Genetics and Molecular Biology, 22, 3, 445-449 (1999)

EXPRESSION OF A METHIONINE-RICH STORAGE ALBUMIN FROM THE BRAZIL NUT (*Bertholletia excelsa* H.B.K., LECYTHIDACEAE) IN TRANSGENIC BEAN PLANTS (*Phaseolus vulgaris* L., FABACEAE)

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ABSTRACT

Bean (*Phaseolus vulgaris*), an important component in the diet of people in developing countries, has low levels of the essential amino acid, methionine. We have attempted to correct this deficiency by introducing a transgene coding for a methionine-rich storage albumin from the Brazil nut via biolistic methods. The transgene's coding sequence was driven by a doubled 35S CaMV promoter and AMV enhancer sequences. The transgene was stable and correctly expressed in homozygous R_2 to R_5 seeds. In two of the five transgenic lines the methionine content was significantly increased (14 and 23%) over the values found in untransformed plants.

INTRODUCTION

The advent of recombinant DNA technology combined with plant tissue culture methods has made it possible to introduce foreign genes into host plants. This has created hopes that nutritionally deficient crops could be corrected through the introduction and expression of suitable transgenes.

In Latin America, India and parts of Africa, the bean (*Phaseolus vulgaris* L., Fabaceae) and broad bean (*Vicia faba* L., Fabaceae) are among the most important grain legumes used for human consumption. However, although beans are rich in some essential amino acids, e.g., lysine, threonine, valine, isoleucine and leucine, their nutritional value is limited because of the small amounts of the essential amino acid methionine and cysteine (Ma and Bliss, 1978). One strategy to correct this deficiency is to introduce transgenes encoding a methionine-rich storage albumin from the Brazil nut (*Bertholletia excelsa* H.B.K., Lecythidaceae) into bean hosts.

We have isolated and characterized one of the Brazil nut's 2S-albumin genes (*be2s1* gene) (Gander *et al*., 1991). This gene codes for an 18-kDa precursor protein, which is processed by a complex series of steps to yield two subunits of 3 and 9 kDa, joined by disulfide bridges (Sun *et al*., 1987). This protein (2S-BN protein) contains 18.8% methionine and is targeted to the seed protein vacuoles (Altenbach *et al*., 1987).

Sequences coding for 2S albumins have been expressed in several species, such as tobacco (*Nicotiana tabacum* L., Solanaceae), canola (*Brassica napus* L., Cruciferae), field bean (*Vicia nabornensis* L., Fabaceae), potato (*Solanum tuberosum* L., Solanaceae) and thale cress (*Arabidopsis thaliana* (L.) Heynh., Cruciferae). Increased levels of methionine in seeds of transgenic *N. tabacum* (30%), *B. napus* (11 to 33%) and *A. thaliana* (20%) have been reported (Altenbach *et al*., 1989, 1992; De Clercq *et al*., 1990; Guerche *et al*., 1990; Conceição *et al*., 1994; Saalbach *et al*., 1994; Tu *et al*., 1994; Pickardt *et al*., 1995). A methionine-rich sunflower 2S-albumin gene has been used to increase the methionine content of grain and pasture legumes (Tabe *et al*., 1993).

We have reported the transient expression of the Brazil nut 2S-albumin gene in cells of the bean embryonic axis after transformation by particle bombardment (Aragão *et al*., 1992). However, a reproducible system for stable transformation and subsequent regeneration of bean plants was not established; thus, no transgenic seeds were obtained. Recently, we have overcome this handicap and transgenic bean plants *(P. vulgaris)* containing and expressing the 2S-albumin gene were obtained through biolistic processes (Aragão *et al*., 1996). Here we report the expression of the 2S-albumin gene from the Brazil nut in several lines of transgenic beans.

MATERIAL AND METHODS

Plant material

Transgenic bean plants (*P. vulgaris*) cv Olathe were obtained via particle bombardment of the apical meristematic region of embryos (Aragão *et al*., 1996). Plants were co-transformed with plasmids pEA23 and pBI426 (circular form). Plasmid pEA23 contains the β-glucuronidase (GUS) coding region (*uidA* gene) under control of the 35S CaMV promoter and the 2S-albumin gene from the Brazil

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nut (*be2s2*) (Gander *et al*., 1991), driven by the doubled 35S CaMV promoter plus the AMV leader sequence (Datla *et al*., 1993). Plasmid pBI426 containing the *uidA-neo* gene fusion (Datla *et al*., 1991), driven by the doubled 35S CaMV promoter plus an enhancer sequence from AMV, was provided by Dr. William Crosby (Plant Biotechnology Institute, Saskatoon, Canada). The *neo* gene confers resistance to the antibiotic kanamycin. Four lines (B34-5, E36-6, F40-4 and G41-14) containing the *be2s1* and *uidA* and one line (D35-11) containing only the *gus* gene were analyzed. The plants were cultivated in a greenhouse at 28ºC and a relative humidity of 70%. The R_1 seeds of 30 plants of each line were planted to obtain R_2 plants. The lines that had *uidA* and *be2s2* genes in all plants were considered homozygous. These plants were self-pollinated until the R_5 generation.

ELISA and Western blot

Seed proteins were extracted from a pooled sample of four mature or immature (15 days after flowering) bean seeds from each line. The seeds were powdered in liquid nitrogen and homogenized in 3 ml of extraction buffer I (10 mM sodium phosphate, pH 7.2, 0.5 M NaCl, 1 mM PMSF and 10 mM 2-mercaptoethanol). The extracts were vigorously shaken at 4ºC for 30 min and centrifuged at 10,000 *g* for 15 min. The supernatant was recovered and used for ELISA and Western blots. Indirect ELISA was carried out according to Hornbeck (1991). Each well was loaded with 200 µg of total protein. Five wells were analyzed for each plant line. Each line was analyzed three times. The Western blot was conducted as described by Grossi de Sá *et al*. (1988). Seventy micrograms of total protein for each sample was fractionated using SDS-PAGE on a 12% acrylamide gel and electroblotted onto a nitrocellulose membrane. The 2S-BN protein was identified using an anti-2S-BN polyclonal antibody raised in mice, and rabbit anti-mouse IgG conjugated to alkaline phosphatase and visualized using 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium. Purified 2S-BN protein (500 ng) was used as a positive control. The mouse antibodies utilized were produced in BALB/c mice according to Grossi de Sá *et al*. (1988), against HPLC- and SDS-PAGE-purified 2S-BN protein.

Extraction of seed proteins and amino acid analysis

Three pooled seeds of each bean line were ground in liquid nitrogen to a fine powder. The powder was defatted three times with 25 ml of hexane for 10 min at room temperature with periodic agitation, followed by centrifugation at 6,000 *g*, for 6 min, at 4ºC. The supernatant was discarded. The defatted material was dried in a Savant Speed Vac Concentrator, and stored at -20ºC. The powder (110 mg) was dissolved in 880 µl of extraction buffer II (0.5 M NaCl in 50 mM phosphate buffer, pH 7.2). The extraction was carried out in a 1.5-ml Eppendorf tube with constant rotation, at 4ºC, for 4 h. The protein extract was clarified through repetitive centrifugation at 9,000 rpm in an Eppendorf centrifuge at room temperature and dialyzed in a microdialyzer system 500 (Pierce, USA) fitted with a 3.5-kDa cutoff dialysis membrane, for 15 h, at 4ºC, against Milli-RO water, at a flow rate of 10 ml/min. The dialyzed sample was dried in the Speed Vac and stored in a freezer. Each sample (0.2 mg) was oxidized with 700 µl of performic acid for 4 h at 0ºC (Moore, 1963). The reaction was stopped with 4 ml of MilliQ water, followed by Speed Vac drying. Each sample was solubilized with 100 µl of 0.1 N HCl, and transferred to a narrow glass tube, which was placed inside a hydrolysis tube in a Pico-Tag workstation (Waters, USA). The sample was dried under vacuum. Gas-phase hydrolysis was performed by adding 200 µl of 6 N HCl at the bottom of the hydrolysis tube. After three cycles of nitrogen and vacuum purge, hydrolysis took place at 150ºC, for 1 h, under vacuum. The hydrolyzed sample was dried, dissolved in 60 µl of 0.1 N HCl, and transferred to a microcentrifuge tube, which was centrifuged at 10,000 rpm for 5 min, at room temperature. Three 10-µl replicas of hydrolyzed supernatant were assayed in a Hitachi L8500 amino acid analyzer. Each line was analyzed three times. The amino acid methionine was determined as methionine sulfone, and cysteine and cystine were determined as cysteic acid.

Statistical testing

The data were analyzed using the program SigmaStat™ (Kuo *et al.* 1992). The Tukey test was used to make comparisons among the mean amino acid composition (percentage) and optical density in ELISA analysis of salt-soluble protein from transgenic and non-transgenic bean seeds.

RESULTS AND DISCUSSION

The 2S-BN methionine-rich albumin comprises 30% of the total seed protein of the Brazil nut (*B. excelsa*). The 18-kDa precursor is synthesized on membrane-bound polysomes, transferred through the secretory pathway into the endomembrane system and, presumably, from the Golgi apparatus, into the protein bodies (Shewry *et al*., 1995). During biosynthesis, at least three stepwise cleavages are involved at the termini and internally. The final protein consists of two polypeptide chains of 9 and 3 kDa, linked by a disulfide bridge (Sun *et al*., 1987).

2S-albumin expression

Expression of the 2S-BN protein in the cotyledon tissues of transgenic bean plants was determined by Western blot (Figure 1) and ELISA (Figure 2). The Western

blot analysis revealed that the 2S-BN protein was correctly expressed in some of the transgenic bean lines (B34-5, E36-6, F40-4 and G41-14), as judged from the presence of the mature 12-kDa protein (Figure 1). No signals were observed in the control D35-11, containing only the *uidA-neo* fusion gene, and in the untransformed control. It was not possible to detect differences in the amount of 2S protein in the transgenic mature seeds. ELISAs were performed on immature and mature seeds (Figure 2). Lines B34-5, D35-11 and F40-4 revealed no significant signal increase when compared to the control plants. Both lines, E36-6 and G41-14, showed 2S-BN protein accumulation in immature and mature seeds, with greater accumulation in immature seeds. During maturation, the 2S-BN protein content in these lines decreased (Figure 2). This suggests that the 2S proteins are either not stored correctly and degraded prematurely or that the 2S-specific mRNA is less stable in the storage cells of beans than in those of the Brazil nut. The Western blot analyses performed in this study were over-incubated and stained, so it is possible that saturation had an influence, making it impracticable to read these results quantitatively.

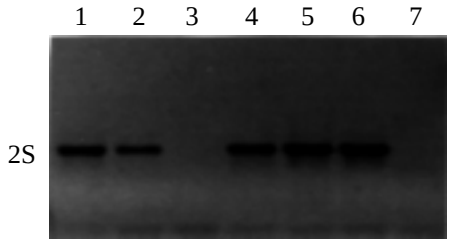

Figure 1 - Western blot analysis using the anti-2S-BN protein antibody to probe protein extracts of soluble protein of mature seeds of transgenic bean plants. Lane 1: 500 ng of purified 2S-BN protein; lane 2: line B34-5; lane 3: line D35-11; lane 4: line E36-6; lane 5: line F40-4; lane 6: line G41-14; lane 7: negative control (non-transgenic plant). Each slot was loaded with 70 μg of total protein.

Amino acid composition of seeds

The amino acid composition of seeds of five transgenic lines and one control was analyzed (Table I). The methionine content significantly increased in all 2S-transgenic lines (B34-5, E36-6, F40-4 and G41-14) from 10 to 23% over the values of the control. As expected, line D35-11, which contains only the *uidA-neo* fusion genes, showed no significant increase.

Transgenic canola and tobacco seeds expressing the *be2s1* sequence driven by the β-phaseolin promoter had an increase of 11 to 33% in methionine content (Altenbach *et al*., 1989, 1992). In *V. nabornensis* transformed with 2S-albumin-coding sequences under the control of the legumin B4 promoter, a 3% increase in total seed proteins was reported (Saalbach *et al*., 1995). Brazil nut 2S sequences driven by the analogous 2S promoter from *A. thaliana* caused a 20% increase in methionine content in transgenic *A. thaliana* (Conceição *et al*., 1994). However, whenever 2S-BN sequences were expressed under the control of the 35S CaMV promoter, the expression levels observed were insufficient to alter the methionine content in the target plants, e.g., *S. tuberosum* (Tu *et al*., 1994), *V. narbonensis* (Saalbach *et al*., 1995) and *N. tabacum* (Marcellino *et al*., 1996).

Since the pioneering work of Murai *et al*. (1983),

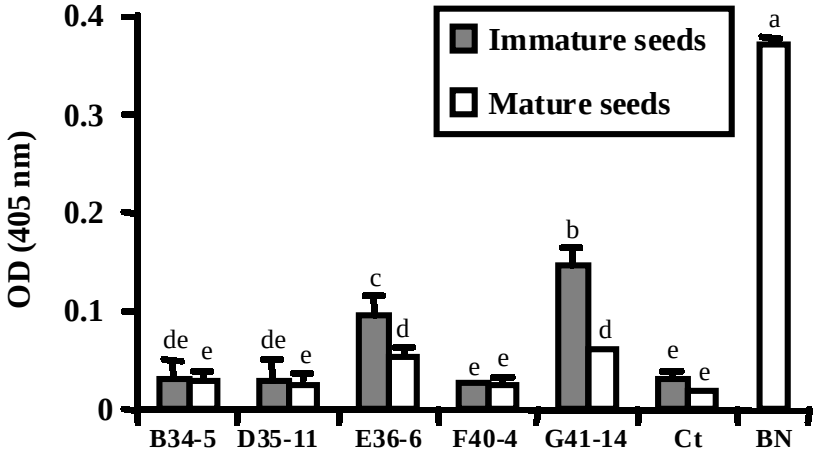

Figure 2 - ELISA for detection of the 2S albumin from the Brazil nut in protein extracts from mature and immature (15 days after flowering) cotyledons from transgenic bean plants. Each well was loaded with 200 μg of total protein and the test was carried out as described in Material and Methods. B34-5, D35-11, E36-6, F40-4 and G41-14: transgenic plants; Ct: non-transgenic; BN: total protein extract from the Brazil nut. Means with different letters are significantly different ($P < 0.05$) according to Tukey's test.

Table I - Amino acid compositions (%) of salt-soluble proteins from transgenic and non-transgenic (control) mature bean seeds and from the Brazil nut. The transgenic lines B34-5, E36-6, F40-4 and G41-14 contain the *be2s1* and *gus* gene. The transgenic line D35-11 contains only the *gus* gene.

Amino acid	B34-5	D35-11	E36-6	F40-4	G41-14	Control	Brazil nut
Ala	6.76[a]	6.78[a]	6.72[a]	6.76[a]	6.79[a]	6.65[a]	4.22[b]
Arg	8.41[b]	8.57[b]	8.55[b]	8.26[b]	8.43[b]	8.53[b]	17.81[a]
Asp	11.90[a]	11.70[a]	12.00[a]	11.67[ab]	11.63[b]	11.97[a]	6.71[c]
$CySO_3H$	1.40[b]	1.27[b]	1.49[b]	1.41[b]	1.06[b]	1.31[b]	2.48[a]
Glu	15.19[c]	17.14[b]	14.97[c]	15.01[c]	14.84[c]	15.13[c]	19.29[a]
Gly	7.73[a]	7.10[b]	7.48[a]	7.54[a]	7.73[a]	7.97[a]	7.12[b]
His	2.55[a]	2.55[a]	2.43[a]	2.60[a]	2.61[a]	2.48[a]	1.58[b]
Ile	3.63[a]	3.66[a]	3.68[a]	3.60[a]	3.64[a]	3.54[a]	2.05[b]
Met	2.27[c]	2.00[d]	2.21[c]	2.19[c]	2.46[b]	1.99[d]	13.10[a]
Phe	6.75[a]	7.43[a]	7.21[a]	6.73[a]	7.50[a]	6.81[a]	3.54[b]
Pro	3.60[bc]	3.43[c]	3.49[cb]	3.55[cb]	3.37[c]	3.86[b]	4.46[a]
Thr	4.59[a]	3.44[a]	4.32[a]	4.59[a]	4.42[a]	4.46[a]	1.74[b]
Val	4.98[a]	5.03[a]	4.98[a]	5.07[a]	5.42[a]	4.97[a]	3.42[b]

a, b, c, d: Mean values in the same columns with different superscripts are significantly different ($P < 0.05$) according to Tukey's test.

expressing the bean phaseolin gene in sunflower (*Helianthus annuus* L., Compositae), numerous attempts to express chimeric genes encoding storage proteins such as the 2S-BN protein have been made. With the exceptions cited above, very low levels of trans-protein accumulation have been achieved (De Clercq *et al*., 1990; Guerche *et al*., 1990; Saalbach *et al*., 1994) and in almost no case could observable changes in seed amino acid composition be observed.

In our study, we transformed bean plants with a chimeric construct containing the doubled 35S CaMV promoter plus the AMV enhancer sequence, assuming that its performance would be superior to the native 35S promoter. Indeed, in two of the transgenic lines a 14 and 23% increase of methionine was achieved.

In view of these results, we are now assessing the possibility of achieving higher levels of methionine in transgenic beans by using homologous and seed-specific promoters such as the β-phaseolin promoter. These results will form the foundation for the production of genetically engineered commercial varieties of beans containing high levels of proteins rich in essential amino acids.

ACKNOWLEDGMENTS

We are grateful to Drs. Juan Arrieta and David John Bertioli for their critical reading of the manuscript and to Nuno Manuel Domingues for technical assistance in protein extraction and amino acid analysis. This work was supported by a grant of PADCT/CNPq.

RESUMO

O feijão (*Phaseolus vulgaris* L.) é um componente importante na dieta da população de países em desenvolvimento. Entretanto, possui um baixo nível de aminoácidos essenciais, como a metionina. Numa tentativa de corrigir esta deficiência, plantas transgênicas de feijão foram produzidas contendo o gene de uma proteína rica em metionina, a albumina 2S da castanha do Brasil. O gene desta albumina (*be2s2*), clonado sob o controle do promotor 35S dobrado do vírus do mosaico da couve-flor e uma seqüência "enhancer" do vírus do mosaico da alfafa, foi introduzido em feijão através do processo biobalístico. O gene foi expressado corretamente em sementes homozigotas desde a segunda até a quinta geração. Em duas linhagens transgênicas o nível de metionina foi incrementado em 14 e 23% nas sementes.

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(Received June 10, 1998)