly provided by Professor N.-H. Chua, was inserted between the *Sal*I and *Sph*I sites of the pUC18 vector, followed by all the other cloning steps described above. This resulted in the insertion of the chloroplast transit peptide coding sequence between the Ω DNA and the *dapA* coding sequence (Figure 2b). The two chimeric genes were excised with *Bam*HI and *Sac*I, inserted between the *Bgl*II and *Sac*I sites of the binary Ti vector pGA492 of *A. tumefaciens* (An, 1986) and introduced into *N. tabacum* cv. Samsun NN by the leaf disc protocol (Horsch *et al.*, 1985).

Analysis of DHPS activity

Leaves from axenic tobacco plants grown for about 1 month on Nitsch minimal medium (Nitsch, 1969) in Magenta boxes were homogenized with a mortar and pestle in an equal volume of cold 100 mM Tris-HCl pH 7.5 containing 2 mM EDTA, 1.4% sodium ascorbate, 1 mM phenylmethylsulphonilfluoride (PMSF) and 0.5 μg ml^{-1} leupeptin. Following 5 min centrifugation (16 000 ***g*** at 4°C) the supernatant was collected. DHPS activity was measured using the *o*-aminobenzaldehyde (*o*-ABA) method of Yugari and Gilvarg (1965). At least two measurements were performed for each transgenic genotype.

Western blot analysis of the E. coli *DHPS in transgenic plants*

Leaves were homogenized and the supernatants collected as described above. Protein (120 μg) was fractionated by SDS–PAGE (Laemmli, 1970) and Western blots were then performed according to Burnette (1981).

Measurements of free lysine level in leaves of transgenic plants

Leaves (100 mg fresh weight) from axenic tobacco plants, grown on Nitsch minimal medium (Nitsch, 1969) in Magenta boxes, were harvested and their free amino acids were immediately extracted as described by Bieleski and Turner (1966). The concentration of free amino acids was determined with the *o*-phthalaldehyde reagent (Hurst, 1984) followed by measuring the 335/447 nm fluorescence. Amino acid composition was determined by loading a sample of 11 nmol on a Hewlett Packard Amino Quant Liquid Chromatograph.

Isolation of intact chloroplasts, measurement of chlorophyll content and analysis of SOD isozymes

Plants B-15 and A-11, expressing, respectively, the chloroplastic- and cytoplasmic-type *E. coli* DHPS, were grown in the greenhouse and transferred to darkness 48 h before harvesting. Intact chloroplasts were then isolated as described previously (Bartlett *et al.*, 1982). Chlorophyll content was measured as previously described (Arnon, 1949). SOD isozymes were analysed by loading extract samples on non-denaturing polyacrylamide gels and staining with riboflavin-nitroblue tetrazolium (Beauchamp and Fridovich, 1971).

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