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Accumulation of a sulphur-rich seed albumin from sunflower in the leaves of transgenic subterranean clover (*Trifolium subterraneum* L.)

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A gene encoding a sulphur-rich, sunflower seed albumin (23% cysteine plus methionine) was modified to contain the promoter for the 35S RNA of cauliflower mosaic virus, in order to obtain leaf expression in transgenic plants. In addition, a sequence encoding an endoplasmic reticulum-retention signal was added to the 3′ end of the coding region so as to stabilize the protein by diverting it away from the vacuole. The modified gene was introduced into subterranean clover (*T. subterraneum* L.) and its expression was detected by northern and western blots and by immunogold localization. The albumin was accumulated in the lumen of the endoplasmic reticulum, and, among six independent, transformed lines, it accumulated in the leaves of T_0 transgenic plants at varying levels up to 0.3% of the total extractable protein. The level of accumulation of the sunflower albumin increased with increasing leaf age, and in the older leaves of the most highly expressing plants of the T_1 generation it reached 1.3% of total extractable protein. Expression of the SSA gene was stable in the first and second generation progeny. These results indicate that there is potential for significantly improving the nutritional value of subterranean clover for ruminant animals such as sheep by expressing genes that code for sulphur-rich, rumen-stable proteins in leaves.

Keywords: transgenic subterranean clover; seed protein gene; leaf expression; sulphur-rich protein

Introduction

In developing seeds of dicotyledonous plants, proteins and other reserve compounds are accumulated in the vacuolar protein bodies where they are stable until such time as the mature seed germinates. In contrast, the vacuole in the cells of expanding leaves is a lytic compartment, analogous to the lysosomes of mammalian cells (Matile, 1978). As a result, when an unmodified seed protein is produced in the leaves of a transgenic plant, the protein is transported through the endomembrane system to the leaf vacuole (Saalbach *et al.*, 1994), where it is exposed to an environment that is unfavourable for high level accumulation of that protein. For example, Wandelt *et al.* (1992) showed that the pea seed storage protein vicilin was rapidly degraded in the leaf vacuoles of transgenic tobacco and lucerne. However, if the protein-coding region of the vicilin gene was first modified by the inclusion of a sequence coding for an endoplasmic reticulum (ER)-retention signal at the carboxyl terminus of the resultant protein, the half-life of the vicilin was extended from 4.5 h to more than 48 h. This indicates clearly that the ER is a more benign environment for the accumulation of this foreign protein, in spite of the fact that this compartment also contains some resident proteases (Klausner and Sitia, 1990).

We have exploited these results to improve the nutritional status of an agronomically important pasture legume, subterranean clover. This is the major pasture legume in southern Australia and is also grown in the Mediterranean regions, the Americas, Africa, New Zealand and Japan (Johnstone and McLean, 1987). Subterranean clover-based pastures are a major contributor to the wool industry in Australia and their beneficial effects on soil N status carry over to subsequent non-leguminous crops in ley farming systems.

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It is well established that wool production by sheep grazing such pastures can be limited by the post-ruminal supply of S-containing amino acids (Marston, 1955). Post-ruminal supplements of cysteine and methionine have been reported to result in pronounced increases (16 to 130%) in wool growth (Reis and Schinckel, 1963; Langlands, 1970; Reis, 1979; Cobon *et al.*, 1988; Morgan *et al.*, 1990; Pickering and Reis, 1993). Similar increases in wool growth were also achieved by feeding with sulphur-rich casein, but only when its degradation in the rumen was avoided or minimized (Dove and Robards, 1974). It is known that unprotected dietary proteins ingested by sheep are rapidly degraded by bacteria in the rumen and converted to microbial proteins, which then form the main source of protein for the animal. Rumen microbial proteins are typically low in S-amino acids (Thompson, 1982) and, as a result, there is a significant loss of cysteine and methionine during the conversion of ingested protein to microbial protein.

In an effort to circumvent this problem of limiting S-amino supply, we have undertaken a programme aimed at using genetic engineering technology to introduce into subterranean clover a protein which is both rich in S-amino acids and resistant to breakdown in the rumen. One such protein, sunflower seed albumin (SSA), has been shown to contain 16% methionine and 8% cysteine (the SFA8 protein of Kortt *et al.*, 1991), and to be resistant to rumen degradation *in vitro* (McNabb *et al.*, 1994). A chimaeric SSA gene was constructed by the addition of a 5′ region that ensured expression in the leaf, and a sequence encoding an ER-retention signal was added to the 3′ end of the protein-coding region. The modified gene was introduced into subterranean clover using our recently developed transformation and regeneration system (Khan *et al.*, 1994). This paper reports the characteristics of SSA gene expression in transgenic subterranean clover, its transmission to subsequent generations, and stable accumulation of the SSA protein in young and old leaves of transgenic plants.

Materials and methods

Source of seed material

Seeds of subterranean clover (*T. subterraneum*, ssp. *yanninicum*) cv. Larisa were obtained from the Western Australian Department of Agriculture.

Gene reconstruction and transfer to subterranean clover

A chimaeric gene encoding the sunflower seed albumin (SSA) was constructed as described in detail by Tabe *et al.* (1995). In brief, the important features of the construct were as follows. The protein-coding region of the SSA gene, including the intron, was modified by the inclusion of a nucleotide sequence encoding seven extra amino acids immediately upstream of the stop codon. The seven amino acids were N-terminal: threonine, serine, glutamic acid, lysine, aspartic acid, glutamic acid and leucine: C-terminal (TSEKDEL). The last four amino acids have been shown to target proteins to the lumen of the ER (Munro and Pelham, 1987). The gene promoter was derived from the cauliflower mosaic virus (CaMV) 35S RNA gene (nucleotides 6909 to 7437 of the CaMV genome) (Pietrzak *et al.*, 1986), and the termination region was from the same CaMV 35S gene (nucleotides 7438 to 7632).

The sunflower albumin gene was cloned into the binary vector pTAB10 (Tabe *et al.*, 1995), which contained the *Agrobacterium tumefaciens* borders flanking a chimaeric gene (*bar*) encoding resistance to phosphinothricin (PPT), and was transferred to *A. tumefaciens* AGL1 (Lazo *et al.*, 1991) by triparental mating (Ditta *et al.*, 1980). The integrity of the gene construct in *A. tumefaciens* was verified by restriction enzyme digestion prior to transfer to subterranean clover. A schematic diagram of the T-DNA is shown in Fig. 1.

The procedure for transferring new genes to subterranean clover has been described in detail (Khan *et al.*, 1994). Briefly, seeds were surface-sterilized, imbibed overnight and the lower hypocotyl together with the radicle (2 to 3 mm) were excised with the aid of a dissecting microscope. *A. tumefaciens* harbouring the recombinant plasmid was grown in Luria broth and was used for inoculation of the hypocotyl explants. After inoculation, the explants were transferred to the cocultivation medium for 7 days. The hypocotyl explants were then cut 1–2 mm below the first cut and the root segment was discarded. These secondary explants were placed with their newly-cut surface in contact with the regeneration medium, which was supplemented with PPT (50 mg l^{-1}). Surviving explants were transferred to fresh medium every three weeks. After 60 to 70 days, vigorous shoots growing on the selection medium were

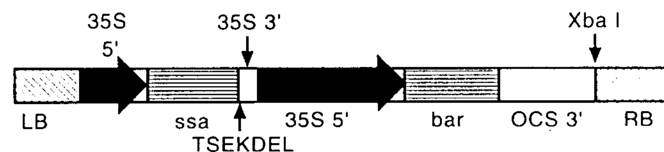

Fig. 1. Schematic structure of the two chimaeric genes transferred into subterranean clover. DNA encoding the sequence: threonine, serine, glutamic acid, lysine, aspartic acid, glutamic acid and leucine (denoted TSEKDEL) was added to the 3′ end of the SSA protein-coding region to ensure retention of the protein in the ER. The promoters (denoted 35S 5′) of the two genes are shown as black arrows, and the terminators (denoted 35S 3′) are shown as clear boxes. The left and right borders (denoted LB and RB) represent the boundaries of the T-DNA. The single *Xba* I restriction site in the T-DNA is shown, and *Xba* I was used to digest genomic DNA for Southern blotting.

excised, rooted, and transferred to pots in the glasshouse. The characteristics of six independent transformants are reported in this paper. One of these (plant 6) was allowed to self-pollinate to produce the T_1 generation, and similarly, one T_1 plant was selected to produce (through self-pollination) a T_2 generation of seeds.

Enzyme assay

Phosphinothricin acetyl transferase (PAT) activity was assayed by the procedure of Spencer *et al.* (1990), with slight modification. Leaf tissue (100 mg) from glasshouse-grown plants was extracted with 1 ml of extraction buffer A (100 mM Na-phosphate, pH 7.0, 20 mM NaCl, 1.0 mM phenylmethyl sulphonyl fluoride [PMSF], and 25 mM ascorbic acid). The protein content of the extract was determined by Bradford (1976) assay. The PAT enzyme reaction mixture (18 μl final volume) contained 30 μg of leaf protein, 0.83 mM glufosinate ammonium (diluted from the commercial preparation Basta™, AgrEvo, Australia) and 0.08 μCi of [^{14}C]- acetyl CoA (50–60 mCi/mmol, Amersham) in the extraction buffer A described above and was incubated at 37 °C for 30 min. The reaction mixture was centrifuged at 10 000 g for 30 s, and 15 μl of supernatant was spotted onto a thin layer silica gel plate (0.2 mm, Merck) and chromatographed in a 3:2 (v/v) mixture of 1-propanol and NH_4OH (28% NH_3). Plates were dipped in a solution of 0.4% 2,5-diphenyloxazole in 1-methyl naphthalene, air-dried, and ^{14}C-acetylated PPT was detected by fluorography.

RNA and protein isolation and detection

Total RNA was isolated as described in Khan *et al.* (1994) and northern blot analyses were done according to Higgins and Spencer (1991). The DNA fragment used for making the SSA probe was obtained by polymerase chain reaction (PCR) amplification of a cDNA clone encoding SSA (pSF8, Kortt *et al.*, 1991) using the nucleotides: 5′GGGGATCCATGGCAAGGTTTTCGATCG3′ (oligo 1) and 5′GGGAATTCCCGGGTTTACATTTGGCATGG3′ (oligo 2) which flank the protein-coding region of the gene. The SSA probe was made by using an aliquot of this PCR as a template in a second PCR containing ^{32}P-dCTP and oligo 2. This should result in the synthesis of a predominantly single-stranded probe. Aliquots of 5 μg of total RNA from plants within each generation were analysed on separate blots. Comparisons of mRNA levels are therefore valid only within, but not between, generations.

Protein was extracted from leaves (100 mg) of glasshouse-grown plants with 1 mL extraction buffer B (0.1 M Tes, pH 7.8, 0.2 M NaCl, 1 mM EDTA, 1 mM PMSF, 25 mM ascorbic acid). Protein concentration was determined by the method of Bradford (1976). SDS-polyacrylamide gels (20%) were prepared according to Laemmli and Favre (1973). Protein samples were diluted in sample buffer (0.25 M Tris-HCl, pH 6.7, 2% SDS, 10% glycerol) before loading onto gels and electrophoresing for 2 to 3 h. The fractionated polypeptides were transferred to nitrocellulose membrane (Laurière, 1993) and SSA was detected with a primary antibody prepared in goats, and a secondary anti-goat immunoglobulin G Fab to which was conjugated alkaline phosphatase. The primary SSA antibody was treated with Sepharose-4B to which a preparation of total leaf protein was covalently bound. This pre-treatment reduced the level of non-specific antibody binding to leaf proteins other than SSA which was evident when leaf extracts from both transgenic and non-transgenic plants were incubated with unfractionated serum. Quantitative estimations of the levels of SSA protein in different transgenic plants were made by densitometry of western blots using a NovaLine Gel Documentation System (NovaLine Inc., Glendale, CA, USA) in conjunction with an Imagequant program.

Immunogold localization of SSA protein in the leaf cells of transgenic subterranean clover was carried out as described in Wandelt *et al.* (1992).

Results and discussion

Expression of the SSA gene

RNA was isolated from the leaves of all primary transgenics (plants 1 to 6 of the T_0 generation) and from four representatives of the T_1 and T_2 generations descended from plant 6 of the T_0 generation (plant T_06). Northern blot analyses showed that there was a ten-fold variation in the level of SSA mRNA in leaves of comparable age taken from the primary transgenics (Fig. 2), and no signal was obtained from untransformed plants (not shown). Four of the T_1 plants derived from plant T_06 showed a two-fold range in their mRNA levels (Fig. 2). The level of mRNA in plant 3 of the T_1 generation (plant T_06–T_13) was twice that of its siblings. Four individual T_2 progeny from this plant showed a uniform level of expression of SSA mRNA. Of this T_2 generation, 300 plants were tested for phosphinothricin resistance and all were resistant, whereas control plants were sensitive. These findings indicated that the T_2 progeny of line T_06–T_13 were homozygous for at least one of the T-DNA loci.

The level of SSA protein in the young leaves of primary transgenics and some of their progeny is shown in Fig. 3. The level of SSA in the primary transgenics correlated closely with the level of its mRNA (compare Figs 2 and 3). Densitometric measurements on western blots containing known amounts of SSA protein as well as extracts of transgenic leaves from each of the T_0 plants showed that SSA level varied between 0.05 and 0.3% of total extractable protein in the T_0 generation. The SSA level in the T_1 generation, derived from plant T_06, varied between 0.3% and 0.6%. The highest level of SSA was

Fig. 2. Northern blots showing SSA mRNA from young leaves of primary transgenics and progeny from single lines. Each lane was loaded with 5 μg of total RNA and probed with an SSA cDNA probe. The top row shows RNA isolated from plants 1 to 6 of the T_0 generation; the second row shows RNA from four of the T_1 progeny from plant T_06; the third row shows RNA from four of the T_2 progeny of plant T_06–T_13. RNA from the three different generations of plants was analysed on separate blots, at different times, therefore comparisons of band intensities are valid within, but not between, generations.

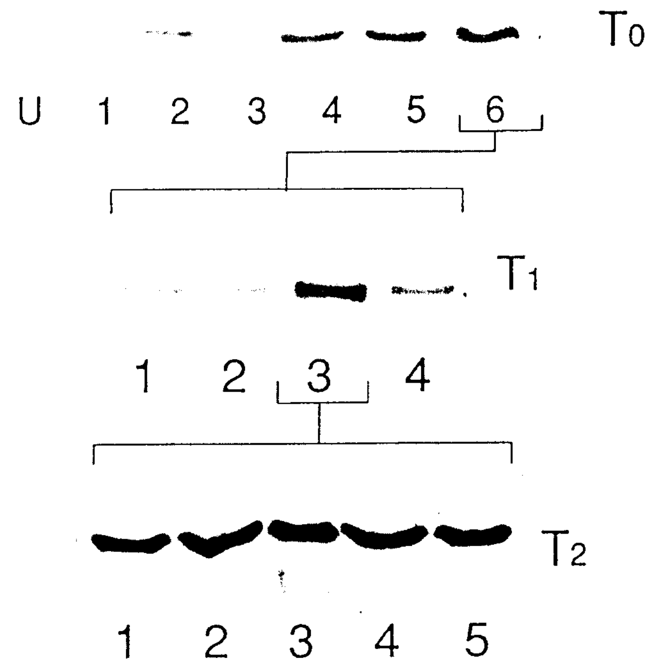

Fig. 3. Western blots showing expression of the SSA gene in the primary transgenics and in selected progeny. Each lane was loaded with 40 μg of total extractable protein from the young leaves of transgenic subterranean clover. For legends to lanes, see Fig. 2. U stands for an untransformed plant. Protein from the three different generations of plants was analysed on separate blots at different times; therefore comparisons of band intensities are valid within, but not between, generations.

from plant T_06–T_13. The progeny of this plant (T_2 generation) showed a uniform level of SSA protein which was equal to that of the parent plant, supporting the conclusion that the T_06–T_13 line was homozygous for at least one of the T-DNA loci.

When 22 progeny of the T_1 generation originating from plant T_06 were analysed for the presence of SSA protein, one was negative. This segregation ratio indicated that the parental plant T_06 contained at least two T-DNA insertions which segregated independently in the T_1 generation. Southern blotting analysis also indicated the presence of two T-DNA inserts in plant T_06 (data not shown).

Intracellular localization of SSA protein in leaves

Our previous studies have shown that vacuolar seed storage proteins such as vicilin (Wandelt *et al.*, 1992) and SSA (Tabe *et al.*, 1993) in the leaves of transgenic plants were rapidly degraded, unless they had been modified to contain an ER-retention signal. For example, when the SSA gene was introduced into tobacco with and without the ER-targetting sequence, the level of SSA protein in the leaves increased from below the limits of detection in the absence of the signal to 0.3% of total extractable protein in its presence (Tabe *et al.*, 1993). Immunogold labelling of leaf sections of transgenic subterranean clover showed that SSA was localized in large electron-dense inclusions within a lumen that was delimited by a ribosome-studded membrane (Fig. 4). We therefore concluded that these deposits were within rough ER cisternae, as seen in tobacco and lucerne (Wandelt *et al.*, 1992). SSA-containing inclusions were observed in palisade and spongy mesophyll cells, and in vascular parenchyma cells. In the xylem parenchyma, they were invariably larger than in the other cell types. In palisade and spongy mesophyll cells, there was not always a labelled inclusion body in any given cell profile. Given the cell diameter (20–150 μm), the inclusion body diameter (< 2 μm) and the section thickness (0.1 μm), it was not unexpected that some cell profiles appeared to lack inclusions. Serial sectioning

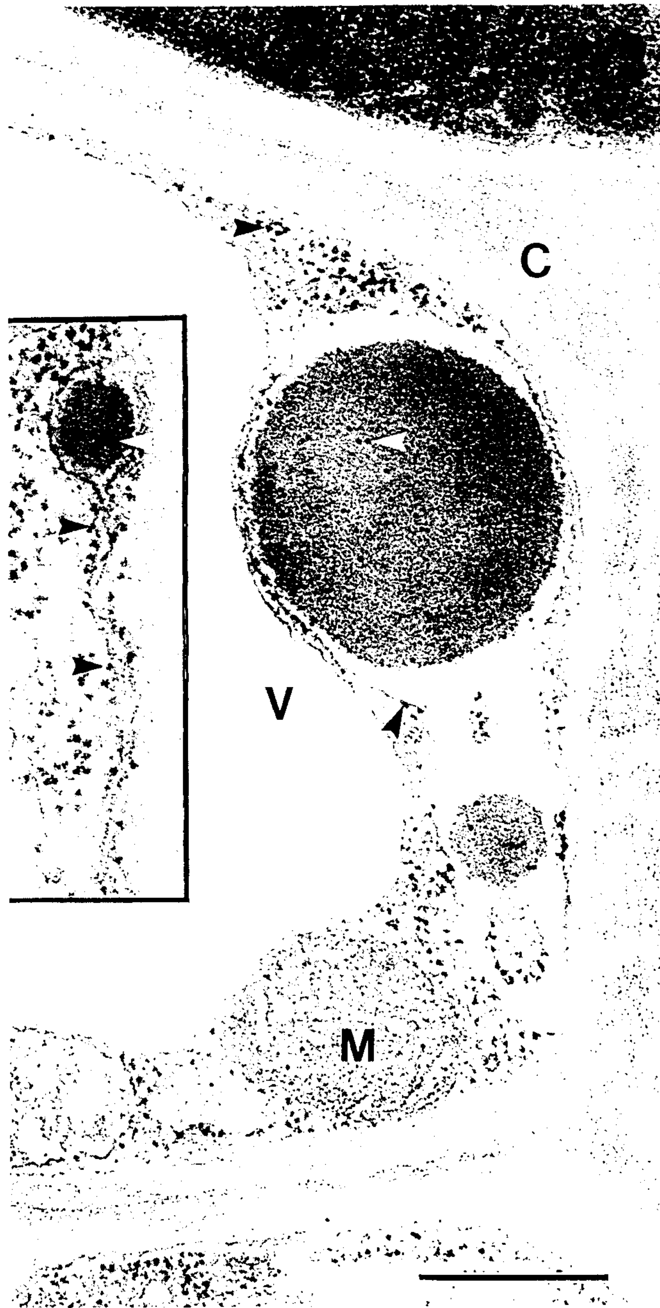

Fig. 4. Electron micrograph showing immunogold localization of SSA within a xylem parenchyma cell in a transgenic subterranean clover leaf. Insert = Mesophyll cell showing an SSA-containing, electron-dense deposit within a rough endoplasmic reticulum cisterna; V = vacuole; M = mitochondrion; C = cell wall; white arrows = gold particle in an electron-dense body; black arrows = cytoplasmic and membrane-bound ribosomes. X 53 000. The scale bar represents 0.5 μm.

would probably establish their presence in every parenchyma cell. Other areas of the leaf cells, such as the cell wall, cytoplasm and vacuole, showed no evidence of SSA accumulation.

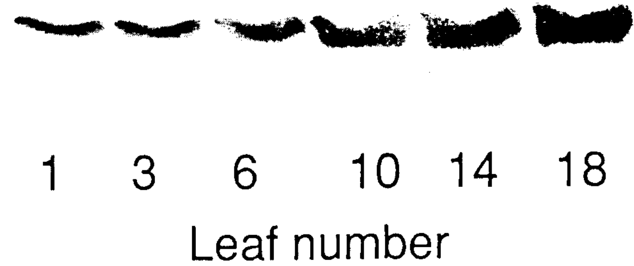

Fig. 5. A western blot showing SSA in leaves of transgenic subterranean clover (plant $T_0$6–$T_1$3). Forty μg of total extractable protein from leaves number 1 (youngest), 3, 6, 10, 14 and 18 (oldest) were loaded on each lane.

SSA level as a function of leaf age

In order to determine the accumulation of SSA as a function of leaf age, extracts were prepared from leaves of plant 3 of the T_1 generation, ranging from the youngest (leaf 1) to the oldest leaf (leaf 18). Leaf 18 was at an early stage of senescence when the harvest was made. Aliquots containing an equal amount of total soluble protein (40 μg) were analysed by western blot procedures to determine the level of accumulation of SSA. There was a steady increase in the relative level of SSA protein with increasing leaf age (Fig. 5). The level of SSA was quantified by densitometric analysis and SSA accumulation was calculated per leaf, per unit fresh weight and per unit extractable protein (Fig. 6). Leaf fresh weight increased steadily with age, and total protein per leaf reached a maximum at leaf 6 then declined with increasing age. There was a consistent decrease in total protein per unit fresh weight from the youngest to the oldest leaf. However, on any of these bases, there was a steady increase in the level of SSA accumulation from the youngest to the oldest leaf. For example, SSA per mg leaf protein increased from 2.5 μg in the youngest leaf to 13.5 μg in the oldest. In spite of the steady decline in total protein per unit fresh weight, the SSA level increased from 82 μg per gram fresh weight to 140 μg per gram fresh weight in the oldest leaf, and on a total leaf basis it increased from 2.6 to 24 μg per leaf. These results indicate that the SSA protein that is accumulated in the ER is more stable throughout most stages of leaf development than the total protein of the leaf. This is a favourable result in situations such as the present one where the aim is to accumulate the transgenic protein to the highest possible level.

SSA and leaf protein quality

The long-term aim of this project is to achieve a level of accumulation of a rumen-stable, sulphur amino acid-rich protein, such as SSA, in the leaves of transgenic

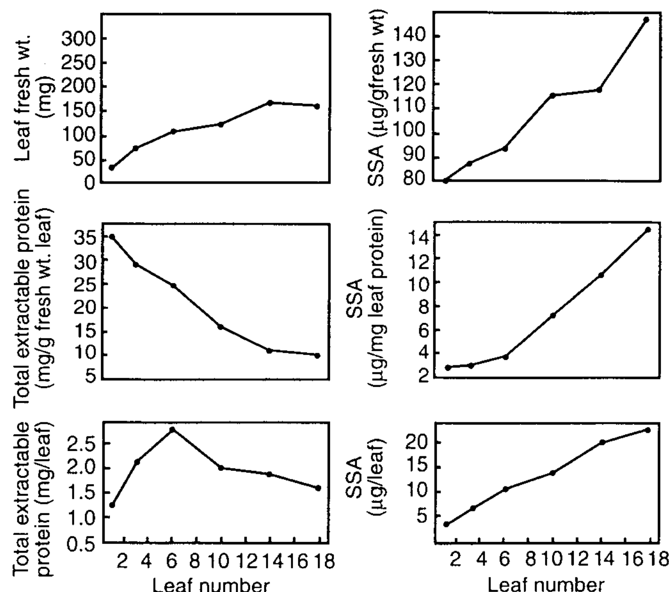

Fig. 6. Changes in levels of SSA and of total extractable protein with age of leaf in transgenic subterranean clover (plant T_06–T_13) expressed per leaf, per unit total soluble protein and per unit fresh weight. Leaf 1 is the youngest and leaf 18 is the oldest.

subterranean clover that would lead to significantly increased wool growth in sheep ingesting those plants. The amounts of extra post-ruminal sulphur-containing amino acids that have been shown to achieve an increase in wool growth vary between studies. When supplementary cysteine or methionine has been supplied post-ruminally, responses in wool growth have been obtained with amounts ranging from 200 mg to 2 g per day. For example, Langlands (1970) recorded an increase of 23% in wool growth in sheep by supplying 360 mg of methionine per day through the abomasum, and Pickering and Reis (1993) obtained a 22% increase with 600 mg of methionine supplied to sheep grazing on pasture which supported less than their full potential for wool growth.

In the present experiments, the targetting of SSA to the lumen of the ER resulted in its accumulation up to 1.3% of total extractable protein in the oldest leaves of the most highly expressing plants (Fig. 6), with an average value for all the leaves of 0.75%. Assuming a soluble protein level of approximately 20% in dry matter and, given that sheep consume approximately 1 kg of dry matter per day, it may be calculated that one kg of dry matter from transgenic subterranean clover leaves contains 1.5 g of SSA or an additional 300 mg of rumen-protected cysteine and methionine. This is at the lower end of the range at which a response in wool growth has been obtained in animal experiments. Added to that is the fact that subterranean clover constitutes only a part of the diet of the grazing sheep: approximately 50% in a good clover-grass pasture. We are endeavouring to increase the accumulation level of SSA still further by crossing the two most highly expressing transgenic lines of subterranean clover. Since they are derived from two independent insertion events, it is anticipated that the progeny should accumulate additive levels of SSA protein. In principle, the present findings indicate that it should be possible to increase significantly the nutritional value of this important pasture plant.

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