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Expression of maize genes in transgenic forage legumes

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SUMMARY – Plants of birdsfoot trefoil (*Lotus corniculatus*) and alfalfa (*Medicago sativa*) were transformed by *Agrobacterium* with binary vectors harbouring genes that code either for wild-type and -zein:KDEL, or for -zein:KDEL. The maize seed storage proteins -zein and -zein are rich in sulphur amino acids and our long-term goal was to improve alfalfa and birdsfoot trefoil forage quality. Significant levels of zeins were detected in leaves of birdsfoot trefoil transformants, ranging up to 0.055% and to 0.30% of total extractable protein for -zein and -zein:KDEL, respectively. In leaves of alfalfa, -zein:KDEL expression level was up to 0.026% of the total extractable protein. Coexpression of the -zein and -zein genes in tobacco proved a useful strategy to increase the content of zein polypeptides and at the moment we are analysing *L. corniculatus* plants derived from the cross of parents expressing different zein genes.

Key words: *Agrobacterium*, forage quality, *Lotus corniculatus*, *Medicago sativa*, sulphur-rich amino acids.

RESUME – *"Expression des gènes du maïs chez des légumineuses fourragères transgéniques". Des plants de lotier corniculé (*Lotus corniculatus*) et de luzerne (*Medicago sativa*) ont été transformés génétiquement par des vecteurs binaires d'*Agrobacterium *contenant des gènes codant soit pour le type sauvage et -zein:KDEL, ou soit pour -zein:KDEL. Les protéines du grain de maïs -zein et -zein sont riches en acides aminés à haute teneur de sulfure, et notre objectif à long terme était d'améliorer la qualité du fourrage à base de luzerne et de lotier. Des teneurs significatives de zéines ont été détectées dans des feuilles de lotier génétiquement transformées, avec des taux respectifs de protéines totales extractibles de 0,055% et 0,30% pour -zein et -zein:KDEL. Le niveau d'expression de -zein:KDEL avait augmenté jusqu'à 0,026% de la quantité totale de protéines extractibles dans les feuilles de luzerne. La co-expression des gènes -zein et -zein chez le tabac s'est révélée une strategie utile pour augmenter le contenu en polypeptides zein et nous analysons à présent des plants de* L. corniculatus *dérivés de parents exprimant des gènes zein différents.*

Mots-clés : Agrobacterium, *qualité des fourrages,* Lotus corniculatus, Medicago sativa*, acides aminés riches en sulfure.*

Introduction

Alfalfa (*Medicago sativa* L.) is the most important protein source in animal fodder, which explains why great efforts have been made to improve the forage quality of this crop. There have been different approaches based on genetic transformation to reach this aim, including the improvement of alfalfa protein composition through expression of genes coding for sulphur-rich proteins in leaves and stems (Tabe *et al*., 1995; Bellucci *et al*., 1997). The use of genes coding for sulphur-rich proteins in the transformation of forage legumes is based on evidence that sulphur-containing amino acids have an important role in the efficiency of dairy, meat and wool production (Barry, 1981; Pickering and Reis, 1993).

Zeins, the foreign proteins used in our studies, are the endosperm storage proteins of maize, they are usually rumen-protected (Sharma *et al*., 1998), and, except for -zeins, are all rich in the essential sulphur amino acids methionine and cysteine. Stable expression of zein transgenes has been achieved in alfalfa and in the model species *L. corniculatus*. *L. corniculatus* was chosen as a model legume because it is particularly suitable for genetic transformation and tissue-culture, as demonstrated by the wide use of this species in other studies (Bavage *et al*., 1997). Forage legumes were transformed with two cDNAs, one coding for a -zein and the other for a -zein. Several gene constructs, with either native or mutated zeins, were used for plant transformation. Mutated zeins were obtained by adding the ER retention signal KDEL to the C-terminal domain in order to improve zein accumulation (Bellucci *et al*., 2000b). Significant -zein accumulation was achieved in alfalfa and birdsfoot trefoil, while -zein-expressing transformants have so far been obtained only in birdsfoot trefoil. Our objective is to coexpress -zein with -zein in alfalfa to maximise the accumulation of sulphur-rich zeins in this species.

Materials and methods

Four binary vectors, all containing the selectable marker *npt*II plus other genes, were used in our transformation experiments. p121.G1L contained a -zein cDNA (G1L) under the control of the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (*nos*) terminator. pROK.TG1L was obtained by replacing the 5' UTR of G1L cDNA with the 5' translation enhancer sequence of the tobacco mosaic virus (TMV) and then inserting the fragment TMV leader-G1L between the rbcS promoter and the *nos* terminator. pROK.TG1LK was the same as pROK.TG1L, except that nucleotides encoding the peptide TSEKDEL, which incorporates the endoplasmic reticulum retention signal KDEL, were added to the G1L protein-coding region. The -zein coding sequence under the control of the CaMV 35S promoter, whose 3'end was fused to the 5'untranslated region of the coat protein mRNA from the alfalfa mosaic virus was cloned into plasmid pBI121.1. The resulting plasmid was named p121.1.G2. The binary vectors were mobilised from *E. coli* strain JM 83 into *Agrobacterium tumefaciens* strain LBA4404 or *Agrobacterium rhizogenes* strain NCPPB 1855. Alfalfa plants were infected with *A. rhizogenes* harbouring pROK.TG1LK. *L. corniculatus* plants were transformed with p121.1.G2, pROK.TG1L, and pROK.TG1LK harboured by *A. rhizogenes.* The p121.G1L *L. corniculatus* transformants analysed derived from seeds harvested on a p121.G1L primary transformant crossed with a non-transformed plant.

Genomic DNA was isolated from leaves according to Saghai-Maroof *et al*. (1984) with minor modifications. Procedures used for DNA blot were identical to those described previously (Bellucci *et al*., 1999). Protein analysis has been described previously (Bellucci *et al*., 2000a), as well as RNA analysis (Bellucci *et al*., 2000b).

Results and discussion

The transgenic plants obtained were characterized by Southern analysis, which revealed the presence of several clonal plants resulting from the same transformation event based on identical restriction fragment patterns. The presence of G1L mRNA was analysed by northern blotting and almost all the transformants had G1L mRNA, albeit its steady-state level for each plant varied greatly. Generally, multi-copy transformants had a high steady-state level of G1L transcript, but the transformant with the highest transcript level was *L. corniculatus* plant p121.G1L-c2, which had only two inverted-repeat G1L copies. Thus, differences in the amount of G1L transcript could be due to the promoter used (CaMV 35S or rbcS) or the position effect, rather than the number of transgene copies. Plants transformed with zein genes were assayed for production of zein polypeptides by "quantitative" western blot analysis (Table 1).

Maximum observed expression levels of zein proteins in tobacco were 0.74% and 0.55% of total extractable protein (Bellucci *et al.,* 2000b). When forage legumes are considered, zein polypeptides were expressed at lower levels based on total protein (Table 1).

Low expression in forage legumes is largely due to the high protein content of their leaf tissue. Alfalfa and birdsfoot trefoil leaves typically yielded about 5 times more protein per unit of fresh weight than was obtained from tobacco leaves. Consequently, in tobacco there are higher relative values of zein accumulation because this species has a lower content of protein than forage legumes. For a practical application, insufficient zein accumulation in these genetically modified plants is certainly a major problem. In other studies, the highest zein expression levels recorded in transgenic leaves were comparable with our own values (Sharma *et al.,* 1998). In order to increase zein accumulation in transgenic plants, we crossed two tobacco plants each expressing a different zein gene. The amount of zeins was 1.1% of the total extractable protein in one plant. For our purposes, this value is nearly adequate and could probably be further increased by passing from a hemizygotic to a homozygotic condition of the zein locus/i. In the forage legume species, it would be very interesting, due to their tetraploid status, to see if an increase in the zein allele number (from one to four) would result in a corresponding increase in the amount of zein polypeptides.

Conclusions

Other strategies must be exploited to maximize transgene expression in forage legumes in order to achieve practical results. One could be the identification of strong promoters other than 35S or rbcS, but, according to us, an even more promising strategy would be that of localizing heterologous proteins in the chloroplast.

Table 1. Protein expression levels of zein genes in leaves of birdsfoot trefoil and alfalfa (pROK.TG1LK-A1, -A2, and -A6) transformants. The values are calculated as the mean of three measurements ± (es)

Transformant	-zein or -zein:KDEL	
	% of alcohol-soluble protein	% of total extractable protein
p121.G1L-c2	2.96 ± 0.21	0.055 ± 0.003
p121.G1L-c7	1.11 ± 0.12	0.022 ± 0.006
pROK.TG1LK-5	1.48 ± 0.07	0.037 ± 0.010
pROK.TG1LK-16	0.74 ± 0.06	0.015 ± 0.008
pROK.TG1L-D	0.14 ± 0.02	<0.001
pROK.TG1L-O	0.22 ± 0.03	<0.002
pROK.TG1LK-A1	0.53 ± 0.02	0.011 ± 0.005
pROK.TG1LK-A2	1.1 ± 0.04	0.021 ± 0.007
pROK.TG1LK-A6	1.0 ± 0.15	0.026 ± 0.010
	-zein:KDEL	
	% of alcohol-soluble protein	% of total extractable protein
p121.1.G2-1	12.9 ± 0.90	0.22 ± 0.02
p121.1.G2-11	12.7 ± 1.20	0.30 ± 0.05
p121.1.G2-30A	0.74 ± 0.03	0.02 ± 0.006
p121.1.G2-33A	3.33 ± 0.42	0.08 ± 0.01
p121.1.G2-34B	1.85 ± 0.06	0.17 ± 0.02
p121.1.G2-56A	6.29 ± 0.54	0.11 ± 0.01

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