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Accumulation of maize γ-zein and γ-zein: KDEL to high levels in tobacco leaves and differential increase of BiP synthesis in transformants

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Abstract Two gene constructs (pROK.TG1L and pROK.TG1LK) were utilized to achieve accumulation of maize γ-zein to high levels in tobacco (*Nicotiana tabacum* L.) leaves. Both the chimaeric genes contained the γ-zein-coding region preceded by the 5′untranslated leader from the coat protein mRNA of TMV, but one of them (pROK.TG1LK) was modified in its protein-coding region by the addition of the ER retention signal KDEL. The accumulation of γ-zein and γ-zein:KDEL in leaves was compared with heterologous protein accumulation in tobacco plants previously transformed with a γ-zein cDNA harbouring a native 5′UTR. Replacement of γ-zein 5′UTR with the TMV leader dramatically increased γ-zein production. Furthermore, γ-zein:KDEL-expressing plants, on average, accumulated twice as much foreign protein in their leaves as pROK.TG1L plants. The two-fold increase in the level of γ-zein:KDEL can probably be attributed to an improvement in the mechanism for ER retention of zeins in the transgenic cells. Transformants also showed increased production of BiP, though to a lesser extent in γ-zein:KDEL-expressing plants compared with pROK.TG1L plants. It is therefore likely that γ-zein:KDEL retention is made less dependent on the chaperone assistance of BiP by the presence of the KDEL signal on the γ-zein mutant.

Key words Gene expression · KDEL · *Nicotiana* · Transgenic plants · γ-Zein

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Introduction

Several research projects are currently concerned with achieving a correct balance of the protein content in food crops through genetic engineering. For example, a chimaeric gene specifying seed-specific expression of a sulphur-rich sunflower seed albumin has been stably transformed in *Lupinus angustifolius* (Molvig et al. 1997). In relation to the nutritional improvement of forage legumes, δ-zein, a sulphur amino acid-rich protein, has been expressed in transgenic white clover (Sharma et al. 1998).

Zeins, the major protein reserves of maize kernels, are extensively utilized in genetic engineering programmes, because the structure and physiology of this kind of prolamin are well documented (Shewry et al. 1995). Zeins can be classified into different relative mobility (M_r) groups, α-, β-, δ- and γ-zeins (Wilson 1991), and they accumulate in protein bodies (PBs) arising from the rough endoplasmic reticulum (ER) (Larkins and Hurkman 1978). A possible role of BiP (binding protein) in zein assembly has been suggested from observing an increase in BiP accumulation in maize mutants and transgenic tobacco plants overproducing β- and δ-zeins (Boston et al. 1991; Bagga et al. 1997).

β-, δ- and γ-zeins are rich in the essential sulphur amino acids methionine and cysteine. It has been demonstrated that sulphur-containing amino acids play an important role in the efficiency of dairy, meat and wool production (Reis 1979; Rogers et al. 1979; Barry 1981). Thus, with the long-term goal of improving forage quality, a stable expression of zein transgenes in forage legumes, and in the model species tobacco, has been attempted (Bagga et al. 1995; Bellucci et al. 1997; Sharma et al. 1998). These studies have indicated that transformed plants accumulate zeins in vegetative tissues and opened up the possibility of using zeins to increase the sulphur-rich amino acid content in forage crops.

In an earlier study, we analysed the expression of a γ-zein cDNA (G1L) in tobacco and in two forage species,

Medicago sativa and *Lotus corniculatus* (Bellucci et al. 1997). γ-Zein accumulated only in tobacco tissues, in very low quantities, and the lack of γ-zein was ascribed to post-transcriptional effects. In the present study, we assessed the expression of two new chimaeric γ-zein genes. In the first gene construct, in order to ascertain whether the 5′ untranslated region (5′UTR) of G1L was an obstacle to γ–zein accumulation, this DNA sequence was replaced with the untranslated leader from the coat protein mRNA of the tobacco mosaic virus (TMV). In the second case, a mutated γ-zein cDNA was constructed by adding a short nucleotide sequence coding for the KDEL signal. Significant increases in γ-zein accumulation were obtained with both gene constructs, and γ-zein:KDEL-expressing plants showed the highest level of foreign protein accumulation. Our data also indicate a difference in the increase in accumulation of BiP between transgenics expressing γ-zein:KDEL or native γ-zein.

Materials and methods

Plasmids and plant transformation

Two chimaeric genes encoding γ-zein were constructed; DNA manipulations to obtain plasmid pROK.G1L (see Fig. 1) have already been described (Bellucci et al. 1997).

Plasmid pROK.TG1L was obtained by replacing the 5′ UTR of G1L cDNA with the 5′ TMV translational enhancer sequence. This was done by amplifying the G1L sequence from plasmid pBSKS.G1L (Bellucci et al. 1997) with two PCR primers, G1L-Bs (5′-CGACATCATGAGGGTGTTGCTCGT-3′) and G1L-Ba (5′-CTCGGATCCAGCGGCTATACTACA-3′). While G1L-Bs was positioned from –7 to +17 of the γ-zein sequence (Prat et al. 1987) and the C positioned at –2 was mutated into T in order to create a *Bsp*HI restriction site, G1L-Ba (from +686 to +702 of the γ-zein sequence plus another seven nucleotides) contained a recognition site for the restriction enzyme *Bam*HI. The PCR product was digested with *Bsp*HI and *Bam*HI and inserted into the *Nco*I/*Bam*HI sites of plasmid pHST244 (forming the plasmid pHST.G1L), which harbours the TMV leader (generously provided by Dr. L. Gehrke, Harvard University, MIT, Cambridge, USA). The G1L sequence was then excised as a *Bgl*II/*Bam*HI fragment and cloned into the *Bam*HI site of plasmid pROK8, itself derived from plasmid pBI131.1 (Jefferson 1987), thereby creating pROK.TG1L in which G1L is driven by the ribulose bisphosphate carboxylase small subunit (rbcS) promoter (see Fig. 1).

Plasmid pROK.TG1LK was created by adding nucleotides encoding the peptide TSEKDEL, which incorporates the endoplasmic reticulum (ER) retention signal KDEL, to the G1L protein-coding region. The method reported by Tabe et al. (1995) using PCR was followed. An oligonucleotide identical to the last 14 nucleotides of the G1L protein-coding region plus those coding for the peptide TSEKDEL, was synthesized. After the stop codon, a recognition site for the restriction enzyme *Sac*I was inserted into the oligonucleotide which had the following sequence: 5′-GGCTCGAGCTCGTTCATAGCTCATCTTTCTCACTAGTGTG GGGGACACCG-3′. The G1L sequence was PCR-amplified from plasmid pHST.G1L using this oligonucleotide and another one (5′-GACTCACTATAGATCTAAG-3′) covering the *Bgl*II site placed just upstream from the TMV leader sequence. The amplified DNA fragment was cleaved with *Bgl*II and *Sac*I and cloned first into those same sites of plasmid pSP72 (Promega) to verify the sequence, then into the *Bam*HI/*Sac*I sites of plasmid pROK8, thus obtaining plasmid pROK.TG1LK.

Transfer of plasmids from *Escherichia coli* strain JM 83 into *Agrobacterium tumefaciens* strain LBA4404, as well as transformation of *Nicotiana tabacum* L. plants, cv Petit Havana SR1 with *A. tumefaciens*, were done according to Bellucci et al. (1997). Thirty six G1L transformants were grown in sterile tissue culture boxes and analysed. They included: Seven previously transformed with plasmid pROK.G1L (Bellucci et al. 1997), 14 transformed with plasmid pROK.TG1L and 15 with plasmid pROK.TG1LK.

Isolation and analysis of nucleic acids and proteins

Procedures used for DNA and RNA isolation from leaves and blots were as described previously, as also were the G1L and *npt*II probes employed (Bellucci et al. 1999).

Protein was extracted, measured, fractionated and blotted as described by Bellucci et al. (1997), with the exception of the extraction buffer [100 mM Tris-HCl pH 7.8, 200 mM NaCl, 1 mM EDTA, 4% 2-mercaptoethanol, 0.2% Triton X-100, 1 mM PMSF, 1× protease inhibitor mix (Boehringer)]. Briefly, after centrifugation of the leaf homogenate for 15 min at 17000 g and 5°C, the supernatant was recovered. The pellet was resuspended in 70% ethanol, 2% 2-mercaptoethanol, 1 mM PMSF, and incubated for 20 min at 65°C. The suspension was then centrifuged under the same conditions as above and the alcoholic supernatant was recovered. The pellet was resuspended again in a diluted acid solution, centrifuged and the supernatant recovered. Proteins of the three supernatants were measured by a Bradford assay (Bradford 1976) to determine the total extractable proteins. Protein samples soluble in the saline extraction buffer were fractionated by SDS-PAGE and transferred onto a nitro-cellulose membrane (Schleicher and Schuell). γ-Zein or BiP were detected by standard Western analysis. Anti-γ-zein antiserum was produced by injection at 15-day intervals of 400-μg of purified maize γ-zein (Vitale et al. 1982) and then utilized at a 1:200 dilution. Anti-BiP antiserum, donated by Dr. A. Vitale (Istituto Biosintesi Vegetali, CNR, Milan, Italy), was used at a 1:4000 dilution. The protein bands were visualized with a peroxidase-linked goat anti-rabbit secondary antibody (Pierce) using 4-chloro-1-naphthol (Sigma). For quantitative Western blots, band intensity was calculated by Phoretix 1D software after scanning the nitro-cellulose membranes. The semi-logarithmic relation between protein loading and staining intensity was used for quantification, as reported by Mogelsvang and Simpson (1998).

An analysis of variance for γ-zein accumulation in the transformants was performed. The means of the two pROK.TG1L and pROK.TG1LK plant groups (see Table 2) were subjected to a *t*-test of significance according to Snedecor and Cochran (1967) to test the difference between the means of two independent samples of equal size. The means were calculated based on the values shown in Table 1, after angular transformation (arcsin√proportion) developed for binomial proportions.

Immunoelectron microscopy

Small pieces of leaves from non-transformed and transgenic plants were fixed in 1.5% glutaraldehyde and 1.6% paraformaldehyde in 0.1 M phosphate buffer pH 6.9 for 1 h at room temperature. After washing with 0.1 M phosphate buffer, the samples were dehydrated in ethanol and embedded overnight in LR white resin (Sigma) at 60°C. Immunogold labeling of ultrathin sections mounted on grids was done according to Gonzalez-Melendi et al. (1998), and normal goat serum diluted 1:10 in PBS was used as a blocking solution. Grids were then incubated with anti-γ-zein antiserum (1:200 dilution). Controls were incubated in preimmune rabbit serum. Sections were then incubated with the secondary antibody (1:25 dilution) conjugated with 10-nm gold particles (BioCell). Grids were examined under an electron microscope (Philips EM 400 T).

Results

Plant transformation and characterization of transgenics by Southern and Northern analysis

Plasmids pROK.TG1L and pROK.TG1LK (Fig. 1B and C) were used to transform tobacco plants, and the transgenic plants so obtained were characterized by Southern analysis. DNA was digested with *Hin*dIII or *Xba*I (which cut only once in the T-DNA between the *npt*II and the G1L genes, Fig. 1), and probed with the *npt*II and the G1L probes. In this way, it was possible to determine the T-DNA copy numbers in each transformant, as well as the presence of contiguously duplicated T-DNA such as inverted or direct repeats (Cluster et al. 1996).

Northern-blot analysis was first conducted to verify G1L expression in the transformants at the transcription level (Fig. 2A, B and C). The blots were further analysed with a 18S rDNA probe to standardize RNA loading. The pROK.G1L transformants (Fig. 2A) produced a 1.28 kb transcript, because the G1L cDNA of this plasmid has a 3′UTR about 100-bp longer than those in the other two plasmids, which instead produced a 1.18-kb transcript, as did maize endosperm (Fig. 2B and C).

To determine whether the three gene constructs could have a different effect on the G1L mRNA steady state level, we analysed, in the same Northern blot, plants transformed with each of these constructs (Fig. 2D), followed by quantification of the autoradiographic bands by scanning using a suitable software (Phoretix 1D). The amounts of G1L mRNA were calculated by the ratio between transgene and 18S signals in each plant and expressed in arbitrary units relative to the amount of G1L mRNA present in plant 2A, which expresses the G1L gene at a high level. The average values of the G1L mRNA steady-state level for each construct are shown in Table 2, while individual measurements of the G1L mRNA steady state level for pROK.TG1L and pROK.TG1LK transformants are shown in Table 1. The seven pROK.G1L transformants were not included in Table 1 because they were not characterized by Southern analysis. No significant differences in G1L mRNA

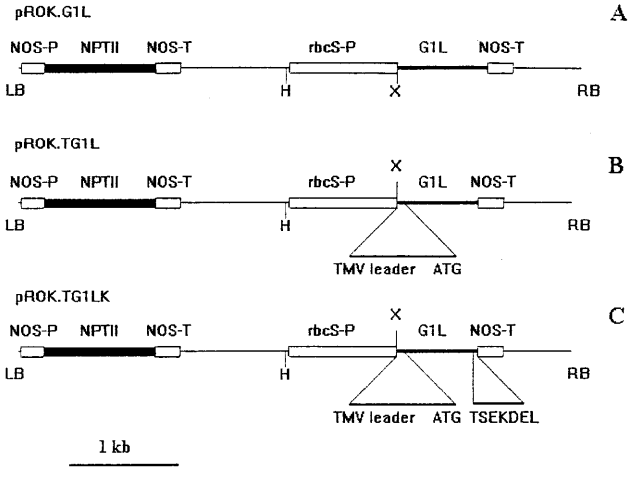

Fig. 1A–C Structures of expression vectors. A T-DNA region of pROK.G1L containing two chimaeric genes. The protein-coding region of the gene neomycin phosphotransferase (*NPTII*) was inserted between the nopaline synthase promoter (*NOS-P*) and the polyadenylation site (*NOS-T*). The G1L cDNA was inserted between the rbcS promoter (*rbcS-P*) and NOS-T. G1L cDNA (880 bp) consists of 67 bp of 5′ untranslated region (5′UTR), 671 bp of coding sequence and 142 bp of 3′UTR. **B** The T-DNA region of pROK.TG1L is the same as that of pROK.G1L except for (1) 5′UTR replacement with the TMV leader (80 bp), and (2) the reduction of 3′UTR length to 30 bp. **C** The T-DNA region of pROK.TG1LK which contained the additional sequence (21 bp) coding for the peptide TSEKDEL. *H Hin*dIII; *X Xba*I; *LB* left border; *RB* right border

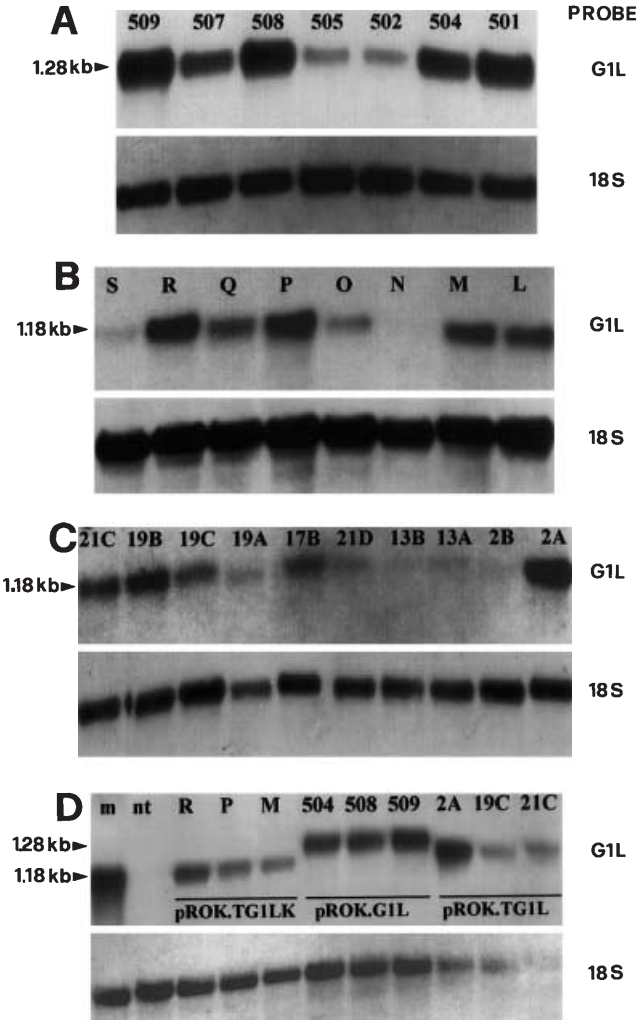

Fig. 2A–D Northern analysis on total RNA (10 µg) from leaves with a G1L probe and a 18S rDNA probe from alfalfa. **A** pROK.G1L transformants. **B** Eight out of fifteen pROK.TG1LK transformants. Plant N was not included in Table 1 because it has a truncated copy of T-DNA with only the *npt*II gene. **C** Ten out of fourteen pROK.TG1L transformants. **D** A comparison between transformants bearing different gene constructs; 0.5 µg of RNA from maize endosperm is loaded in *lane m*, together with 10 µg of non-transformed tobacco RNA. This kind of comparison (Fig.2D) was made twice for all the transformants and plant 2A was always present in each comparison-RNA gel blotting. m maize endosperm; nt non-transformed

Table 1 Characterization of pROK.TG1L and pROK.TG1LK transformants for T-DNA organization, G1L mRNA steady state level and γ-zein accumulation. DR, direct repeat; IR, inverted repeat; nd, not determined; nf, not found

Plasmid	Plant	Number of T-DNA copies	Adjacent T-DNA structures	G1L mRNA, %[a]	γ-Zein, %[b]
pROK.TG1LK	A	nd	nd	2	nd
–	B	3	DR	12	0.74
–	C	3	nf	29	0.53
–	D	3	nf	5	0.12
–	E	2	IR	2	0.31
–	F	4	IR	4	0.04
–	H	3	IR	4	0.44
–	L	3	nf	4	0.03
–	M	3	IR	9	0.43
–	O	2	IR	2	0.02
–	P	4	IR	9	0.08
–	Q	nd	nd	4	0.05
–	R	3	nf	15	0.16
–	S	nd	nd	1	0.14
PROK.TG1L	2A	3	IR	100	0.55
–	2B	8	IR	2	0.05
–	13A	2	nf	5	0.04
–	13B	1	nf	2	nd
–	17B	3	nf	18	0.06
–	17C	2	IR	84	0.03
–	19A	3	IR	7	0.03
–	19B	nd	nd	32	0.02
–	19C	2	IR	20	0.03
–	19E	nd	nd	nd	0.21
–	21C	4	DR	22	0.17
–	21D	4	nf	5	0.02
–	21E	6	DR	28	0.03
–	21F	nd	nd	30	0.02

[a] G1L mRNA steady state levels are expressed as a percentage of the G1L mRNA steady state level (fixed to 100 arbitrary units) in plant 2 A. Each value is the mean of two measurements (see legend to Fig. 2D)

[b] γ-Zein accumulations are expressed as a percentage of total extractable leaf protein. Each value is the mean of three measurements

steady state level were found between plants transformed with constructs pROK.G1L and pROK.TG1L (38.30±8.0 and 27.08±9.3, respectively, Table 2), while the pROK.TG1LK transformants exhibited a markedly lower value (7.28±2, Table 2) than other transformants. This characteristic of pROK.TG1LK transformants was not due to their T-DNA organization; that is, the T-DNA copy number or the presence of inverted or direct repeats which may be involved in repeat-induced silencing (Matzke and Matzke 1995). Actually, the correlation between copy number and gene expression in the transformants of each gene construct, as well as between transformants from different gene constructs, appeared to be indeterminate. In fact, some plants showing the same T-DNA copy number (plants 17C, 19C, E, Table 1) differed in their levels of G1L mRNA; in some cases multi-copy transformants contained less transgene mRNA than low-copy plants (plants 2B and 19C, Table 1); or, conversely, low-copy plants expressed lower G1L mRNA steady state levels than multi-copy plants (plants O and 21C, Table 1). However, the presence of inverted or direct repeats was spread equally across the two groups of transformants; thus, if gene-silencing phenom-

Table 2 Expression levels of chimaeric G1L genes and accumulation patterns of BiP in tobacco transformants. The values are calculated as mean ± (es)

Gene construct	G1L mRNA[a]	γ-Zein[b]	BiP[c]
pROK.G1L	38.30±8.0	–	nd
pROK.TG1L	27.08±9.3	0.10±0.04[d]	301.50±22
pROK.TG1LK	7.28±2	0.24±0.06	127.10±6.3
Non-transformed plants	–	–	83.75±9

[a] The values utilized to calculate the mean are those shown in Table 1, except for pROK.G1L transformants whose G1L mRNA steady state levels are not shown

[b] The values utilized to calculate the mean are those shown in Table 1. The amounts of γ-zein accumulated by pROK.G1L transformants were too low to be measured.

[c] BiP accumulation is expressed as a percentage of BiP accumulation (fixed to 100 arbitrary units) in one non-transformed plant (Fig. 5A, lane 3). In order to calculate the mean, the individual values of all the plants transformed with pROK.TG1L and pROK.TG1LK plasmids were considered, as well as those of ten non-transformed plants (data not shown). Individual values of BiP accumulation originating from quantitative Western blots were made in duplicate (Fig. 5)

[d] The two means of γ-zein accumulation (0.10±0.04 and 0.24±0.06) are significant at $P \leq 0.05$

ena occurred, they probably did not affect the differentiation of the two gene constructs of Table 1 based on G1L expression at the RNA level. The variation in the accumulation of G1L mRNA may have been caused by other factors, for example the location of T-DNAs in the genome (position effect).

G1L expression at protein level

The transformants were also tested for the production of γ-zein by Western blot analysis (Fig. 3A and B). When 40 µg of protein was loaded on the gel, pROK.G1L transformants did not produce detectable amounts of γ-zein (Fig. 3B), since the level of G1L protein in leaves of the highest expressing transformant reached only 0.05% of the alcohol-soluble proteins (Bellucci et al. 1997). On the other hand, replacement of the G1L leader with the TMV leader significantly increased γ-zein accumulation in the pROK.TG1L transformants (Fig. 3B), as well as in the pROK.TG1LK transformants (Fig. 3A). In the latter case, addition of the peptide TSEKDEL raised the molecular mass of the γ-zein mutant from 28 kDa to about 29 kDa. This difference in size was determined by the TSEKDEL amino-acid sequence, because a similar result was observed when a β-zein was mutated by addition of the same seven amino acids (unpublished results).

In order to compare the levels of both native γ-zein and γ-zein:KDEL in transformed plants, quantitative Western blots were attempted (Fig. 3C). Western analysis was carried out in triplicate for each plant using proteins from transformed plants, and purified γ-zein as a standard loaded in different amounts. Both the pROK.TG1L and pROK.TG1LK transformants were analysed in this manner. The amount of γ-zein in each plant was calculated as the mean of the three values arising from three identical blots and expressed as a percentage of total extractable leaf protein. The levels of γ-zein detected in each of the transformants are shown in Table 1, and the mean values for each gene construct in Table 2. Transformants displayed considerable variability in their level of γ-zein and γ-zein:KDEL accumulation. This, however, is hardly surprising because in at least another study a similar degree of intra-variability was observed within several groups of transformants (Ni et al. 1995). Expression of native γ-zein in pROK.TG1L transformants ranged between 0.02 and 0.55% of total extractable protein, and expression of γ-zein:KDEL in pROK.TG1LK transformants ranged between 0.02 and 0.74% of total extractable protein (Table 1). These two ranges of γ-zein accumulation were comparable, but the average values for γ-zein- and γ-zein:KDEL-expressing plants were 0.1 and 0.24% of total extractable protein, respectively (Table 2). The analysis of variance indicated that the difference between these two means is significant at $P \leq 0.05$. The value of P is just within significance limits but, if we leave aside plant 2A which is an outsider to its group of pROK.TG1L plants, the difference between means becomes significant at $P \leq 0.01$.

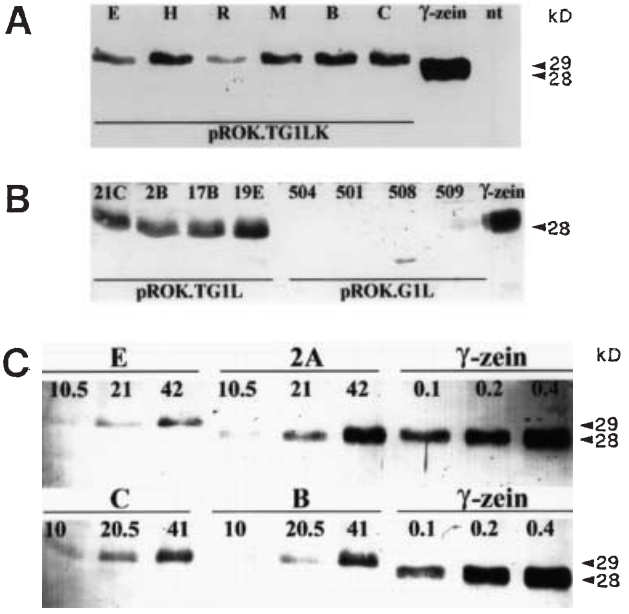

Fig. 3A–C Western analysis of tobacco transformants with the anti-γ-zein antibody. **A** Six pROK.TG1LK transformants and one non-transformed plant; 40 µg of leaf proteins soluble in saline extraction buffer with 4% 2-mercaptoethanol were separated by SDS-PAGE and subjected to Western analysis; 0.4 µg of purified γ-zein extracted from maize endosperm was used as a standard. **B** Four pROK.G1L and four pROK.TG1L transformants. The same analysis as in **A** was performed, except that only 0.2 µg of purified γ-zein was loaded. **C** Quantitative Western blots. Proteins soluble in saline buffer with 4% 2-mercaptoethanol from plants B C, E (pROK.TG1LK transformants) and plant 2A (pROK.TG1L transformant) were loaded in 10, 20 and 40 µg, but in the Figure the corresponding µg of total extractable proteins are indicated. Each blot has three lanes with 0.1, 0.2 and 0.4 µg of purified γ-zein. The two blots were made in triplicate and subjected to Western analysis. *nt* non-transformed

γ-Zein protein accumulated to high levels in both pROK.TG1L and pROK.TG1LK transformants. This fact, together with the extremely low quantity of γ-zein found in pROK.G1L transformants despite their high G1L mRNA steady state levels, suggest that mRNA translational efficiency was a rate-limiting step to G1L protein expression in tobacco, and that the TMV leader enhanced translation of G1L mRNA.

Concerning transformed plants expressing native γ-zein or γ-zein:KDEL, although transformants which were able to accumulate a high level of G1L mRNA usually accumulated a high level of zein protein as well (Table 1, plants B, C, R, 2A, 21C), there was not always a direct correlation between the mRNA level and γ-zein production (Table 1, plants E, H, S, 17C). Thus, the steady state level of mRNA was not as important as mRNA translational efficiency. The data reported in Table 2 support this view because the pROK.TG1LK transformants showed not only the highest value of γ-zein accumulation but also the lowest value of G1L mRNA. The question remains why the addition of the KDEL re-

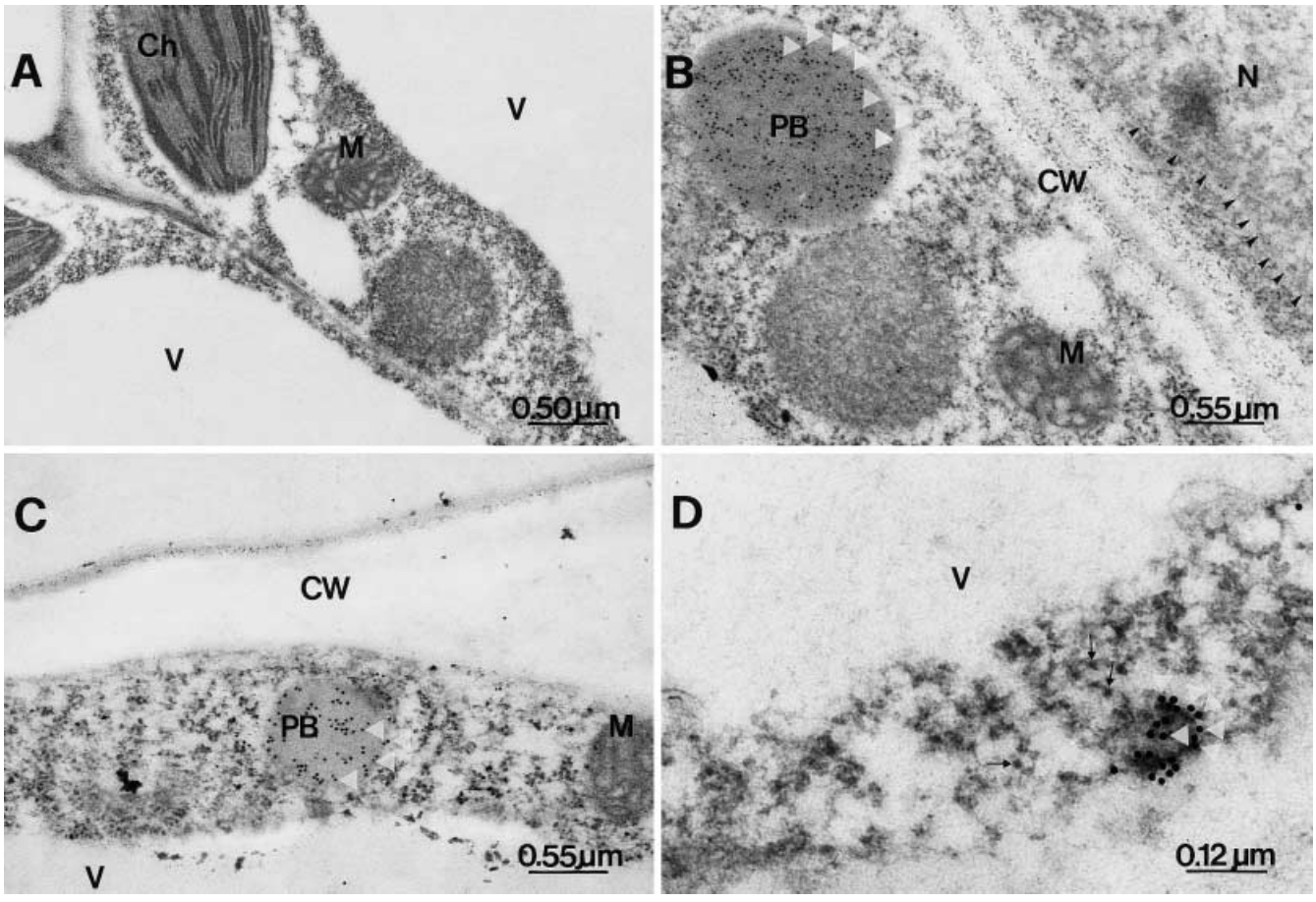

Fig. 4A–D Immunogold localization of γ-zein in transformants. Thin sections of transgenic tobacco leaves were treated with preimmune serum (**A**) or anti-γ-zein antiserum. (**B**, **C** and **D**). **A** Two mesophyll cells of a transformed plant. **B** A typical γ-zein protein body of a mesophyll cell belonging to a pROK.TG1LK transformant (plant B). The *white arrowheads* point to the 10-nm diameter gold-conjugated goat anti-rabbit IgG, while the *black arrowheads* indicate the nuclear membrane. **C** An example of a γ-zein protein body located in an epidermal cell of a pROK.TG1L transformant (plant 2A). **D** Pre-PB inside a leaf cell. Black arrows indicate ribosomes. *Ch* chloroplast; *CW* cell wall; *M* mitochondrion; N nucleus; PB protein body; V vacuole

tention signal caused a two-fold increase in the accumulation of γ-zein in pROK.TG1LK transformants over pROK.TG1L transformants. Therefore, protoplast labeling of zein protein was attempted to evaluate the stability of zein proteins in the transgenic cells. Protoplasts from mesophyll of transformants (pROK.TG1L and pROK.TG1LK) and non-transformed plants were pulse-labelled for 3 h and then chased for different periods of time in the presence of unlabelled amino acids. The fluorograph of immunoprecipitated radioactive proteins from protoplast extracts suggests that γ-zein and γ-zein:KDEL have a similar stability (data not shown). Based on these results, there is evidence that γ–zein accumulation is regulated at both the post-transcriptional level (mRNA translational efficiency) and the post-translational level (retention in the ER facilitated by the KDEL signal).

Immunolocalization studies

In order to elucidate the possible reasons for the two-fold γ-zein:KDEL accumulation in pROK.TG1LK transformants compared with native γ-zein in pROK.TG1L transgenics (Table 2), we analysed the subcellular localization of these proteins in leaf cells of one non-transformed plant (data not shown) and two transformed plants (Fig. 4). In both the pROK.TG1LK (plant B, Fig. 4B) and pROK.TG1L (plant 2A, Fig. 4C) transformants, but not in the non-transformed plant, zein proteins accumulated in spherical or elliptical electron-dense bodies in the cytoplasm, similar to those observed by Coleman et al. (1996) in transgenic tobacco seeds expressing γ-zein. The PBs were 0.6 to 1.5 µm in diameter, were present in either mesophyll cells (Fig. 4B) or epidermal cells (Fig. 4C), and a distinct membrane surrounded them. Pre-PBs structures were also visible that may represent an initial phase of protein body formation (Fig. 4D). γ-Zein antiserum gave specific labeling reactions on the sections, even if background gold particle labeling was present on subcellular structures, such as the nucleus, the vacuoles, the cell walls (Fig. 4B and C), and the chloroplasts (data not shown), both in transformed or non-transformed plants. Fig. 4A shows two transformed mesophyll cells treated with preimmune serum. Our conclusion was that the difference in the level of γ-zein and γ-zein:KDEL accumulation cannot be explained by a differential sequestration of these proteins in the cell.

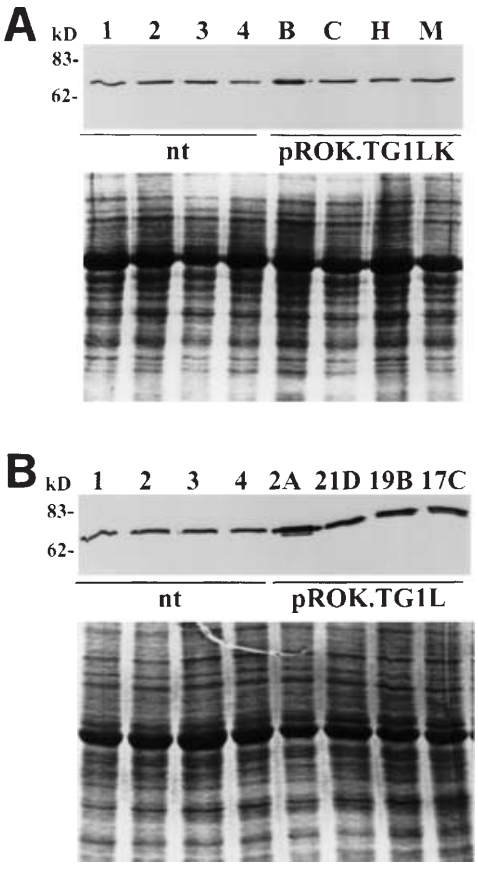

Fig. 5A, B Quantitative Western analysis of tobacco transformants with the anti-BiP antibody. **A** Four non-transformed plants and four pROK.TG1LK transformants; 40 µg of leaf proteins soluble in saline buffer with 4% 2-mercaptoethanol were separated by SDS-PAGE. Three identical gels were made in this manner: one was stained with a solution of 0.1% Coomassie brilliant blue and the other two were subjected to Western analysis in duplicate. **B** Western analysis and staining for protein loading were performed as in **A** on the same four non-transformed plants and four pROK.TG1L transgenics. nt non-transformed

A negative correlation between BiP induction and γ-zein:KDEL accumulation

It has recently been shown that the synthesis of zein proteins in transgenic tobacco plants induces the synthesis, or stable accumulation, of BiP (Bagga et al. 1997). This result is consistent with the idea that BiP may play a role in the formation of zein protein bodies in tobacco transformants, and it is also supported by the implication of BiP in prolamin protein body biogenesis as observed by Li et al. (1993). In pROK.TG1L and pROK.TG1LK transformants BiP accumulation was also increased, as demonstrated by the mean values obtained by quantitative immunoblot analysis using a BiP antibody (Table 2). We measured BiP accumulation in plants transformed with these two gene constructs and in ten non-transformed plants as a control. Western analysis (Fig. 5) and quantification were performed in the same way as for γ-zein (Fig. 3C), except that each protein sample on the blots was loaded in one amount instead of three different amounts. The increase in the level of BiP in γ-zein-expressing plants was almost four-fold higher than in non-transformed plants, while in γ-zein:KDEL-expressing plants the corresponding increase was only 1.5-fold. Thus, although the pROK.TG1LK transformants had the higher level of γ-zein accumulation, they showed a lower increase in BiP accumulation than pROK.TG1L transformants.

Discussion

Poor accumulation of heterologous proteins is often a problem with transgenic plants, thus making it necessary to modify the original gene construct in order to increase protein expression. Actually, in the tobacco plants transformed with the pROK.TG1L construct, γ-zein accounted for up to 0.55% of total extractable protein, while protein was expressed at very low levels in plants transformed with the pROK.G1L construct. γ-Zein was correctly expressed and stored in tobacco seeds (Coleman et al. 1996) and in leaves (between 0.01 and 0.1% of total protein) of *Arabidopsis thaliana* (Geli et al. 1994) by using the native 5′UTR. This indicates that the 5′UTR of the γ-zein gene assures gene expression, but if protein accumulation is to be increased further, it is necessary to replace the native 5′UTR with a sequence such as the TMV leader which, like the alfalfa mosaic virus (AMV) leader, enhances the translatability of messenger RNA (Jobling and Gehrke 1987).

Maize γ-zein is retained within the ER lumen and assembled with other zeins, giving rise to PBs. Geli et al. (1994) have shown that γ-zein possesses a N-terminal proline-rich tandem repeat necessary for ER retention. We generated γ-zein:KDEL to verify if the addition of the KDEL motif could further enhance γ-zein accumulation in tobacco plants by increasing the efficiency of the mechanism that directs protein retention in the ER.

On average, transgenic pROK.TG1LK plants, accumulated twice as much γ-zein in their leaves as did pROK.TG1L transformants (0.24% vs 0.1% of total extractable protein, respectively). Many factors may contribute to the expression of a foreign gene in the host cell. These include site and copy number of gene integration, and the degree of gene silencing (Matzke and Matzke 1995). We can exclude that the difference in γ-zein expression between pROK.TG1LK and pROK.TG1L transformants was due to the factors mentioned above. In fact, tobacco transformants of these two groups of plants had a variable number of T-DNA copies, from 1 to 8, and the T-DNA copy number and amount of γ-zein transcript were not always correlated. Furthermore, measurements of mRNA levels showed that it was not possible to attribute enhanced accumulation of γ-zein:KDEL to an increase in the corresponding mRNA. The occurrence of gene-silencing phenomena was not completely ruled out, but, considering the relatively high number of plants analysed and the variability in T-DNA rearrangements within the two groups of transgenics, we can exclude

that gene silencing, if it occurred at all may have had a different effect on the average transgene expression of the two populations.

With reference to the influence of zein accumulation on BiP synthesis, our data, in accordance with those reported by Bagga et al. (1997), show that there is a higher accumulation of BiP in tobacco transformants expressing the zein genes. Furthermore, there is a differential accumulation of BiP in tobacco transformants, because in pROK.TG1LK plants the increase in BiP synthesis is less than in pROK.TG1L plants. If we assume that the γ-zein retention mechanism in the ER and the subsequent zein protein body formation in tobacco are mediated by BiP, it is reasonable to conclude that, while the presence of native γ-zein in transformed tobacco plants increases BiP synthesis, the γ-zein:KDEL protein is probably less dependent on the chaperone assistance of BiP because the mutated γ-zein has the same canonical ER retention sequence as BiP. In this case, the native γ-zein will be retained in the ER via its tandem-repeat proline-rich domain as a result of γ-zein/BiP association (Geli et al. 1994; Williamson 1994). The fact that γ-zein:KDEL possesses its own retention signal, though not likely to prevent the association of some γ-zein:KDEL molecules with BiP through the tandem-repeat proline-rich domain, might allow the ER retention of other γ-zein:KDEL polypeptides simply by the interaction of the KDEL placed in their sequence with the membrane-bound receptor localized outside the ER (Galili et al. 1998), and without requiring BiP assistance. This could explain why the stimulation of BiP synthesis in pROK.TG1LK transformants is reduced compared with pROK.TG1L transformants, but always present in comparison to non-transformed plants. The two-fold increase in the level of γ-zein:KDEL could probably be attributed to an improvement in the mechanism for ER retention of zeins in the transgenic tobacco cells. Since ER is the natural environment for zein deposition, the increase in γ-zein:KDEL accumulation was not so conspicuous as in the case of the seed protein vicilin exposed in transgenic plants to the proteases of the leaf vacuole, which showed a 20 to 100-fold increase in accumulation after being re-targeted to the ER (Wandelt et al. 1992).

In this study, some tobacco transformants accumulated γ-zein in their leaves at up to 0.74% of total extractable protein. Though this may be considered a good starting point, further improvements must still be made to significantly increase the nutritional quality of forages, considering that foreign proteins must account for 1–5% of the total protein in the leaf (Wandelt et al. 1992). The highest level of sulphur-rich proteins ever achieved in transgenic leaves ranged from 0.1 to 0.8% (Tabe et al. 1995; Khan et al. 1996; Sharma et al. 1998) and all those foreign proteins stored in PBs derived from ER membranes. ER is considered a favourable environment for a significant level of protein accumulation; however, up to now, the highest levels of expression of transgenes in plants have been achieved for genes coding for cytosolic enzymes (Ku et al. 1999). Apart from the intracellular location involving protein stability, other strategies can be exploited to maximize transgene expression, including crossing the best γ-zein-expressing plants or co-expressing different zeins (Coleman et al. 1996; Bagga et al. 1997). Furthermore, it is also possible to modify the amino-acid composition of the zein proteins through the introduction of methionine/cysteine-rich coding sequences or the conversion of some amino-acidic residues into methionine or cysteine.

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