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The sulphur-rich Brazil nut 2S albumin is specifically formed in transgenic seeds of the grain legume *Vicia narbonensis*

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Summary

Epicotyl explants were co-cultivated with *Agrobacterium tumefaciens* EHA101 to transfer a chimeric 2S albumin gene construct carried in the binary Ti plasmid vectors pGSGLUC1 or pGA472 into the grain legume *Vicia narbonensis*. This gene encoding the sulphur-rich Brazil nut albumin was under the control of either the CaMV 35S promoter which permits gene expression in all organs, or the *Vicia faba* legumin B4 promoter which elicits seed-specific gene expression. After callus formation and selection for kanamycin resistance, somatic embryos were induced which, in the case of transformation with the vector pGSGLUC1, were screened for GUS activity. Embryos that produced GUS were in addition analysed for 2S albumin formation. Selected transgenic embryos were cloned by multiple shoot regeneration. Rooted and fertile plants were obtained by grafting transgenic shoots on the appropriate seedlings. R_1 and R_2 generations were raised and analysed for GUS as well as 2S albumin gene expression.

Expression of the 35S promoter/2S albumin gene fusion took place in all organs of the transgenic plants including the cotyledons of seeds, whereas seed-specific gene expression was found in transformants with the legumin promoter/2S albumin gene fusion. The 2S albumin accumulated in the 2S protein fraction of transgenic seeds and its primary translation product was processed into the 9 and 3 kDa polypeptide chains. The foreign protein was localised in the protein bodies of the grain legume. Analysis of the R_2 plants indicated Mendelian inheritance of the 2S albumin gene. In homozygous *V. narbonensis* plants the amounts of 2S albumin were twice that present in the corresponding heterozygous plants. Whereas only low level formation of the foreign protein was achieved if the gene was under the control of the 35S promoter, approximately 3.0% of the soluble seed protein was 2S albumin if seed-specific gene expression was directed by the legumin B4 promoter. Some of these transformants exhibited a three-fold increase in the methionine content of the salt-soluble protein fraction extracted from seeds.

Abbreviations: 35S – cauliflower mosaic virus 35S protein gene, GUS – β-glucuronidase, NPTII – neomycin phosphotransferase II, LeB4 – *Vicia faba* legumin B4 gene, 2S albumin – Brazil nut (*Bertholletia excelsa*) 2S albumin, ER – endoplasmic reticulum, rER – rough endoplasmic reticulum, HPLC – high pressure liquid chromatography

Introduction

One long-standing economic goal for the genetic manipulation of maize, cereals and grain legumes is to improve their nutritional quality. The amino acid composition of the total seed protein does not correspond to the dietary needs of humans and monogastric animals. The very low level of lysine in the most important cereal crops and the very limited methionine content of legume grains, decreases the biological value of their seed protein to 50 to 75 percent when compared with a balanced amino acid diet or chicken egg protein. In cultures with primarily vegetarian diets such as India, this imbalance can lead to forms of malnutrition in which children less than 4 years of age suffer from retarded mental and physical development (Waterlow

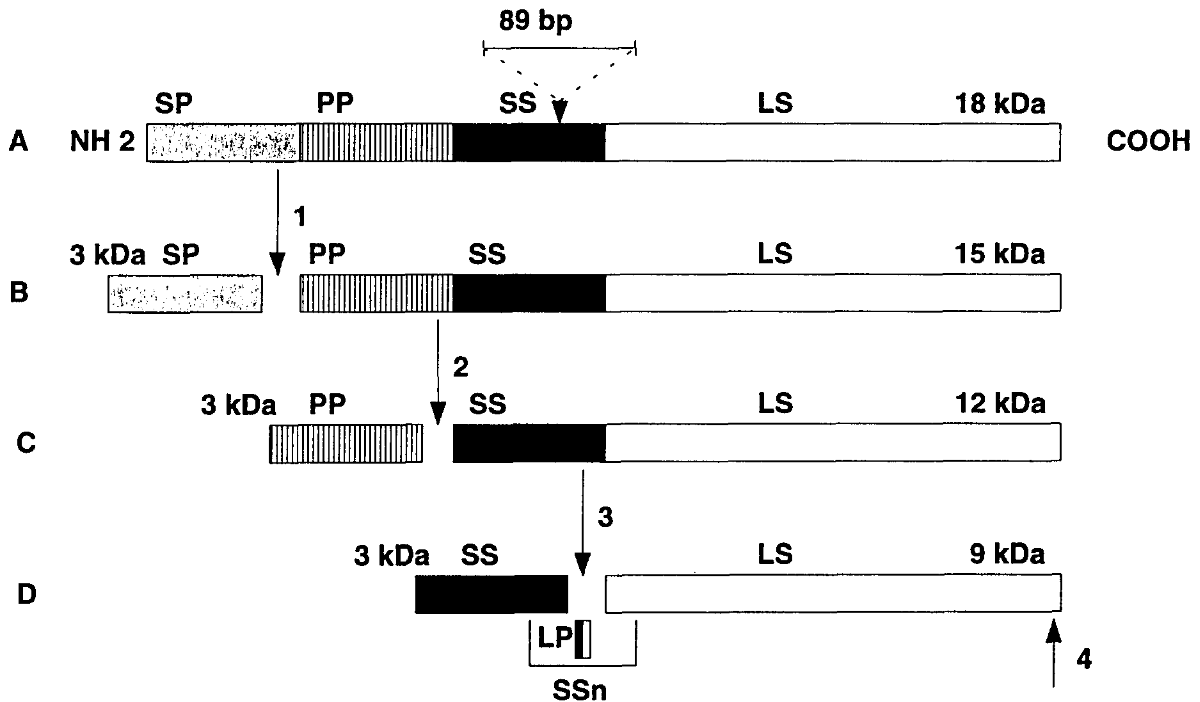


Fig. 1. The 2S albumin of Brazil nut (*Bertholletia excelsa* H.B.K.) and its precursors. A: The pre-propolypeptide encoded by the 2S albumin-specific cDNA (Altenbach et al., 1987; Sun et al., 1987); the position of the only intron in the corresponding gene is indicated by an arrow head (Gander et al., 1991). B–D: Processing steps 1 to 3 and the different products of the 2S albumin precursor maturation by limited proteolysis. Step 1 detaches the signal peptide (SP) from the 18 kDa propolypeptide and step 2 the propeptide (PP) from the 15 kDa precursor that still comprises the small (SS) and large (LS) subunit. A peptide with 5 amino acid residues (LP) links the small and large subunit and is cleaved off during step 3 of the limited proteolysis. Step 4 indicates the cleavage of a tetra peptide from the C-terminus of the large subunit. SSn, disulphide linkages between SS and LS.

& Payne, 1975). The imbalanced amino acid composition of the above-mentioned crops also affects strongly the efficiency of livestock fattening. The more closely the amino acid composition corresponds to the nutritional demands, the more efficient the transformation of seed protein nitrogen into human or animal protein nitrogen becomes. This not only alleviates human malnutrition and improves the efficiency of livestock fattening but concomitantly it decreases the amount of secreted nitrogen that can lead to environmental pollution (Kirchgessner et al., 1994). Without doubt the genetic improvement of the nutritional quality of the most important cereals like maize, rice, wheat or barley, and of many grain legumes remains one of the challenges for breeding and genetic engineering.

Despite the discovery of high-lysine mutants of maize and barley beginning in the sixties (Nelson et al., 1965; Munck, 1970; Green & Phillips, 1974; Hibberd & Green, 1982), progress has been slow in achieving the above-mentioned goals. New hopes have been inspired with the advent of the genetic engineering of crop plants. One promising strategy for improving the nutritional quality of legumes is to introduce into their genomes a gene encoding the extremely methionine-rich 2S albumin from Brazil nut, *Bertholletia excelsa* H.B.K. (Altenbach et al., 1987). The practicality of this approach was demonstrated in transformation experiments carried out with tobacco (Altenbach et al., 1989) and rape (Altenbach et al., 1992). The foreign protein accounted for at most 8 and 4%, respectively, of the salt-soluble protein in the transgenic seeds which represents an increase in the methionine content of the seed protein of approximately one third.

The stable transformation of grain legumes along with subsequent regeneration of fertile plants and germ line transmission of the foreign gene has been reported only few times in the literature. Transgenic pea plants (*Pisum sativum* L.) were obtained by Puonti-Kaerlas et al. (1992). They were able to demonstrate Mendelian inheritance of the hygromycin phospho-

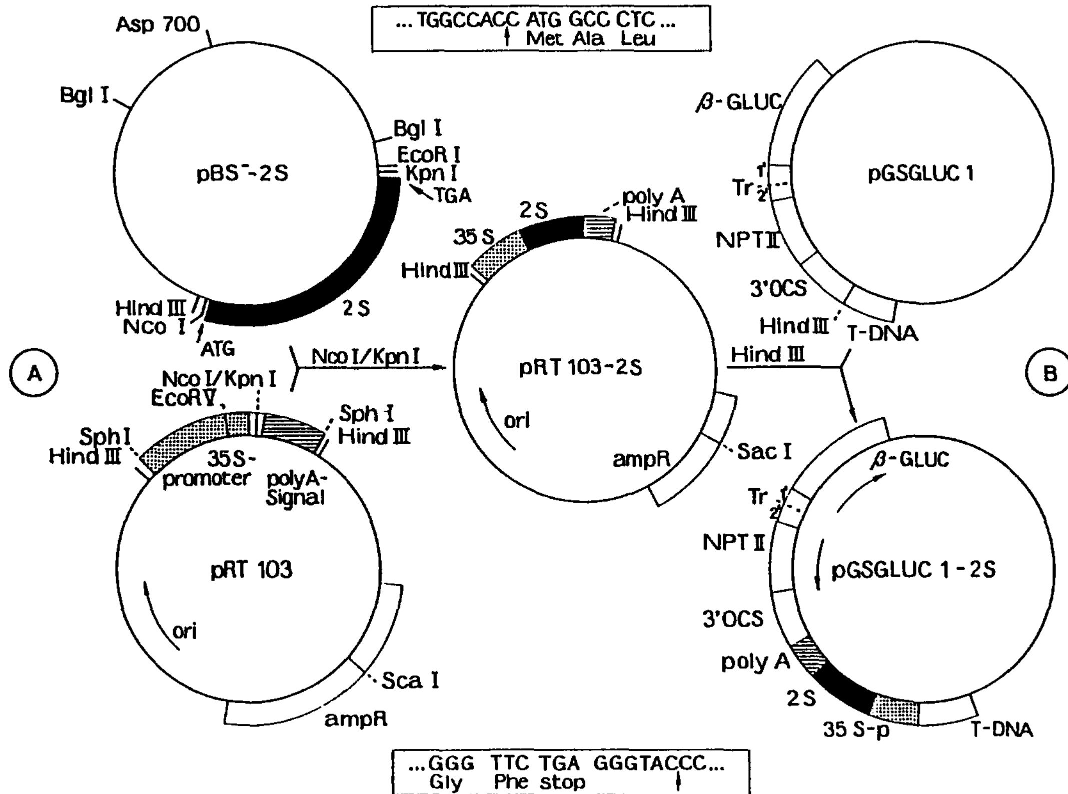


Fig. 2. Assembly of the CaMV 35S promoter and terminator with the DNA encoding the 2S albumin pre-propolypeptide and subsequent insertion of the completed fusion gene into the binary vector plasmid pGSGLUC1. A: The 2S albumin gene (filled bar) was taken from pBS⁻-2S and inserted into the polylinker of plasmid pRT103 (Töpfer et al., 1987) giving plasmid pRT103-2S. B: The HindIII fragment of pRT103-2S, which comprises the complete gene construct, was inserted into the HindIII site of pGSGLUC1, leading to the binary vector pGSGLUC1-2S which carries the 2S albumin gene together with the CaMV 35S gene promoter (stippled) and polyA addition signal (hatched). The inserts at the top and bottom of the figure indicate the junction sequences at the start and stop codons, respectively, between the 2S albumin-coding DNA and the CaMV 35S gene sequences in plasmid pRT103. Other structural elements in the various constructs are indicated by open boxes.

transferase gene that had been used as a selection marker to obtain the transgenic plants. Recently, Schroeder et al. (1993) demonstrated the expression and Mendelian inheritance of the bacterial phosphinotricin acetyltransferase gene which conferred resistance to the herbicide Basta in stably-transformed peas. Aragao et al. (1992) reported transient expression of the Brazil nut 2S albumin gene in cells of mature *Phaseolus vulgaris* embryos after particle bombardment. Stably-transformed *Pisum sativum* and *Vicia faba* tissues expressing the 2S albumin gene were produced by Saalbach et al. (1994), however no fertile plants could be regenerated from these tissues. Seed-specific expression of the 2S albumin gene in stably-transformed grain legume (*V. narbonensis*) regenerants that inherit the gene in the germ line has recently been achieved (Saalbach et al., 1995).

Materials and methods

Transformation and regeneration systems

We have synthesised a DNA encoding the 12 kDa precursor of the 3 and 9 kDa polypeptides (Saalbach et al., 1994) following the published cDNA sequence of the Brazil nut 2S albumin (Altenbach et al., 1987). This DNA was brought under the control of either the 35S promoter and terminator (Fig. 2) taken from plasmid pRT103 (Töpfer et al., 1987) or the legumin B4 promoter and terminator (Bäumlein et al., 1986, 1991, 1992). Whereas the former directs the expression of foreign genes in all tissues, although to different extents (Benfey et al., 1990), the latter imposes seed-specific and developmentally-regulated gene expression (Bäumlein et al., 1987, 1988). The fusion genes were inserted into the appropriate sites of the bina-

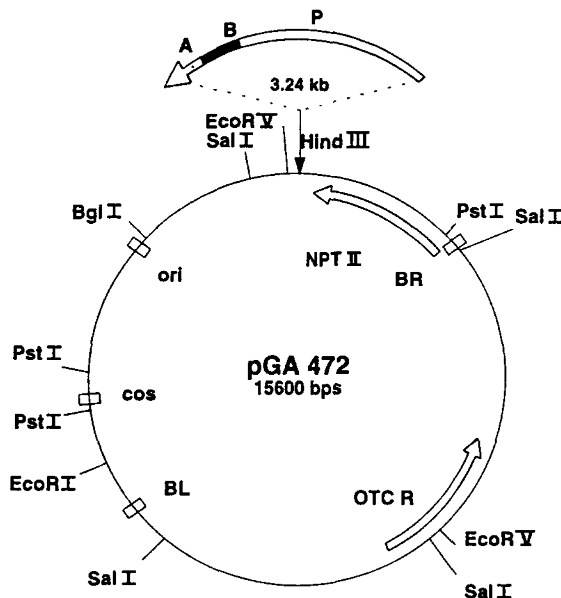


Fig. 3. Insertion of the 2S albumin gene into the HindIII site of the binary vector plasmid pGA472. The inserted fragment comprises the 2S albumin gene (B, 0.441 kb) under the control of the legumin B4 promoter (P, 2.4 kb) and terminator (A, 0.4 kb) both from *Vicia faba*. In order to test the inserted T-DNA for recombination events 3 different DNA probes were used in Southern blotting: 1 – 2S albumin gene fragment, 2 – *nptII* gene fragment, and 3 – a fragment specific for the region containing the origin of replication (ori).

ry vector plasmid pGSGLUC1 giving pGSGLUC1-2S (Fig. 2), or pGA472 (An et al., 1985) giving pGA472-2S (Fig. 3), both with the bacterial *npt II* gene as a selectable marker. The former plasmid contains the screenable *gus* gene whereas the latter does not. These binary Ti-plasmid vectors were used for *Agrobacterium*-mediated gene transfer to *V. narbonensis*.

Transformation and regeneration of the legume were achieved following the protocol of Pickardt et al. (1991). Epicotyl explants were co-cultivated with the *Agrobacterium tumefaciens* strain EHA101 (Hood et al., 1986; McGranahan et al., 1990) carrying the corresponding binary vector plasmid. After callus formation and selection for kanamycin resistance in the presence of 30 μg/ml geneticin, somatic embryos were induced which in transformation experiments with pGSGLUC1-2S were screened for GUS activity using the histochemical method of Jefferson (1987). Selected GUS-positive embryos were cloned by multiple shoot regeneration. Regenerated shoots had to be grafted onto roots of 5 to 10-day-old *V. narbonensis* seedlings in order to overcome rooting difficulties, as mentioned by Pickardt et al. (1991). Transgenic R_1 and R_2 plants were raised after self pollination.

In some cases transgenic tobacco was used for comparison. This plant was transformed according to the leaf disc protocol of Horsch et al. (1985).

DNA purification and Southern blotting

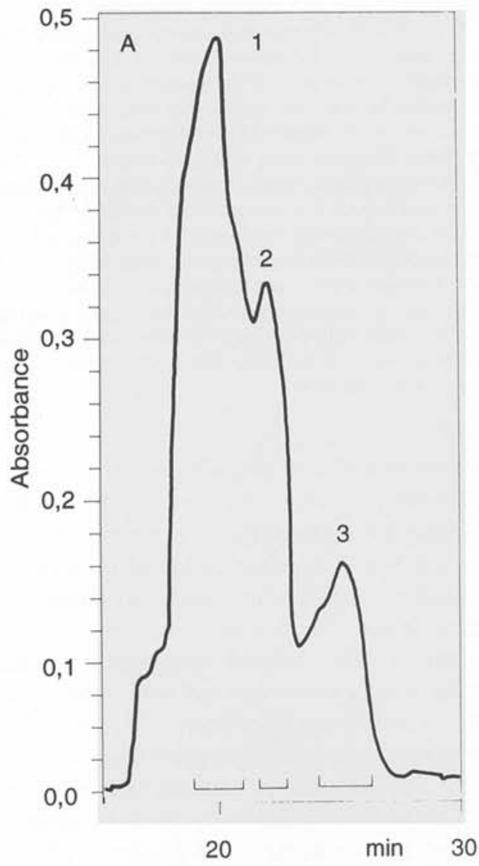
DNA was extracted from young leaves according to Bäumlein et al. (1987) and used for Southern blotting (Sambrook et al., 1989). The HindIII fragment from pRT103-2S was used as a probe in tests with DNA from *V. narbonensis* which was transformed with pGSGLUC1-2S. Since extensive recombination was observed with transformants bearing the LeB4/2S albumin fusion gene, probes specific for the *nptII* gene, for the 2S albumin gene and for the replication origin were used.

Segregation analysis

Self pollination of R_0 and R_1 plants respectively, was used to generate R_1 and R_2 plants for inheritance analysis. Leaf or seed extracts were tested for 2S albumin expression using protein fractionation by SDS polyacrylamide gel electrophoresis (Laemmli, 1970) with subsequent immunoblotting (Karey & Sirbasku, 1989). In addition, screening for *gus* gene expression (Jefferson, 1987) was performed with transformants bearing the 35S/2S albumin gene fusion.

2S-albumin purification and localisation

Different organs or tissues of the transformants were extracted with buffered SDS solutions and the extracts were analysed as follows (see also Saalbach et al., 1994). Fractionation of the legume seed protein extracts by HPLC on a LKB Ultropack TSK G3000 column (7.5 × 600 mm) separated the albumin-containing 2S protein fraction from other storage proteins (Figs 4A and B), like legumin and vicilin, which turned out to be free of 2S albumin when analysed by Western blotting (Fig. 4C). When the SDS gels were run under reducing conditions, the 12 kDa holoprotein was fractionated into the 9 and 3 kDa polypeptides (Fig. 4D). This indicates that the precursors underwent correct processing as far as this can be seen from molecular weight determination in SDS gels. The result confirms that the machinery for limited proteolytic processing of this type of 2S storage protein must have been highly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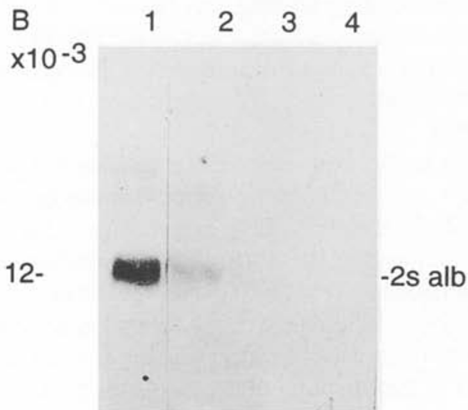
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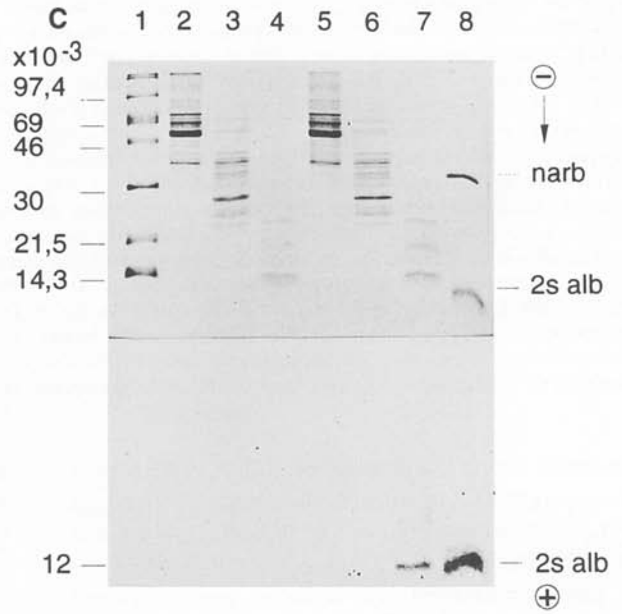
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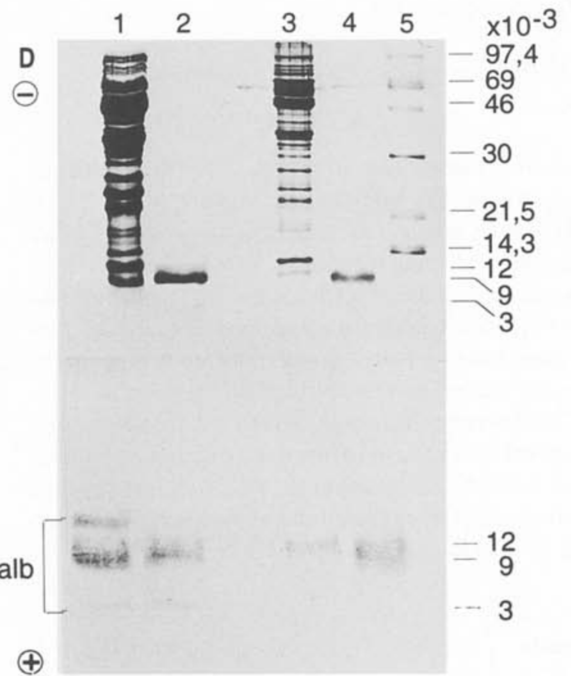
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Fig. 4. Analysis of SDS-extracted proteins from transgenic *Vicia narbonensis* seeds. A: Elution profile of a 100 μl extract prepared from a seed of a plant that had been transformed with the 35S promoter-controlled 2S albumin gene giving only low level expression in this organ. The extract was fractionated by HPLC on a LKB Ultropac TSK G 3000SW column (7.5 × 600 mm) in SDS extraction buffer. Fractions indicated by brackets were electrophoretically analysed. B: Fractions of peak 3 were pooled before running the SDS polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions with subsequent immunoblotting (lane 2). Purified 2S albumin (20 ng, lane 1) and extracts from seeds of wild-type plants (lane 4) were used as controls. For comparison the original seed extract was included (lane 3). C: Comparative electrophoretic analysis of the three fractions from the gel filtration elution profile presented in A. After pooling each fraction and concentrating to 50 μl, aliquots were run in SDS gels according to Laemmli (1970). Peak eluates from wild-type seeds (lanes 2–4) were compared with corresponding eluates from transgenic seeds (lanes 5–7). Lane 1, molecular weight markers, lane 8, mixture of authentic narbonin (narb), an endogenous 2S globulin from *V. narbonensis* seeds, with 2S albumin (2S alb) preparations. The upper panel shows a Coomassie stained gel slab on which no differences in banding patterns are visible in a comparison of the corresponding peak eluates from wild-type and transgenic seeds. The lower panel represents the corresponding immunoblot. 2S albumin appears only in peak 3 of extracts from transgenic seeds. D: The presence of mature 3 and 9 kDa polypeptides indicates the regular processing of 2S albumin precursors in seeds of plants that had been transformed with the 2S albumin gene under the control of the LeB4 promoter and terminator. Extracts from transgenic seeds (lanes 1 and 3) as well as authentic 2S albumin preparations were compared by SDS-PAGE under reducing (lanes 1 and 2) and non-reducing conditions (lanes 3 and 4); lane 5, molecular weight markers. Bands specific for the 2S albumin are already visible in the stained gel slab (upper panel) but are specifically labelled by immunoblotting (lower panel). The 12 and 9 kDa polypeptides exhibit only small mobility differences. The 3 kDa band reacts much less strongly with the polyclonal antibodies raised against the holoprotein and stains much less than the 9 kDa polypeptide. Therefore, the corresponding bands are barely visible in the photographs of the stained gel and of the blot.

conserved during higher plant evolution, since correct processing was found in transgenic tobacco (Altenbach et al., 1989), rape (Altenbach et al., 1992) and legume seeds (Saalbach et al., 1994).

The compartmentation analysis was performed by immuno-gold labelling of electron micrographs (Müntz et al., 1993b).

Quantification of 2S-albumin and methionine content

Since it is much easier to find the 12 kDa 2S albumin precursor on the gels than the mature 9 and 3 kDa polypeptide chains, electrophoresis was performed under non-reducing conditions. Standard probes of 2S albumin preparations were always included for comparison. Semi-quantitative analysis was based on comparative analysis with a series of different amounts of the standard protein for calibration.

To determine the methionine content, seeds were extracted with buffered neutral salt solutions of suitable ionic strength (Altenbach et al., 1992), dialysed against distilled water, lyophilised and hydrolysed. The amino acid composition was kindly performed by BASF.

Results

The 2S albumin gene was detected in R_0, R_1, and R_2 plants following transformation with either the 35S/2S albumin or the LeB4/2S albumin fusion gene (data not shown). Several R_2 plants were obtained that no longer expressed or had lost the foreign gene. Inheritance analysis with 35S/2S albumin gene transformants is given for the progeny of R_0 plant no. 15/1 (Table 1). Seedlings were analysed for 2S albumin formation as well as GUS activity. Segregation of the markers analysed was consistent with Mendelian inheritance of one gene insert. Two homozygous lines expressing 2S albumin were obtained (seedlings no. 3 and 10). Similar results were obtained with corresponding tobacco transformants (Saalbach et al., 1994). The genetic analysis of R_2 plants provided clear evidence for the 2S albumin gene integration into the plant genome. This was confirmed by Southern blotting for transformants containing the LeB4/2S albumin fusion gene. No such unequivocal conclusion could be drawn from corresponding blotting experiments with DNA extracted from transformants carrying the 35S/2S albumin fusion gene, since the probes used only detected sequences inside the T-DNA without extension to the plant DNA.

Seed-specific expression of the 2S albumin gene under the control of the legumin B4 promoter was examined in transformation experiments with the pGA472-2S vector. Of 70 transgenic *V. narbonensis* plants obtained so far, 40 R_0 plants contained the 2S albumin gene. Inheritance of the foreign gene, copy number and stability of its expression have so far been analysed in the offspring of 18 R_0 plants. Sixteen of these transformants formed the 2S albumin in their seeds whereas the seeds of the remaining two did not contain the foreign protein. Homozygous lines were obtained from two transformants. Gene expression was often unstable in the offspring of transformants with

Table 1. Inheritance of the 2S albumin gene in the progeny of *Vicia narbonensis* transgenic plant 15/1. n.d., not determined

R_1			R_2							
Seedling	GUS	2S albumin	GUS segregation		Fit to Mendelian expectations for segregation of one allele		2S albumin segregation		Fit to Mendelian expectations for segregation of one allele	
			+	-	χ^2	P	+	-	χ^2	P
1	+	+	23	10	0.49	0.48	8	2	0.13	0.72
3	+	+	40	0	–	–	10	0	–	–
4	-	-	0	30	-	-	n.d.	n.d.	–	–
8	+	+	17	6	0.01	0.90	n.d.	n.d.	–	–
10	+	+	50	0	–	–	20	0	–	–

Table 2. Levels of 2S albumin formation in different organs of transgenic *Vicia narbonensis*; n.d. not determined

Organ	R_0 generation		R_1 generation	
	2S alb (%)	GUS	2S alb	GUS
Leaf	0.2	(+)	0.2	(+)
Root	grafted	grafted	0.3	++
Seed coat	0.08	++	0.1	++
Cotyledon	0.01	+	n.d.	+

more than one gene insertion, and in the offspring of plants containing recombinant insertions.

Tissue specificity of gene expression and intracellular localisation of the gene product

Where the 2S albumin gene was under control of the 35S promoter, expression was observed in all analysed organs and tissues of the transgenic plants though at different levels (Table 2). Seeds of the transgenic legume as well as of corresponding tobacco transformants exhibited only low levels of 2S albumin formation which is in agreement with the known low-level activity of this promoter in embryonic tissue. Weak expression was also registered in the leaves of transgenic tobacco even in those plants containing three copies of the foreign gene. In contrast, the *V. narbonensis* transformants with only one gene copy exhibited high levels of gene expression in leaves (Table 2). The highest levels of 2S albumin production took place in the roots of the legume transformants.

As was to be expected from the findings of Bäumlein et al. (1987, 1988, 1991, 1992), the legumin B4 promoter directed seed-specific 2S albumin gene expression and this at a much higher level than that achieved using the 35S promoter (see section 6). No 2S albumin formation was detected in other organs.

Analysis of vacuoles of leaf mesophyll cells from tobacco plants transformed with the 35S promoter/2S albumin/35S terminator fusion gene, revealed vacuolar targeting of the 2S albumin (Saalbach et al., 1994). In transgenic seeds of *V. narbonensis*, the 2S albumin was located in the protein bodies that belong to the vacuolar system of the storage tissue cells (Fig. 5).

Expression level and its effect on the methionine content of transgenic seeds

Expression levels of GUS and 2S albumin differed between organs and tissues of *V. narbonensis* transformed with the vector pGSGLUC1-2S. This could be due to differences in either the stability of mRNA or protein, or in the controlling elements directing the expression of the different genes, or a combination of the above. Whereas expression of the 2S albumin gene was directed by the 35S promoter, the *gus* and *nptII* genes were controlled by the T-DNA Tr1′,2′ 'double-headed' promoter. The differences in the level of 35S promoter-controlled 2S albumin gene expression in different organs and tissues were described in the previous section.

Western blots with extracts from different organs of tobacco plants as well as from heterozygous and homozygous transformed tobacco and *V. narbonensis* plants (Saalbach et al., 1994), revealed a close relation-

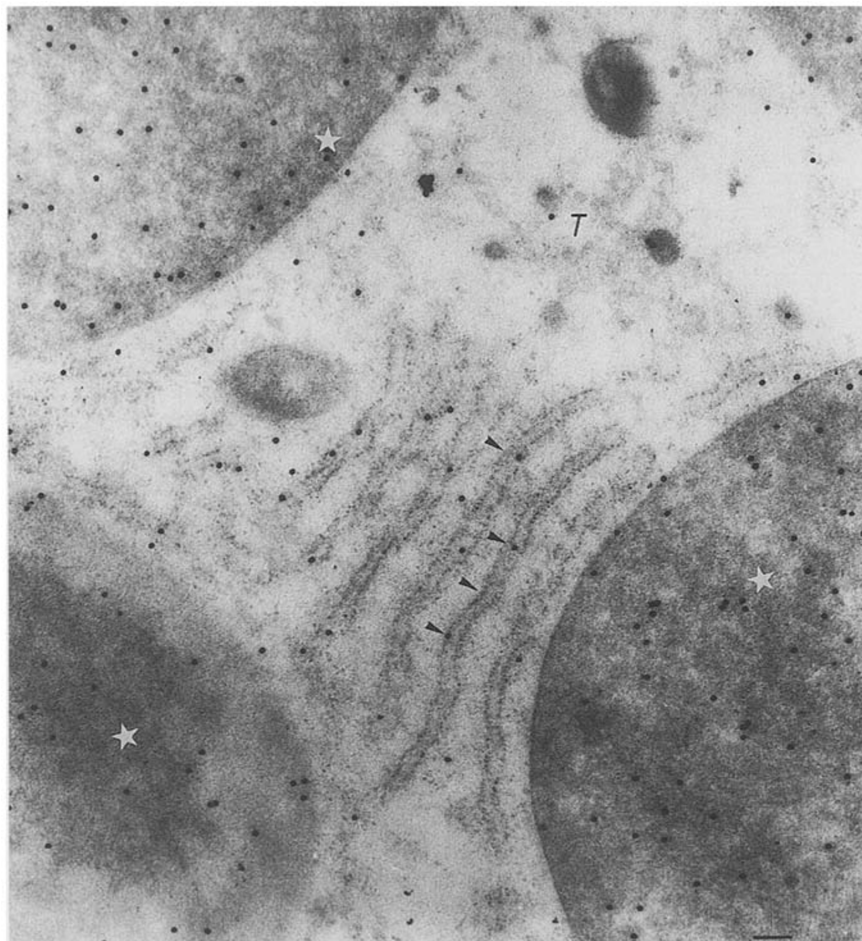

Fig. 5. Electron micrograph with immunogold-labelled Brazil nut 2S albumin in the protein bodies (stars), rough endoplasmic reticulum (arrow heads), and trans-Golgi network (T) of cells from cotyledons of developing seeds from *V. narbonensis* plants that were transformed with the 2S albumin gene under the control of the seed-specific LeB4 promoter and terminator from *V. faba*. The localisation of the foreign protein in the different cell compartments indicates that it must be synthesised on membrane-bound polysomes, transferred through the endoplasmic reticulum and Golgi apparatus, which belong to the endomembrane system, and targeted into the vacuole that is converted into protein bodies during seed maturation. The bar corresponds to 0.2 μm.

ship between the amount of accumulated 2S albumin and the number of integrated genes. Although variability of gene expression in different transformants can not be excluded as a factor influencing the accumulation level of the foreign protein, it does not seem to be very probable that this influence could act so as to mimic gene dosage effects in all cases. A gene dosage effect has already been described for the expression of the legumin gene from *Vicia faba* in transgenic tobacco (Bäumlein et al., 1988). Therefore, we feel justified in concluding that gene dosage effects also act in our *V. narbonensis* plants that express the foreign 2S albumin-specific Brazil nut gene.

Whereas up to 0.01% of the SDS-soluble protein was found to be 2S albumin in the cotyledons from seeds of transgenic *V. narbonensis* plants carrying the 35S/2S albumin gene fusion, the level of 2S albumin in cotyledons of transformants carrying the seed-specific LeB4/2S albumin construct was between 0.5 and 3 percent, a 50–300 fold increase.

The methionine content of the salt-soluble protein fraction was determined in the seeds of a series of selected R_0 transformants to find out whether the increased amounts of methionine-rich foreign protein influences the quantity of this limiting essential amino acid in transgenic seeds. The salt-soluble proteins rep-

resent the major quantity of proteins in the legume's seeds. Transgenic seeds of R_0 plants were found to exhibit up to a threefold increase in methionine content over that of the wild-type seeds of *V. narbonensis*. Analysis of methionine content in the seeds of the R_1 offspring is now under way.

Discussion

The Brazil nut 2S albumin and its gene

The hypocotyl of Brazil nut seeds contains approximately 50 percent of the total seed protein by dry weight. Thirty percent of this fraction is accounted for by the 2S protein fraction in which the water-soluble 12 kDa sulphur-rich protein, called Brazil nut 2S albumin, predominates (Sun et al., 1987). The purified protein contains 18% methionine and 8% cysteine, and it is primarily responsible for the high methionine content of Brazil nut seed protein (8.3% by weight). Electrophoretic analysis under denaturing and disulphide bridge-reducing conditions, revealed that the albumin is composed of polymorphic 9 kDa and 3 kDa polypeptide chains linked by one or more disulphide bonds. These polypeptides are formed from a larger precursor that also contains a 3 kDa propeptide located N-terminally from the 3 kDa chain and an N-terminal 3 kDa signal peptide. The 9 kDa polypeptide of the mature holoprotein forms the C-terminus of the precursor (Fig. 1).

This precursor is synthesised on membrane-bound polysomes (rough endoplasmic reticulum, rER). The mature holoprotein accumulates inside protein bodies belonging to the vacuolar compartment of the storage tissue cells in the hypocotyl (Harris et al., 1993). Consequently, the 2S albumin polypeptides are transferred from the rER through the so-called secretory pathway into the endomembrane system and finally, presumably from the trans-Golgi network, into the protein bodies which are extracytoplasmic storage vacuoles. On its way from the site of formation to the site of deposition the precursor undergoes at least 3 successive steps of limited proteolytic tailoring (Fig. 1): 1 – The co-translational cleavage of the signal peptide which is known to take place at the inner surface of the ER membrane inside the ER lumen; 2 – the detachment of the propeptide; and 3 – the separation of the 3 and 9 kDa mature polypeptides. In addition, by comparing the cDNA-derived amino acid sequence (Altenbach et al., 1987) with that determined by direct amino acid sequencing (Ampe et al., 1986), a fourth step of limited proteolysis that seems to eliminate the four C-terminal amino acid residues from most of the known polymorphic 9 kDa polypeptides, has been revealed. Presumably, processing steps 2, 3 and 4 occur in the vacuolar compartment by analogy with the processing of 2S albumins and other storage globulins from different plants (Hara-Nishimura et al., 1991; Müntz et al., 1993a).

Tissue-specific expression of foreign genes in seeds

Heterologous transfer of storage protein genes between angiosperms began more than 10 years ago (Murai et al., 1983). The numerous transformations that have been performed since then have shown that the mechanisms of control and regulation of storage protein gene expression have been highly conserved throughout plant evolution. During the period of continuous protein accumulation in seeds, tissue-specificity and developmental pattern of expression are determined by the promoter region of the storage protein genes and hence controlled at the transcriptional level. This implies that the pattern of expression of the seed storage genes can be conferred upon foreign genes by fusing these controlling elements before the protein coding regions of the foreign gene. Furthermore, the structural signals that determine the biosynthesis of the molecular precursors of such proteins on membrane-bound polysomes and their deposition as mature holoproteins inside the protein bodies – specialised vacuolar compartments of the cell – as well as those signals involved in intracellular transfer and targeting from the site of formation to the compartment of deposition, are universally recognised by the cells of different plant seeds.

The results of our experiments, in which the protein-coding region of a methionine-rich 2S albumin from *Bertholletia excelsa*, the Brazil nut, was put under the control of the seed-specific LeB4 promoter and the fusion gene successfully transferred into the grain legume *V. narbonensis*, have confirmed the conservative nature of the mechanisms of transcriptional control of seed-storage gene expression as well as those involved in the processing of their gene products. Seed-specific expression of this gene was documented and its Mendelian inheritance shown (Saalbach et al., in press). The protein was formed on the rough endoplasmic reticulum and transported into the protein bodies of the legume cotyledon cells where it accumulated. Furthermore, we were able to show by comparing het-

erozygous and homozygous offspring, that the amount of protein produced in the storage tissue was correlated with the number of genes.

The methionine content of the salt-soluble proteins increased threefold in the seeds of R_0 transgenic plants over that of corresponding wild-type seeds. This appears to be a very large increase when it is compared with the increases of 30 to 40% that have been reported in similar transformation experiments with tobacco (Altenbach et al., 1989) and rape (Altenbach et al., 1992). However, the level of methionine in the salt-soluble protein fraction of wild-type *V. narbonensis* seeds is only 0.5% (mol/mol), whereas the corresponding protein fraction of the wild-type winter variety of *Brassica napus* that was used by Altenbach et al. (1992) contains 2.64% (mol/mol) methionine. The 1.5% (approx.) methionine found in this protein fraction in transgenic *V. narbonensis* seeds corresponds to an increase of 1% (mol/mol) of methionine and is comparable with that found in the seeds of the rape transformants. The similar absolute increases result from increases in the percentage of 2S albumin that were found in the salt-soluble protein fractions in the transgenic seeds of both species. The methionine content of this protein fraction in transgenic rape reaches 3.52%, and thereby achieves the recommended level of methionine and cysteine for a balanced amino acid diet for monogastric animals. The methionine level we have so far achieved with transgenic *V. narbonensis* corresponds to 42% of this standard; it reaches 80% the cysteine values are added (1.3%, mol/mol).

Improvements in the amino acid composition of proteins via genetic engineering are based upon the evolutionary conservation of the pattern of gene expression as well as the elements for translational control and intracellular protein sorting. So far two strategies have predominated in this field:

1) The codon composition of suitable genes has been changed by site-specific *in vitro* mutagenesis, frame shift mutation and/or insertion of suitable fragments from other genes. The modified genes were transformed into receptor plants that in most cases belonged to the *Solanaceae*. Unfortunately, in most cases, the protein stability was affected by these alterations and the accumulation of these proteins was reduced when compared with the accumulation of wild-type protein (e.g. Hoffmann et al., 1988; Saalbach et al., 1990). Recently, methionine codons were generated at 8 different sites in a 50 kDa-vicilin gene from *Vicia faba*, a gene that was originally free of methionine and cysteine codons. The *in vitro*-mutated gene was transferred into tobacco where stable gene expression was achieved. No differences have so far been observed in the stability of the wild-type and the mutant vicilin in transgenic tobacco seeds (Christov, 1993).

2) Foreign genes encoding proteins with suitable amino acid composition have been transferred into the target species. The methionine-rich 2S albumin from Brazil nut became the favourite protein for transformation experiments aimed at increasing the methionine content in legume seeds where this sulphur-containing amino acid is the most limiting essential amino acid from a nutritional point of view.

So far, the second strategy has turned out to be much more successful than the first one. The experiments we have reported here successfully employed this strategy for the first time in a grain legume.

In the near future we plan to determine whether further increases in the expression levels of the 2S albumin in transgenic *V. narbonensis* can be achieved by crossing transgenic lines with independent 2S albumin gene insertions, which has already been achieved in experiments with transgenic tobacco (Hobbs et al., 1993). In addition, transgenic *V. narbonensis* seeds forming suitable amounts of the methionine-rich 2S albumin will enable us to investigate the effect of the foreign methionine sink on the regulation of methionine biosynthesis as well as on the formation of endogenous storage proteins. When sufficient quantities of transgenic seeds become available we will be able to test the feeding efficiency with rats. We also plan to transform grain legumes that have been adapted to the moderate climates of Europe and North America like peas or field beans, as well as the economically-important legumes grown in tropical regions, like chickpeas, with the Brazil nut 2S albumin. The practical application of this technology will require investigation of genetic stability, growth and yield of the transgenic legumes in field trials.

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