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ACCUMULATION OF MUTATED MAIZE ZEINS IN TRANSGENIC FORAGE LEGUMES

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Abstract

Accumulation of zeins, the endosperm storage proteins of maize, in a heterologous plant expression system was attempted. Plants of birdsfoot trefoil (*Lotus corniculatus)* and alfalfa (*Medicago sativa)* were transformed by *Agrobacterium* with binary vectors harboring genes that code for γ-zein and β-zein, two proteins rich in sulphur amino acids. Adding the ER retention signal KDEL to the C-terminal domain modified zein polypeptides. Our long-term goal was to improve birdsfoot trefoil and alfalfa forage quality. Significant levels of γ-zein:KDEL and β-zein:KDEL were detected in primary transformants of birdsfoot trefoil. Moreover, alfalfa plants expressing γ-zein:KDEL in the leaves were obtained. γ-zein:KDEL accumulated in spherical or elliptical electron-dense bodies of birdsfoot trefoil leaves. The protein bodies were present in the cytoplasm of either mesophyll cells or epidermis cells.

Keywords: *Agrobacterium*, forage quality, KDEL, *Lotus corniculatus*, *Medicago sativa*, recombinant proteins, sulphur-rich amino acids

Introduction

Recently, with the long-term goal of improving forage quality by increasing its sulphur-rich amino acid content, a stable accumulation of heterologous proteins was attempted in forage legumes, as well as in the model species tobacco (Bellucci et al., 1997; Sharma et al., 1998). Zeins, the foreign proteins used in our studies, are the endosperm storage proteins of maize. They can be classified into different relative mobility (M_r) groups, α-, β-, δ- and γ-zeins, and, except for α-zeins, are all rich in the essential sulphur amino acids methionine and cysteine.

In this study, zein polypeptides were modified by adding to the C-terminal domain a short peptide incorporating the ER retention signal KDEL, in order to improve zein accumulation (Bellucci et al., 2000). We focused our attention on the level and cellular site of γ-zein:KDEL and β-zein:KDEL accumulation in transgenic plants of birdsfoot trefoil (*Lotus corniculatus)* and alfalfa (*Medicago sativa).*

Material and Methods

The construction of the two chimaeric genes encoding γ-zein:KDEL and β-zein:KDEL has been described previously (Bellucci et al., 1999). pROK.TG1LK harbored the γ-zein:KDEL DNA sequence under the control of the tobacco ribulose bisphosphate carboxylase small subunit (rbcS) promoter, and p121.1.G2 contained the β-zein:KDEL coding sequence under the control of the cauliflower mosaic virus (CaMV) 35S promoter.

Alfalfa plants were transformed only with pROK.TG1LK harboured by *A.rhizogenes* (Damiani and Arcioni, 1991), while both vectors were used to infect birdsfoot trefoil. Embryogenesis and regeneration of plantlets from *L.corniculatus* hairy roots were achieved as described in Arcioni et al. (1988).

Plant material (0.5 g) was homogenized in liquid nitrogen with sand. Proteins were extracted using 1.2 ml extraction buffer [100 mM Tris-HCl pH 7.8, 200 mM NaCl, 1 mM

EDTA, 0.2% Triton X-100, 1 mM PMSF, 1x protease inhibitor mix (Boehringer)]. After centrifugation for 15 min at 17,000 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. Proteins of both the aqueous and alcoholic extracts were measured by Bradford assay (Bradford 1976). Protein samples were fractionated by SDS-PAGE in a 16% gel, and electrophoretically transferred onto nitro-cellulose membrane (Schleicher and Schuell). Zeins were detected by standard western analysis with rabbit polyclonal antisera (anti-γ-zein antiserum at 1:200 dilution and anti-β-zein antiserum at 1:400 dilution). The protein bands were visualized with peroxidase-linked goat anti-rabbit secondary antibody (Pierce) using 4-chloro-1-naphthol (Sigma).

Small pieces of leaves from non-transformed and transgenic plants were fixed in 1.6% (w/v) paraformaldehyde mixed with 1.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer pH 6.9 for 1 h at room temperature. Procedures used for embedding samples and immunogold labelling were described in Gonzalez-Melendi et al. (1998). The grids were examined under an electron microscope (Philips EM 400 T).

Results and Discussion

L.corniculatus plants transformed with plasmid p121.1.G2 showed a marked variability in β-zein:KDEL accumulation. The molecular mass of the mutated zein was slightly higher than expected, likely due to the addition of the peptide TSEKDEL: 16 kD instead of 15. Regarding *L.corniculatus* transformants expressing γ-zein:KDEL, some plants accumulated significant amounts of the heterologous protein, while others had extremely low levels of it (Figure 1). The molecular mass of the immunoreactive bands was higher than expected. Specifically, plants 5 and 16 had one main band 32 kD in molecular mass and a

minor one, more evident in plant 16 than in plant 5, of a molecular mass between 60 and 70 kD, while plants 24 and 20 had a 35 kD main band. Such differences in the γ-zein:KDEL molecular mass inside the group of *L.corniculatus* transformants, could be generated by post-translational modifications such as those observed for some γ-zein mutated forms in *Arabidopsis* (Alvarez et al., 1998). The immunoreactive bands in Figures 1 were diffused, due to the presence of some compounds in the ethanol-soluble fraction of the leaves that retards the migration of the zein proteins in SDS-PAGE. Preliminary quantitative Western blots indicated that expression of both γ-zein:KDEL and β-zein:KDEL in *L.corniculatus* transformants ranged between 0.01 and 0.3 % of total extractable protein. γ-zein:KDEL was also highly expressed in alfalfa.

For immunolocalization of zeins, we examined young leaves of non-transformed and *L.corniculatus* pROK.TG1LK plants. γ-zein:KDEL accumulated in elliptical electron-dense bodies in the cytoplasm of mesophyll cells in transformants (Figure 2). A distinct membrane surrounded these organelles, of a diameter ranging from 0.6 to 1.5 μm, and sometimes a partial coating of ribosomes appeared on their surface.

Our results demonstrated that γ- and β-zein:KDEL can be efficiently accumulate in *L.corniculatus*, and γ-zein:KDEL in alfalfa. This encouraged us to follow the strategy of coexpress in the same plant these two polypeptides, and at the moment we are crossing *L.corniculatus* plants with different zein genes. Moreover we are transforming alfalfa with β-zein gene.

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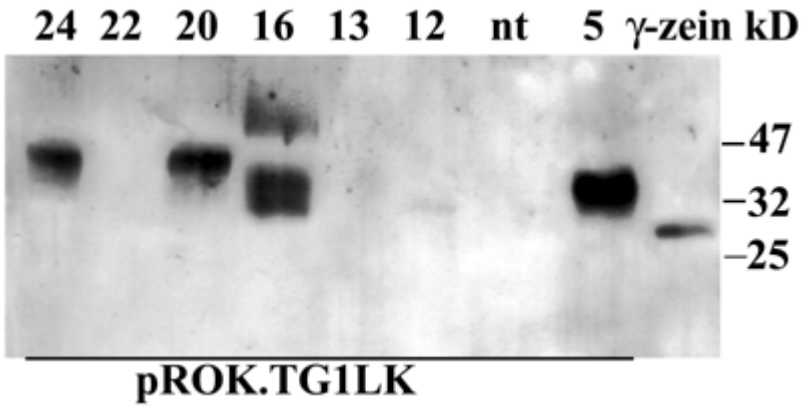

Figure 1 - Expression of γ-zein:KDEL in transgenic *L.corniculatus* plants. Immunoblot using γ-zein antibody of proteins extracted from leaves of pROK.TG1LK transformants. 25 µg of ethanol-soluble proteins was loaded on the gel and a non-transformed plant was used as negative control. A positive control containing 0.2 µg of purified γ-zein was loaded together with 25 µg of non-transformed plant proteins. Molecular-mass markers (in kD) are indicated at right.

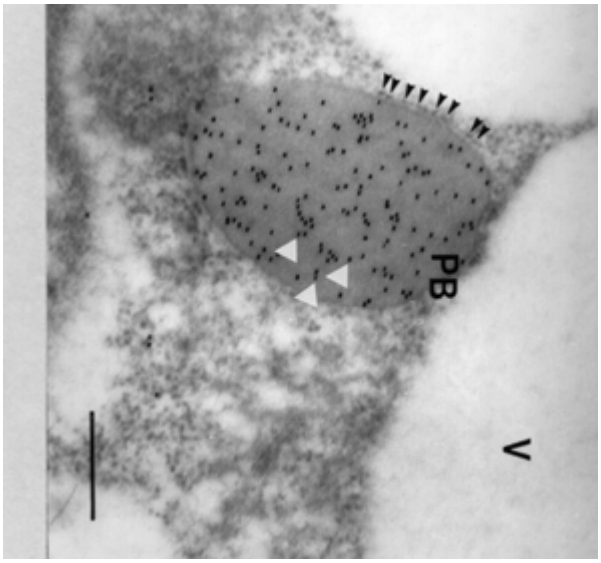

Figure 2 - Immunogold localization of γ-zein:KDEL. Ultrathin sections of leaves were treated with γ-zein antibody. A typical γ-zein protein body is evident in a mesophyll cell of a transformed *L.corniculatus* plants. Black arrowheads indicate ribosomes, while the 10 nm in diameter gold-conjugated goat anti-rabbit IgG are indicated by white arrowheads. PB, protein body; V, vacuole. Bars = 0.3 µm.