IMPROVEMENT OF METHIONINE-DEFICIENT LEGUMES THROUGH GENETIC ENGINEERING

LUCILIA HELENA MARCELLINO AND EUGEN SILVANO GANDER
EMBRAPA – Recursos Genéticos e Biotecnologia Parque Estação Biológica PqEB. Final W5 Norte 70770-900 Brasília-DF- Brazil
e-mail: lucilia@cenargen.embrapa.br

Abstract

A review of research aiming at the improvement of methionine-deficiency in legumes is presented. We discuss three possible strategies to reach this objective and present data on the legume crops where recombinant DNA technology has been applied to introduce and express genes coding for methionine-rich proteins. Some considerations concerning the possibility of introducing allergenic proteins into host plants are discussed.

1. Introduction

Jonathan Swift put it in a nutshell: in "Gulliver's Travels", the king of Brobdingnag declares that "... whoever could make two ears of corn, or two blades of grass to grow upon a spot of ground where only one grew before; would deserve better of mankind, and do more essential service to his country, than the whole race of politicians put together" (J. Swift, 1726).

These lines were written in 1726, when the world's population was under 1 billion; they are more true now, when actually close to 7 billion people have to be sustained. It is quite obvious: the necessary sustainment is mostly, if not completely, based on agriculture.

Within the agricultural commodities used for feeding humans and animals, legumes, in particular the grain legumes, i.e. black beans, mung beans, chickpeas, common beans and soybeans, occupy an important place, and in particular in developing countries.

According to the FAO the average daily protein intake of an adult should be around 0.35 g/kg of body weight while a 2 year old needs an average of 2.4 g/kg of body weight. In developed countries these needs are, to a large part, satisfied through the intake of animal proteins, while in developing countries vegetable proteins are a major component of the diet. Animal proteins from meat, milk and eggs are, from a nutritional point of view, more adequate for human nutrition since their amino acid composition, in particular

P.K. Jaiwal and R.P. Singh (eds.), Improvement Strategies for Leguminosae Biotechnology, 281–290.

their content of essential amino acids, is more balanced. Indeed, the major drawback of vegetable proteins is the fact that they are poor in the essential sulfur amino acids methionine and cysteine as well as tryptophan. This is illustrated in Table 1 (Aykroyd and Doughty, 1964) where the amino acid content of some of the more important grain legumes, in comparison with a hypothetical "ideal" protein, is shown.

So far there is no indication that this low content in sulfur amino acids and tryptophan of grain legumes can be overcome by classical breeding methods. The advent of recombinant DNA technology in the beginning of the 80s, however, offered a promising alternative based on the isolation of high-methionine storage protein genes and their introduction and expression in suitable, methionine-deprived and economically interesting host plants.

*Table 1. Amino acid content of legumes (mg/gN)**

Legume species Dry seeds	Isoleu-cine	Leu-cine	Ly-sine	Phenyl-alanine	Tyro-sine	Sulfur (total)	Methi-onine	Cystin	Threo-nine	Tryp-phan	Valine
FAO-hypothetical ideal protein	270	306	270	180	180	270	144	126	180	90	270
Arachis hypogaea	260	380	220	320	220	150	60	90	170	70	310
Cajanus cajan	380	490	450	540	210	160	70	90	240	30	330
Canavalia ensiformis	280	570	370	390	240	180	110	80	310	–	330
Glycine max	340	480	400	310	200	200	80	110	250	90	330
Lens esculenta	330	440	380	280	170	100	50	50	220	50	340
Mucuna utilis	300	480	390	300	320	130	80	60	250	–	240
Parkia biglobosa	300	500	440	380	310	150	100	50	210	–	–
Phaseolus acutifolius	280	480	410	330	200	150	60	90	250	–	360
P. angularis	280	490	440	340	210	180	110	70	240	–	340
P. aureus	350	560	430	300	100	110	70	40	200	50	370
P. lunatus	360	520	420	370	160	190	100	90	300	60	390
P. mungo	270	490	460	410	210	140	90	60	230	–	370
P. vulgaris	360	540	460	350	240	120	60	60	270	60	380
Pisum sativum	350	520	460	320	250	160	80	80	240	70	350
Vicia faba	390	540	350	260	170	70	30	40	200	60	410
Vigna unguiculata	260	450	410	340	210	230	120	110	220	–	340

*Aykroyd and Doughty (1964).

During the last decade several excellent reviews on the subject of genetic engineering of the sulfur amino acid content of grain legumes have been published (e.g. Altenbach and Simpson, 1990; Tabe *et al.*, 1993; Müntz *et al.*, 1998). Therefore, here we first give an overview of the different approaches used to achieve this goal, the problems encountered, the state of the art of research and then discuss one of the major problems in food-orientated biotechnology, i.e. the possible introduction of allergenic components into food.

2. Biotechnological approaches for protein quality and quantity improvement

Recombinant DNA Technology offers many approaches that may result in a change of the protein profile and/or amino acid composition of model plants or socio-economically important crops. This review is limited to the discussion of three of them: the delivery and expression of suitable transgenes into hosts, the site-directed mutagenesis and subsequent re-introduction of the mutated endogenous or heterologous genes and the engineering of the pathways involved in the biosynthesis of essential amino acids.

2.1. INTRODUCTION OF HETEROLOGOUS HIGH-METHIONINE GENES

Historically the first method to be employed to alter amino acid composition was also the most obvious: introduction of heterologous genes coding for high methionine proteins. So far not many such genes have been isolated: according to Altenbach and Simpson (1990) and Müntz *et al.* (1998) those presently available code for two 10 and 15 kDa maize zeins (22% and 11% methionine, respectively), a 10 kDa rice prolamin (20% methionine), a 10 kDa sunflower albumin containing 15% methionine (Molvig *et al.*, 1997) and a 18% methionine 2S albumin from Brazil nut. Another gene, from wild maize, coding for a 18 kDa zein is quite promising since it contains 25% methionine (Swarup *et al.*, 1995). Furthermore, a gene coding for a nutritionally well balanced seed albumin from *Amaranthus hypochondriacus* has become available (Raina and Datta, 1992), and one can hope that in the near future still more genes will be at hand. Recently, for instance, the primary structure of a methionine-rich albumin from *Cannabis sativa* has been reported (Odani and Odani, 1998).

Supposedly the best (albeit, recently, most controversial) candidate for recombinant DNA technology applied to legumes was one gene of the 2S albumin multigene family from Brazil nut (*Bertholletia excelsa*) coding for an albumin containing 18% methionine and 8% cysteine. Since the discovery and description of this protein by Rotenberg and Iachan (1975) and Youle and Huang (1981), several groups have successfully isolated and characterized physically-chemically as well as biologically the 2S albumin proteins (Ampe *et al.*, 1986). cDNA, genomic sequences and *in vivo* biosynthesis of these proteins have also been investigated extensively (Altenbach *et al.*, 1987; De Castro *et al.*, 1987; Gander *et al.*, 1991). This, true to the dogma that in order to do biotechnology one has to know the product of the gene of interest and the physical-chemical and biological properties of the gene itself.

Most of these groups attempted to introduce this gene into different plant species in order to achieve improvement of their nutritional value. In 1989, Altenbach *et al.* reported

for the first time the enhancement of the methionine content of seed proteins after introducing and expressing Brazil nut (BN) 2S albumin cDNA sequences in transgenic tobacco. In this study, the levels of methionine in the seed proteins increased 30%. This strongly indicated that it is possible to improve the nutritional value by means of recombinant DNA technology. Tobacco, however, is a model plant and soon or in parallel several other attempts using similar strategies with different nutritionally and economically important crops were reported by other groups. Table 2 summarizes these efforts.

Table 2. Plant species transformed with transgenes coding for methionine-rich protein

Crop	Transgene	Promoter	Method of transformation	Methionine increase	Authors
N. tabacum	15 kDa zein	ß-phaseolin	*A. tumefaciens*	ND	Hoffman *et al.*, 1987
N. tabacum	BN2S	Phaseolin	*A. tumefaciens*	30%	Altenbach *et al.*, 1989
N. tabacum, A. thaliana, B. napus	BN 2S/AT2S1	AT2S1	*A. tumefaciens*	0.1–0.2%	De Clercq *et al.*, 1990
B. napus	BN2S	Lectin	Electroporation	ND	Guerche *et al.*, 1990
B. napus	BN2S	Phaseolin	*A. tumefaciens*	33%	Altenbach *et al.*, 1992
S. tuberosum	BN2S	CaMV 35S	*A. tumefaciens*	ND	Tu *et al.*, 1994
N. tabacum, V. narbonensis, V. faba and *P. sativum*	Synthesized BN2S gene	CaMV 35S	*A. tumefaciens, A. rhizogenes*	ND	Saalbach *et al.*, 1994
N. tabacum, V. narbonensis	BN2S	CaMV 35S and LeB4	*A. tumefaciens*	3 X [wt]	Saalbach *et al.*, 1995a
V. narbonensis	BN2S	CaMV 35S and LeB4	*A. tumefaciens*	3 X [wt]	Saalbach *et al.*, 1995b
L. angustifolius	SSA	Vicilin	*A. tumefaciens*	94%	Molvig *et al.*, 1997
S. tuberosum	BN2S, modified	CaMV 35S	*A. tumefaciens*	ND	Tu *et al.*, 1998
P. vulgaris	BN2S	CaMV 35S	Particle bombardment	23%	Aragão *et al.*, 1999
S. guianensis	BN2S	*Ats1A*	Particle bombardment, electroporation	ND	Quecini, 1999

BN2S = Brazil nut 2S albumin; LeB4 = legumin B4 from *Vicia faba*; CaMV 35S = cauliflower mosaic vírus 35S; SSA = sunflower seed albumin; ND = not determined; [wt] = wild type concentration.

It is evident from this table that, with the exception of one group (Movig *et al.*, 1997), all others used the Brazil nut 2S albumin gene sequences as transgenes. It is also evident that the concept of introducing and expressing a transgene in order to overcome nutritional deficiencies is correct. However, there exists a puzzling variability with respect to the actual increase of the methionine content: the results vary from 0.1% in *A. thaliana* transformed with BN2S (De Clercq *et al.*, 1990) up to 94% in *L. angustifolius* transformed with SSA (Molvig *et al.*, 1997). It is difficult if not impossible to discuss these discrepancies since not only different promoters were used but, in addition, different host plant species were transformed.

2.2. INTRODUCTION OF *IN VITRO* ENGINEERED TRANSGENES

The *in vitro* modification of available genes, prior to the introduction and expression in host plants, is another possibility, useful for the correction of amino acid deficiencies. This was first shown with the tobacco model by Hoffman *et al.* (1988). These authors increased the methionine codons of ß-phaseolin from three to nine by inserting an oligonucleotide of 45 bases and transforming *N. tabacum* via *A. tumefaciens*. The modified sequence was expressed and processed in the host, however, no accumulation of the transgene's product in the protein bodies could be shown using immunocytochemical techniques. The authors speculate that the modified protein was degraded somewhere between the Golgi complex and the protein bodies.

It is worthwhile to mention here another attempt, although its goal was not the improvement of methionine deficiency but the demonstration that storage proteins can be used as protein factories for the synthesis of pharmaceutically important compounds. Vanderckhove *et al.* (1989) used a 2S albumin gene from *A. thaliana* as a carrier for the sequences coding for the neuropeptide Leu-enkephalin. The chimeric gene was introduced into *A. thaliana* and *Brassica napus*. Leu-enkephaline was recovered from seeds of the transgenic plants in very satisfactory amounts, i.e. 200 nmol/gram of *Arabidopsis* seeds and 50 nmol/gram of *Brassica* seeds. Besides showing the usefulness of storage proteins in plant molecular pharming, this article also demonstrates the high biological plasticity of 2S seed storage albumin genes.

To a certain degree this is also reflected in the work of De Clercq *et al.* (1990) who modified *Arabidopsis* 2S sequences. All these modifications were done in the *Arabidopsis* 2S large subunit hypervariable region, between cysteins # 6 and 7, and consisted of synthetic oligonucleotides. The modified 2S proteins contained 7, 10 and 14 methionine residues, respectively. The modified sequences were introduced into *A. thaliana, B. napus* and *N. tabacum* and the authors determined that between 100 to 200 nmol of modified 2S proteins are present per gram of *Arabidopsis* seed.

It is possible, at least in this hypervariable region, to introduce foreign sequences without affecting transcription or protein processing. Based on this notion and aware of the fact that although 2S from Brazil nut is methionine-rich, it is deficient in tryptophan, another essential aminoacid, we applied site-directed mutagenesis in order to compensate for this deficiency. In a first construct, 5 tryptophan codons were introduced into the hypervariable region of the BN2S cDNA sequence (Marcellino *et al.*, 1996). The modified transgene, under control of the 35S CaMV promoter, was introduced in *N. tabacum*

via *A. tumefaciens*. Western analyses have shown that the modified protein was expressed and correctly processed in tobacco seeds.

In addition, two other modifications were introduced into the 2S albumin cDNA. Based on a theoretical model of the tertiary structure, two modifications were planned so that the tryptophan residue would occupy an internal (leucine 44 substitution) or an external position (arginine 80 substitution) in the 2S molecule (Fig. 1). These last two mutants could be used for further structural analyses of the tertiary conformation of the 2S protein by fluorescence quenching techniques. We could show that all the mutants were correctly transcribed, expressed in tobacco seeds and were accumulated in protein bodies, as expected for storage proteins in general. Semi-quantitative analyses indicated that between 0.15 and 0.3% of salt-extractable seed proteins are mutated 2S proteins. This result was somewhere between the values reported by Guerche *et al.* (1990) and Altenbach *et al.* (1992) for transgenic *B. napus*.

Another work that takes advantage of the Brazil nut gene's hypervariable region as an acceptor for additional codons, albeit not for the engineering of legumes but of potatoes, is described by Tu *et al.* (1998). The four mutations constructed by these authors and introduced into the variable region of the Brazil nut 2S large subunit increased the methionine content from an original 18% to 21, 23, 24 and 25% in the coding region. These mutants were put under the control of the CaMV 35S promoter and introduced into *S. tuberosum* via *Agrobacterium*-mediated transformation. All mutant genes were expressed and the proteins correctly processed. The authors emphasize, however, that no increase of the methionine concentration in the transformed plants was obtained and they state that in order to do so, a ten-fold increase of the expression level would be necessary.

The most obvious message here is doubtless the fact that the 2S albumins and in particular the Brazil nut 2S sequences, which, *a priori* are already rich in methionine, can be modified in their variable region, and can be expressed successfully in host plants. These modifications do not seem to influence transcription efficiency nor do they interfere with the correct processing and storage of the modified proteins.

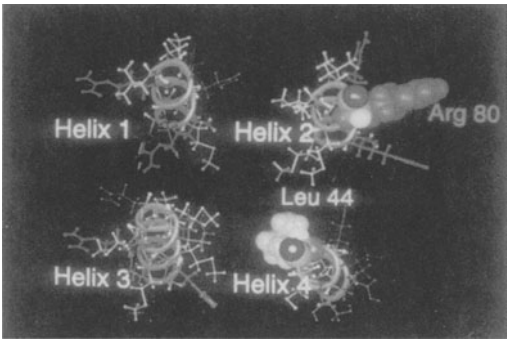

Figure 1. Theoretical structure of the 2S albumin. The photo shows four of the five helices presumably present in Brazil nut 2S albumin. Note that Leu^{44} faces the interior of the molecule while Arg^{80} faces outwards – both amino acids are represented as ball model.

2.3. ENGINEERING OF THE BIOSYNTHESIS PATHWAYS OF METHIONINE

The biosynthesis of methionine is dependent on the citrate-cycle and, consequently, on aspartate as the point of origin. This, in turn, means that oxaloacetate and therefore the citric acid cycle stand, invariably, at the root of methionine biosynthesis. If the assumption is made that in methionine-deficient crops the limiting factor is methionine as a free amino acid, attempts to interfere with methionine's biosynthesis pathway would be justified. The idea would then be to engineer one of the key enzymes that might regulate methionine accumulation through a feed-back mechanism. This type of approach is indeed valid since high-lysin soybeans have been obtained with this strategy (Falco *et al.*, 1995). In addition the biosynthesis of lysine in bacteria, algae, ferns as well as in higher plants also starts from aspartic acid. The perspective, however, to interfere with some of the 9 steps involved in the citrate cycle and/or the 7 steps that are necessary to synthesize methionine from aspartic acid seems to us, for the time being at least, an approach much too complex to be of any practical value in the near future. This, of course, says nothing about the scientific insights and merits that could result from investigations along these lines.

3. Conclusions and further considerations

The examples presented so far of recombinant DNA technologies applied towards the compensation of methionine deficiency of model plants or of species of agro-commercial and/or nutritional importance leave no doubt that genetic engineering of seed protein composition is possible and a reality. However, with the exception of the case of the *Lupinus* transformed with the sunflower SSA gene, no animal feeding tests have been performed in order to evaluate the actual nutritional effect of the transgenics. One reason might very well be that in most of the cases, the fraction of proteins derived from transgene transcription is too small to provoke a nutritionally significant change of the methionine concentration. This indicates that one of the more important topics of future research most probably has to focus on the understanding of promoter and enhancer elements and their interactions with regulatory proteins as well as the identification of strong seed promoters or enhancer-like elements that could be used to construct more effective seed promoters. Along these lines one might mention the work of Vincentz *et al.* (1997) who identified, in the promoter of the Brazil nut 2S albumin gene, boxes containing binding sites for the maize trans-acting factor opaque-2 and showed that this regulatory protein can transactivate promoter constructs consisting of the duplicated boxes and of a 35S minimal promoter in tobacco leaves.

Another important point emerges from the work of Hoffman *et al.* (1988) who showed that the products of modified phaseolin transgenes are not stable in tobacco seeds. The reasons for this instability are not clear, however, it could be a problem of misdirectioning to a wrong cellular compartment. Consequently another important aspect arises: the directioning of the transgene's product to an appropriate cell compartment. This requires the inclusion, in transgenes, of the adequate signal sequences. The work of Tabe *et al.* (1993) illustrates this fact: these authors included, at the 3′ end of the vicilin gene under the control of the 35S promoter, sequences representing a signal for the retention of proteins in

the endoplasmic reticulum. They showed that tobacco leaves from plants transformed with this construct accumulated 100 times more vicilin than leaves from plants transformed with a similar construct that did not contain the signal sequence.

Last but not least, it is important to realize that within the programs that regulate the ordinate expression of genes and gene batteries during development and during an organism's lifetime, there must also exist modules responsible for correct structural dimensions. Consequently it is not necessarily obvious that a quantitative increase of proteins in , say, seeds, can simply be achieved through the expression of one or several transgenes. One possibility to cope with this notion would be to knock-out an undesired gene concomitantly with the introduction of a desirable transgene. Evidence for the correctness of this line of reasoning comes from the work of Kohno-Murase *et al.* (1994, 1995) who introduced and expressed either an anti-sense napin or anti-sense cruciferin construct in *Brassica*. In the first case, production of cruciferin was increased whereas cruciferin knockouts produced more napin than wild type plants.

3.1. FOOD FROM TRANSGENIC CROPS – THE ALLERGEN PROBLEM

As mentioned before, when improvement of methionine content is envisioned, the Brazil nut 2S gene was, and still is, one of the most obvious transgenes but, on the other hand, it has become also one of the most controversial.

In 1996 Nordlee *et al.* published a study, supported by Pioneer Hi-Bred International Inc., showing that sera from individuals with the documented allergy to Brazil nuts contain IgE that binds to the Brazil nut 2S albumin present in protein extracts from transgenic soybean. This result confirms the findings of Arshad *et al.* (1991) that Brazil nut proteins are allergenic and establishes that the allergenic principle present in the Brazil nut 2S albumin can be transferred to host soy plants via recombinant DNA technology. These results have been widely used as an argument against transgenic food and biotechnology. The fact is that numerous non-manipulated native proteins used for human consumption, e.g. proteins from peanuts (not only Brazil nuts!), crustaceans, cow milk, legumes, cereals, etc. can be allergenic for a certain segment of the population. Now this, of course, is common knowledge and nobody would dare, for this reason, to take a stance against, say, crustaceae. So why take a stance against the use of Brazil nut 2S albumin in manipulation of food crops? All it takes in order to get into the clear with this admittedly serious aspect are regulations stipulating:

1. a label indicating if the food or crop contains a known allergen and
2. the execution of routine-tests, long since a common practice in the cosmetic industry, to verify if proteins of unknown allergenic properties are to be introduced in the nutritional line.

Tests of this kind should or could include the demonstration that, after processing and/or cooking, the allergenic properties are still maintained, a test not done, to the best of our knowledge, in the case of the soybeans transformed with the BN2S gene. Furthermore, with the introduction of transgenes into food crops being a reality, it is essential to fund more programs that are aimed at the understanding of the molecular basis of allergenicity and at the identification of useful genes in organisms of less antigenic notoriety.

In summary, we are convinced that biotechnology applied to the qualitative improvement of food for human and animal consumption is a valid alternative to the "classical" plant breeding methods. The successful introduction and expression of genes coding for high-methionine storage proteins in crops of nutritional importance illustrate this notion.

However, we are also aware that, parallel to the rapid increase of available genes with desirable traits and the fact that, by now, virtually any crop can be transformed, it is essential to establish risk-assessment procedures and biological tests as foolproof as possible and to inform the consumer clearly on the components of a given food.

Acknowledgements

We would like to thank Drs. I. Miranda dos Santos, F. J. L. Aragão and L. A. B. de Castro for critical reading of the manuscript.

References

Altenbach S B and Simpson R B (1990) Manipulation of methionine-rich protein genes in seeds. *TIBTECH*, **8**: 156–160.

Altenbach S B, Pearson K W, Leung F W and Sun S S M (1987) Cloning and sequence analysis of a cDNA encoding a Brazil nut protein exceptionally rich in methionine. *Plant Mol. Biol.*, **8**: 239–250.

Altenbach S B, Pearson K W, Meeker G, Staraci L C and Sun S M (1989) Enhancement of the methionine content of seed proteins by the expression of a chimeric gene encoding a methionine-rich protein in transgenic plants. *Plant Mol. Biol.*, **13**: 513–522.

Altenbach S S, Kuo Ch-Ch, Staraci L C, Pearson K W, Wainwright C W, Georgescu A and Townsend G (1992) Accumulation of a Brazil nut albumin in seeds of transgenic canola results in enhanced levels of seed protein methionine. *Plant Mol. Biol.*, **18**: 235–245.

Ampe C, Van Damme J, De Castro L A B, Sampaio M J, Van Montagu M and Vandekerckhove J (1986) The amino-acid sequence of the 2S sulphur-rich proteins from seeds of Brazil nut (*Bertholletia excelsa* H.B.K.). *Eur. J. Biochem.*, **159**: 597–604.

Aragão F J L, Barros L M G, De Souza M, Grossi De Sá M F, De Almeida E R P, Gander E S and Rech E L (1999) Expression of a methionine-rich storage albumin gene from Brazil nut (*Bertholletia excelsa* H.B.K.) in transgenic bean plants (*Phaseolus vulgaris* L.). *Genet. Mol. Biol.*, **22**: 445–449.

Aragão F J L, Grossi de Sá M F, De Almeida E R P, Gander E S and Rech E L (1992) Particle bombardment-mediated transient expression of a Brazil nut methionine-rich albumin in bean (*Phaseolus vulgaris* L.). *Plant Mol. Biol.*, **20**: 357–359.

Aykroyd W R Y and Doughty J (1964) Las leguminosas en la nutrición humana. Organización de las Naciónes Unidas para la Agricultura y la Alimentacíon. Roma.

De Castro L A B, Lacerda Z, Aramayo R A, Sampaio M J A M and Gander E S (1987) Evidence for a precursor molecule of Brazil nut 2S seed proteins from biosynthesis and cDNA synthesis. *Mol. Gen. Genet.*, **206**: 338–343.

De Clercq A, Vanderwiele M, Van Damme J, Guerche P, Van Montagu M, Vandekerckhove J and Krebbers E (1990) Stable accumulation of modified 2S albumin seed storage proteins with higher methionine contents in transgenic plants. *Plant Physiol.*, **94**: 970–979.

Falco S C, Guida T, Locke M, Mauvais J, Sanders C, Ward R T and Webber P (1995) Transgenic canola and soybean seeds with increased lysine. *Bio/Technology*, **13**: 577–582.

Gander E S, Holmstroem K O, De Paiva G R, De Castro L A B, Carneiro M and Grossi de Sá M F (1991) Isolation, characterization and expression of a gene coding for a 2S albumin from *Bertholletia excelsa* (Brazil nut). *Plant Mol. Biol.*, **16**: 437–448.

Guerche P, De Almeida E R P, Schwarzstein M A, Gander E, Krebbers E and Pelletier G (1990) Expression of the 2S albumin from *Bertholletia excelsa* in *Brasica napus. Mol. Gen. Genetics.*, **210**: 510–520.

Hoffman L A, Donaldson D D and Herman E M (1988). A modified storage protein is synthesized, processed and degraded in seeds of transgenic plants. *Plant Mol. Biol.*, **11**: 717–729.

Hoffman L M, Donaldson D D, Bookland R, Rashka K and Herman E M (1987) Synthesis and protein body deposition of maize 15 kd zein in transgenic tobacco seeds. *EMBO J.*, **6**: 3213–3221.

Kohno-Murase J, Murase M, Ichikawa H and Imamura J (1994) Effects of an antisense napin gene on seed storage compounds in transgenic *Brassica napus* seeds. *Plant Mol. Biol.*, **26**: 1115–1124.

Kohno-Murase J, Murase M, Ichikawa H and Imamura J (1995) Improvement in the quality of seed storage protein by transformation of *Brassica napus* with an antisense gene for cruciferin. *Theort. Appl. Genet.*, **9**: 672–631.

Molvig L, Tabe L M, Eggum B, Moore A E, Craig S, Spencer D and Higgins T J V (1997) Enhanced methionine levels and increased nutritive value of seeds of transgenic lupins (*Lupinus angustifolius* L.) expressing a sunflower seed albumin gene. *Proc. Natl. Acad. Sci. USA*, **94**: 8393–8398.

Müntz K, Christov V, Saalbach G, Saalbach I, Waddell D, Pickardt T, Schieder O and Wuestenhagen T (1998) Genetic engineering for high methionine grain legumes. *Nahrung*, **42**: 125–127.

Nordlee J A, Taylor S L, Townsend J A, Thomas L A and Bush R K (1996) Identification of a Brazil-nut allergen in transgenic soybeans. *N. Engl. J. Med.*, **334**: 688–692.

Odani S and Odani S (1998) Isolation and primary structure of a methionine- and cysteine-rich seed protein of *Cannabis sativa. Biosci. Biotech. Biochem.*, **62**: 650–654.

Quecini V M (1999) Transferência direta de genes para plantas de *Stylosanthes guianensis* (Aubl.) Sw. Ph.D. thesis, University of São Paulo, School of Agriculture "Luiz de Queiroz", Piracicaba-SP.

Raina A and Datta A (1992) Molecular cloning of a gene encoding a seed-specific protein with nutritionally balanced amino acid composition from *Amaranthus. Proc. Natl. Acad. Sci. USA*, **89**: 11774–11778.

Rotenberg B E and Iachan A (1975) Estudo da proteína da Castanha do Pará. *Informativo do Instituto Naciónal de Tecnologia*, **8**: 22–24.

Saalbach I, Pickardt T, Machemehl F, Saalbach G, Shieder O and Müntz K (1994) A chimeric gene encoding the methionine-rich 2S albumin of the Brazil nut (*Bertholletia excelsa* H.B.K.) is stably expressed and inherited in transgenic grain legumes. *Mol. Gen. Genet.*, **242**: 226–236.

Saalbach I, Pickardt T, Waddell D R, Hillmer S, Schieder O and Müntz K (1995) The sulphur-rich Brazil nut 2S albumin is specifically formed in transgenic seeds of the grain legume *Vicia narbonensis. Euphytica*, **85**: 181–192.

Saalbach I, Waddell D R, Pickardt T, Schieder O and Muntz K (1995) Stable Expression of sulphur-rich 2S albumin gene in transgenic *Vicia narbonensis* increases the methionine content of seeds. *J. Plant Physiol.*, **145**: 674–681.

Swarup S, Timmermans M C, Chauduri S and Messing J (1995) Determinants of the high-methionine trait in wild and exotic germplasm may have escaped selection during early cultivation of maize. *Plant J.*, **8**: 359–368.

Swift Jonathan (1993) Gulliver's Travels. Wordsworth Classics, Wordsworth Editions Limited, St. Albans, Hertfordshire, UK.

Tabe L M, Higgins C M, McNabb W C and Higgins T J V (1993) Genetic engineering of grain and pasture legumes for improved nutritive value. *Genetica*, **90**: 181–200.

Tu H M, Godfrey L W and Sun S S M (1998) Expression of the Brazil nut methionine-rich protein and mutants with increased methionine in transgenic potato. *Plant Mol. Biol.*, **37**: 829–838.

Tu H M, Godfrey L W and Sun S M (1994) Expression of the Brazil nut methionine-rich protein in transgenic potato plants. In: *The Molecular and Cellular Biology of the Potato* (Eds Belknap W R, Vayda M E and Park W D), CAB International, Wallingford, pp. 209–220.

Vandekerckhove J, Van Damme J, Van Lijsebettens M, Botterman J, De Block M, Vandewiele M, De Clercq A, Leemans J, Van Montagu M and Krebbers E (1989) Enkephalins produced in transgenic plants using modified 2S storage proteins. *Bio/Technology*, **7**: 929–932.

Vincentz M, Leite A, Neshich G, Vriend G, Mattar C, Barros L, Weinberg D, De Almeida E R, Paes de Carvalho M, Aragão F and Gander E S (1997) ACGT and vicilin core sequences in a promoter domain required for seed-specific expression of a 2S storage protein gene are recognized by the opaque-2 regulatory protein. *Plant Mol. Biol.*, **34**: 879–889.

Youle R and Huang A H C (1981) Occurrence of low molecular weight and high cysteine containing albumin storage protein in oil seeds of diverse species. *Am. J. Bot.*, **68**: 44–48.