

MODIFYING THE SULPHUR AMINO ACID CONTENT OF PROTEIN IN TRANSGENIC LEGUMES

LINDA TABE, LISA MOLVIG, RAFIQUK KHAN, HART SCHROEDER, STEPHANIE GOLLASCH, ¹TERESE WARDLEY-RICHARDSON, ANDY MOORE, STUART CRAIG, DON SPENCER, ²BJORN EGGUM and T. J. V. HIGGINS

CSIRO Division of Plant Industry, GPO Box 1600, Canberra, A.C.T. 2601, Australia

¹ Plant Science CRC, GPO Box 475, Canberra, A.C.T. 2601, Australia,

² Danish Institute of Animal Science, Research Centre, Foulum, Box 39, DK8830, Tjele, Denmark³

Abstract

Chimeric genes encoding a sunflower seed albumin (SSA) rich in the nutritionally essential amino acids, methionine and cysteine, were transferred to tobacco, lucerne and subterranean clover using *Agrobacterium tumefaciens*. In the vegetative parts of transgenic subterranean clover plants, SSA accumulated to levels estimated to supply rumen-protected sulphur amino acids in quantities similar to those reported to result in increased wool growth in sheep. A gene encoding SSA was transferred to lupin, and accumulation of the protein in transgenic seeds was associated with increases in seed sulphur amino acid content and nutritional value.

Introduction

Animals are incapable of synthesising ten of the twenty amino acids needed for protein synthesis. These so-called essential amino acids must consequently be obtained from the diet. In order to obtain maximal efficiency of feed protein utilisation, the intake of the essential amino acids must be balanced with respect to each other. For this reason, animal feed formulations usually include protein from diverse sources. Cereal grains, commonly used as the principal energy source in the diet, contain protein poor in lysine (1). Conversely, legume seed protein generally contains an adequate supply of lysine, but is deficient in the sulphur amino acids, methionine and cysteine (2). Deficits in essential amino acids which can consequently occur in formulated diets for animals are often rectified by the addition of pure, crystalline amino acids.

With the advent of gene transfer technology, it is now possible to think of enhancing the levels of essential amino acids in some of the plant material commonly used as animal feed. For example, proteins with unusually high contents of methionine and cysteine have been identified, and their structural genes cloned (3, 4). Genes encoding a methionine- and cysteine-rich Brazil nut seed 2S storage protein have been transferred to canola (5) and narbon bean (6) resulting in seed-specific expression of the introduced genes, and concomitant increases in sulphur amino acid content of seed protein. One concern regarding the use of the Brazil nut protein for nutritional enhancement, particularly of material for human consumption, is the recently demonstrated allergenicity of the protein in some subjects (7).

³ We dedicate this report to the memory of Dr. Bjorn Eggum who died after a short illness on March 12th 1996

We report the transfer of a constitutively-expressed chimeric gene encoding a methionine- and cysteine-rich sunflower seed storage protein to the pasture plant, subterranean clover (*Trifolium subterraneum*) in order to improve the nutritional value of the leaf and stem protein for ruminant animals. In addition, we have transferred a seed-specific gene encoding the sunflower protein to the narrow leaf lupin, (*Lupinus angustifolius*), pea (*Pisum sativum*), and chickpea (*Cicer arietinum*) for enhancement of the sulphur amino acid content of the seed protein, and hence its nutritional value.

Adding proteins rich in sulphur amino acids to the leaves of pasture legumes

It is especially difficult to accurately formulate the diets of ruminant animals. The protein ingested by ruminants is largely converted to microbial protein during fermentation in the rumen. This conversion process utilises the ingested protein relatively inefficiently, with significant losses of nitrogen and sulphur occurring before the eventual degradation and assimilation of the microbial protein in the lower gastro-intestinal tract of the animal (Fig. 1). A small number of proteins have been shown to be naturally resistant to degradation by the rumen microflora, by virtue of their secondary structures (10). Such proteins, referred to as "bypass protein", travel to the true stomach, or abomasum, of the animal in a relatively intact form. There, they are degraded and their amino acids are absorbed directly into the animal's bloodstream.

Nutritional studies involving feeding of protein that is chemically protected against degradation in the rumen demonstrate that ruminant animal production is often limited by amino acid supply (11, 12). Furthermore, post-ruminal infusion of specific amino acids, has been reported to improve animal production. In particular, post-ruminal infusion of methionine and cysteine in sheep can result in large increases in wool growth rate (Fig 2, and refs 13 and 14).

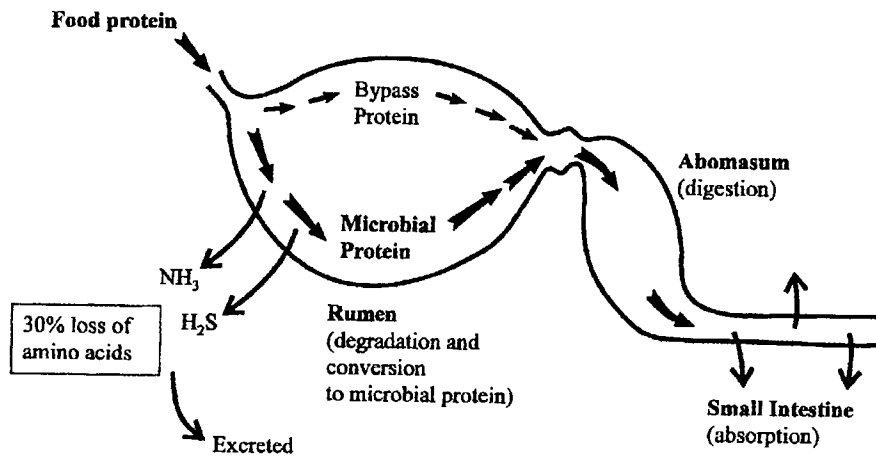


Fig. 1. The fate of dietary protein in sheep. In the rumen, ingested protein is degraded to ammonia, approximately 70% of which is converted to microbial protein. The remaining 30% is absorbed across the rumen wall into the bloodstream, and eventually excreted in the form of urea (8). A similar proportion of unutilised sulphur from degraded protein is lost from the rumen as hydrogen sulphide (9).

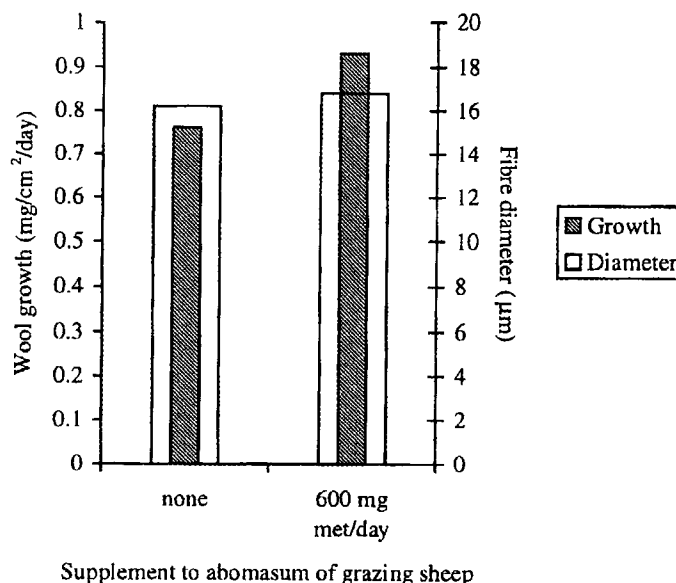


Fig. 2. Effects of methionine on wool growth and fibre diameter. Modified from Pickering and Reis (14). Sheep received supplements in the form of gelatin capsules via an abomasal cannula once daily. Wool growth was measured by clipping a midside area of about 100 cm², at three-week intervals. Values given are the means for 5 animals. Maximum effective standard error was 1.25 mg cm⁻² day⁻¹. Fibre diameters are the means for 5 animals. Maximum effective standard error was 0.75 µm.

We have identified proteins that are both rich in the sulphur amino acids, and naturally resistant to degradation in the rumen (15). One of these, sunflower seed albumin 8 (SSA), which contains 23.3 percent methionine plus cysteine (4), was degraded from a polypeptide of 12 kilodaltons, with a half-life of three hours, to a polypeptide of 8 kilodaltons that persisted in an *in vitro* rumen experimental system with a half-life of 69 hours (16). SSA has therefore been the basis for an approach designed to supplement pasture legumes with rumen-protected sulphur amino acids, via expression of introduced genes in transgenic plants.

We constructed a chimeric gene incorporating the protein-coding information for SSA fused to a strong, constitutive plant promoter from the 35S gene of cauliflower mosaic virus (CaMV). The 35S-ssa gene was transferred to tobacco and lucerne using *Agrobacterium tumefaciens*-mediated transformation. A number of transgenic plants were generated in each case, and northern blotting of total leaf RNA showed the presence of readily detectable levels of SSA mRNA in most transformants. However, no SSA protein was found in the leaves of the transgenic plants, indicating a post-translational limitation to protein accumulation (17). Dicot seed storage proteins contain targeting information which directs their intra-cellular transport from the endoplasmic reticulum (ER) to the vacuole of the developing seed. In this organ, the vacuole is specialised for protein storage, whereas the function of the vacuole in leaves is thought to be analogous to that of the hydrolytic lysosome of mammalian cells. It was concluded that the SSA protein was unstable in its vacuolar location in leaves (17), as had been previously observed for a pea seed storage protein, vicilin (18).

It was demonstrated in the case of vicilin, that re-direction of the protein to different sub-cellular locations resulted in increases in the half-life of the protein, and in the consequent accumulation of the protein in leaves. The protein was found to accumulate to the highest levels when targeted to the ER (18). Similarly, when the *ssa* gene was modified to include a nucleotide sequence encoding a carboxy-terminal ER retention signal (lysine-aspartate-glutamate-leucine, KDEL, see Fig. 3a), the SSA protein was readily detectable in leaves of transgenic tobacco and lucerne. However, the maximum levels of SSA expression in lucerne were estimated to be too low to result in significant nutritional enhancement of the transgenic plant material (15, 17).

The *35S-ssakdel* gene was also transferred to subterranean clover, the major pasture legume grown in southern Australia, using an *A. tumefaciens*-mediated transformation system (19). A progeny line homozygous with respect to the transgene was selected from the transgenic plant expressing the highest level of SSA. Under glasshouse conditions, the sunflower albumin accumulated in progressively older leaves of the transgenic plants, indicating that SSA was more stable than the average leaf protein (Fig 3b). SSA was estimated to comprise 0.3% of the total extractable protein of young leaves, increasing to approximately 1.3% in the oldest leaves, with an average over the aerial parts of the plant (leaves and stems) equal to 0.75% of total extractable protein.

It has recently been demonstrated that the above levels of SSA expression are maintained under field trial conditions. Assuming that the dry matter of subterranean clover is 20% protein, it can be calculated that a sheep consuming 1 kg of dry matter per day would ingest approximately 1.5 g of SSA, containing approximately 300 mg of rumen-protected methionine plus cysteine. This level of sulphur amino acid supplementation is at the lower end of the range of levels reported to result in significant increases in wool growth rate in sheep. Langlands (20) recorded an increase of 23% in wool growth in sheep supplied 360 mg of methionine per day direct to the abomasum, while Pickering and Reis (14) reported a 22% increase in wool growth in response to 600 mg of methionine per day supplied via an abomasal cannula (see Figure 2).

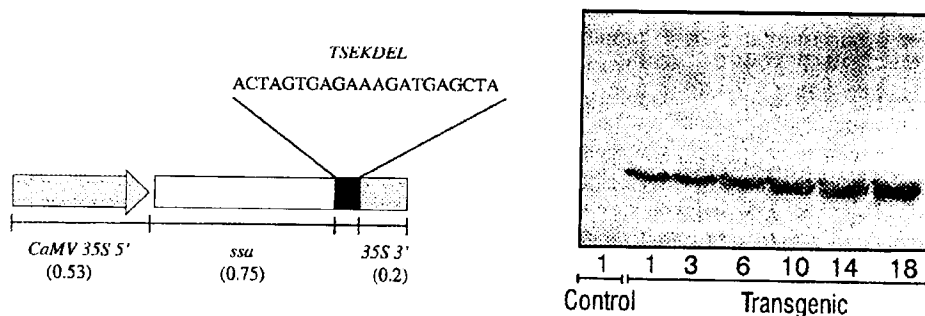


Fig. 3. Expression of SSA in transgenic subterranean clover.

a) Structure of the *35S-ssakdel* gene. Gene construction was as described in reference 17. The stop codon of the SSA protein-coding region was replaced with the nucleotide sequence shown, encoding a seven amino acid extension, which ends with the ER retention signal. Numbers in brackets refer to the lengths of DNA fragments in kilobases.

b) Accumulation of SSA in progressively older leaves of transgenic subterranean clover. Modified and reproduced with permission from reference 19. A western blot with 40 µg total leaf protein per lane, probed with antiserum to SSA. The numbers refer to the leaf number, starting with the youngest leaf, from control or transgenic subterranean clover plants grown in the glasshouse.

Given that subterranean clover usually constitutes 50% or less of the diet of grazing sheep, we would predict that sheep would obtain an additional 150 mg of cysteine and methionine from the transgenic subterranean clover in a field situation. We therefore aim to increase the level of SSA in transgenic subterranean clover by expression of modified SSA-encoding genes in the transgenic plants. It should be noted that increases in wool growth rate resulting from sulphur amino acid supplementation are accompanied by increases in wool fibre diameter (14). Too large an increase in fibre diameter will result in a reduction in the quality of the wool. Therefore our ultimate aim is to achieve relatively modest increases in wool growth rate, accompanied by only slight increases in fibre diameter. For example, as shown in Figure 2, Pickering and Reis (14) recorded a 22% increase in wool growth accompanied by only a 3.7% increase in fibre diameter.

Adding proteins rich in sulphur amino acids to the seeds of pulses

The pulses, lupin, pea and chickpea are widely used as sources of protein in human and animal diets. The seed protein of pulses is regarded as being of high nutritional quality because of its relatively high content of lysine (around 4.5 to 7% of total protein). The limitation to utilisation of pulse seed protein lies in the low content of the sulphur amino acids (around 2.2 to 3% of total protein) which is well below the estimated dietary requirement of non-ruminant animals (approximately 5% of total protein). In order to supplement the seed protein of pulses with extra cysteine and methionine, we have constructed and transferred seed-specific chimeric genes encoding SSA to the narrow-leaf lupin, field pea and chickpea.

A chimeric gene was constructed incorporating the promoter from a pea vicilin gene (*vic*), which is normally expressed only in the developing pea seed between approximately 11 and 21 days after flowering (21). The vicilin gene promoter was fused to the DNA encoding an unmodified SSA protein (Fig. 4a). The gene was transferred to the three pulse species using *A. tumefaciens*-mediated transformation protocols established recently in our laboratory (22, Molvig *et al*, unpublished). In all cases, transgenic lines expressing readily detectable amounts of SSA in seeds were produced (Fig. 4b).

A transgenic lupin progeny line (from cv Warrah) which was homozygous with respect to the transgene, was estimated to contain SSA equivalent to over 5% of extractable seed protein. The physical composition of grain from this line was compared with that of control, non-transgenic lupin seed (Molvig *et al*, in preparation, summarised in Table 1). Seed weights and total protein content did not differ between the two samples. The sulphur in each seed sample was estimated by an X-ray spectrometry technique that can be used to resolve two major fractions corresponding to sulphate (oxidised sulphur) and sulphur amino acids (carbon-bonded sulphur) in plant material (23). The total sulphur content in each sample was very similar, however the partitioning of that sulphur between the oxidised and carbon-bonded fractions was markedly different between the two samples. In the transgenic grain, the carbon-bonded sulphur fraction represented 79.6% of the total seed sulphur, in contrast to 62.7% in the case of the control seed. This equated to an increase in carbon-bonded sulphur of approximately 27% in the transgenic seed. Analysis of amino acid composition of the two samples showed a more than 100% increase in the methionine content of the transgenic seed. As far as can be assessed under glasshouse conditions, seed viability and seed yield of the transgenic plants was not altered in comparison to controls.

A rat feeding trial was performed with the transgenic lupin seed, and it was found that the increased sulphur amino acid content correlated with improved efficiency of utilisation

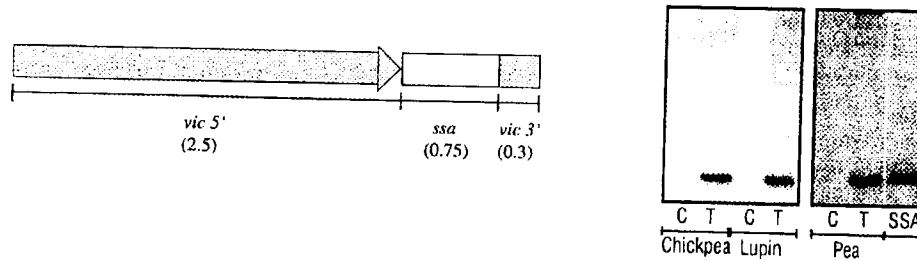


Fig. 4. Expression of SSA in seeds of transgenic chickpea, lupin and pea.
 a) Structure of the *vic-ssa* gene. The numbers refer to lengths of DNA fragments, in kilobases.
 b) Detection of SSA in seed extracts from transgenic pulses. A composite of two western blots showing: on one blot, the presence of SSA in 1 µg of total protein from a single control (C) or transgenic (T) seed of chickpea or lupin, and in the other, SSA in 20 µg of protein from a single control (C) or transgenic (T) seed from pea, as well as 50 ng of pure SSA (SSA).

Table 1. Characteristics of transgenic lupin seeds

Determination	Non-transgenic lupin	Transgenic lupin
Average seed weight (mg)	157	154
Protein (% DW)	34.3	35.8
Sulphur Total (ppm)	3800	3900
Oxidised (ppm)	1411	790
Carbon-bonded (ppm)	2350	3085
Methionine (µg/16gN)	0.65	1.40
Biological Value (%)	69.9	77.6

tion of the seed protein, as measured by biological value of the diet (Table 1). We have therefore demonstrated the feasibility of improving the nutritional quality of plant protein by addition of an exogenous protein rich in amino acids often present in limiting quantities in the diets of ruminant and non-ruminant animals. An SSA-containing lupin line is currently undergoing field trial to investigate its agronomic performance and to obtain large enough seed stocks for feeding trials with pigs, chickens and sheep. Transgenic field peas and chickpeas expressing SSA are also being evaluated.

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