Delivery of grasses with high levels of unsaturated, protected fatty acids

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Abstract
Our goal is to increase the metabolisable energy of forage species (such as *Lolium perenne*, perennial ryegrass) via accumulation of lipids in the leaves, and further improve upon this by the delivery of polyunsaturated fatty acids whilst maintaining a low input agricultural system. To date we have generated transgenic ryegrass plants by over expressing the *Arabidopsis thaliana* triacylglyceride synthesising gene (DGAT1) which accumulate up to 40% more lipid in the leaf. We have also demonstrated that our invention, polyoleosin (designed to protect lipids from biohydrogenation), is correctly assembled and targeted to the oil bodies in the model plant *A. thaliana*. This paper reports on the preliminary findings of these studies.
Keywords: lipid protection, triacylglyceride, DGAT, oleosin, biohydrogenation

Introduction
New Zealand forage grasses such as *Lolium perenne* (perennial ryegrass) often lack sufficient nutritive value to achieve the levels of meat and milk productivity seen in non-pasture based systems. Particularly, pasture plants are relatively rich in protein in comparison to their overall energy contents. As a result, much of the ingested protein is degraded by the rumen micro-organisms and lost from the animal in the form of urea (Ulyatt *et al.* 1988). In addition, primary and secondary fermentation within the rumen leads to the release of methane gas, a product believed to act as an electron sink for unwanted hydrogen (McAllister *et al.* 1996). It has been reported that poor quality feeds with relatively high fibre levels (insoluble non-digestive carbohydrates) can lose up to 15% of the gross energy intake to the ruminant through methane production (Sauer *et al.* 1998).

In comparison, an increase in dietary lipids has been shown to provide an environmental benefit by reducing methane production (Ellis *et al.* 2007; Grainger *et al.* 2008). This agrees with the previous suggestion that improving the energy content of the forage by raising the concentration of fatty acids would lead to improved nitrogen utilisation and less methane production (Ulyatt 1981; Ulyatt *et al.* 1988). Elevated lipid levels in the diet (up to 10% of the dry matter), mostly in the form of triacylglyceride (TAG), have been shown to have a significant positive influence on the feed conversion efficiency of sheep (33%) and beef (6-11%) as well as increase milk production by enabling a higher stocking rate (10%) and reproductive performance by 5% (Cosgrove *et al*. 2004; Felton & Kerley 2004; Schroeder *et al*. 2004; Jenkins & McGuire 2006). However, given the relatively low level of lipid accumulation in the bulk of plant tissue the efficacy of this diet is rarely achieved without supplementation.

The concentration of fatty acids in the temperate grasses was found to be influenced by species, cutting date and cutting interval and, while it was concluded that there was potential to produce high lipid grasses, it was clear that both season and management practices had a substantial impact (Dewhurst *et al*. 2001). At any one time the majority of leaf lipid is in the form of membranes and the degree to which this can be altered is physically limited by space constraints. In comparison, most plants synthesise and store significant amounts of lipid as TAG in the developing seeds and pollen where it is subsequently utilised to provide energy during germination and pollen tube growth. TAG production is of tremendous commercial value with food, nutraceutical, and industrial applications. Consequently, numerous conventional and molecular genetic strategies have been explored in attempts to increase TAG content and modify the fatty acid composition of plant seed oils. Dicotyledonous plants can accumulate up to approximately 60% of their seed weight as TAG. Ordinarily, this level is considerably lower in the monocotyledonous seeds where the main form of energy storage is carbohydrates (e.g. starch). A key bottleneck in the biosynthesis of TAG *in planta* is the addition of a third fatty acid to an existing diacylglycerol, predominantly performed by: acylCoA:diacylglycerol acyltransferase (DGAT1) and an unrelated acyl DGAT2. A third plant enzyme, phosphatidylcholine-sterol O-acyltransferase (PDAT), can also generate TAG; however, it does not use acylCoA. A major part of our research presented here is the over expression of ryegrass and Arabidopsis DGAT1 and Arabidopsis DGAT2 and the analysis of fatty acid in ryegrass leaves.

The second aspect of our work has been investigating the potential to protect the ingested unsaturated lipids from becoming saturated in the rumen, a process termed biohydrogenation. This process dramatically influences the final lipid make up of ruminant meat and dairy

products (Demeyer & Doreau 1999; Lock & Bauman 2004; Firkins *et al*. 2007; Jenkins & McGuire 2006). Biohydrogenation can be prevented/reduced by encapsulating the lipids in a protein or proteins that provide resistance to microbial degradation (Jenkins & Bridges 2007). In nature, both pollen and seeds accumulate TAG in the form of micelles (oil bodies) which consist of a TAG core surrounded by a spherical phospholipid monolayer and one or several species of oleosin proteins. We are seeking to exploit this phenomenon by manipulating the properties of oleosin to protect the encapsulated TAG from biohydrogenation. The topology of oleosin is attributed to its physical properties which includes a folded hydrophobic core flanked by hydrophilic domains. This arrangement confers an amphipathic nature to oleosin resulting in the hydrophobic domain being embedded in the phospholipid monolayer and projecting into the TAG core (Roberts *et al*. 2008) while the flanking hydrophilic domains are exposed to the aqueous environment of the cytoplasm. At the laboratory level, we have modified oleosin by engineering the head to tale fusion of two or more oleosin units to create polyoleosin (Scott *et al.* 2006; Roberts *et al.* 2008). Altering the number of oleosin units enables the properties (thermal stability and degradation rate) of the oil bodies to be tailored.

This work is now progressing towards engineering both polyoleosin and elevated TAG biosynthesis into the leaves of forage plants to deliver polyunsaturated fatty acids whilst maintaining our commitment to New Zealand's low input agricultural systems based on ryegrass. The results to date and our projection for future work are presented here.

Methods

Expression of DGAT1 in ryegrass

Wild type ryegrass calli was transformed by particle bombardment with a variety of promoters and nucleotide sequences encoding for Arabidopsis or ryegrass DGAT1 and Arabidopsis DGAT2 (Table 1). The coding sequences were optimised for expression in rice (the closest neighbour to ryegrass which has regularly been transformed) and were synthesized by GENEART (www.geneart.com). All transformed calli were analysed for the corresponding transgenes by PCR and/or Southern blotting.

Analysis of fatty acids in ryegrass leaves

Leaf material was harvested and snap frozen in liquid nitrogen and then stored at -80°C until required. Samples were ground to a fine powder in liquid nitrogen and 25-65 mg of frozen material was accurately weighed into a glass vial, extracted for methyl esterified fatty acids (FAMEs) and quantified by gas chromatography/mass spectroscopy (GC/MS) described by Browse *et al.* (1986) using the 50mQC2/BPX70-0.25 GC capillary column (part# 054603, SGE). Each sample was extracted in duplicate. The lipid content of each sample was semiquantified based on the internal C15:0 standard added prior to methyl esterification. These total fatty acids are presented as percentage of leaf fresh weight as well as the proportion made up from C16:0, C16:1, C18:0, C18:1, C18:2, and C18:3 (The shortest descriptions of fatty acids include the number of carbon atoms and double bonds).

Transformation and analysis of oleosin/polyoleosin accumulation in *Arabidopsis thaliana*

Constructs containing either a single, trimeric or hexameric tandem repeat encoding for the sesame seed oleosin (Accession number AF091840) were optimised for expression in Arabidopsis and synthesised by GENEART. These were placed under the control of either the CaMV35s promoter or the Arabidopsis oleosin seed promoter (Plant *et al*. 1994) and transformed into Arabidopsis using agrobacterium mediated transformation (Cookson *et al*. 2007).

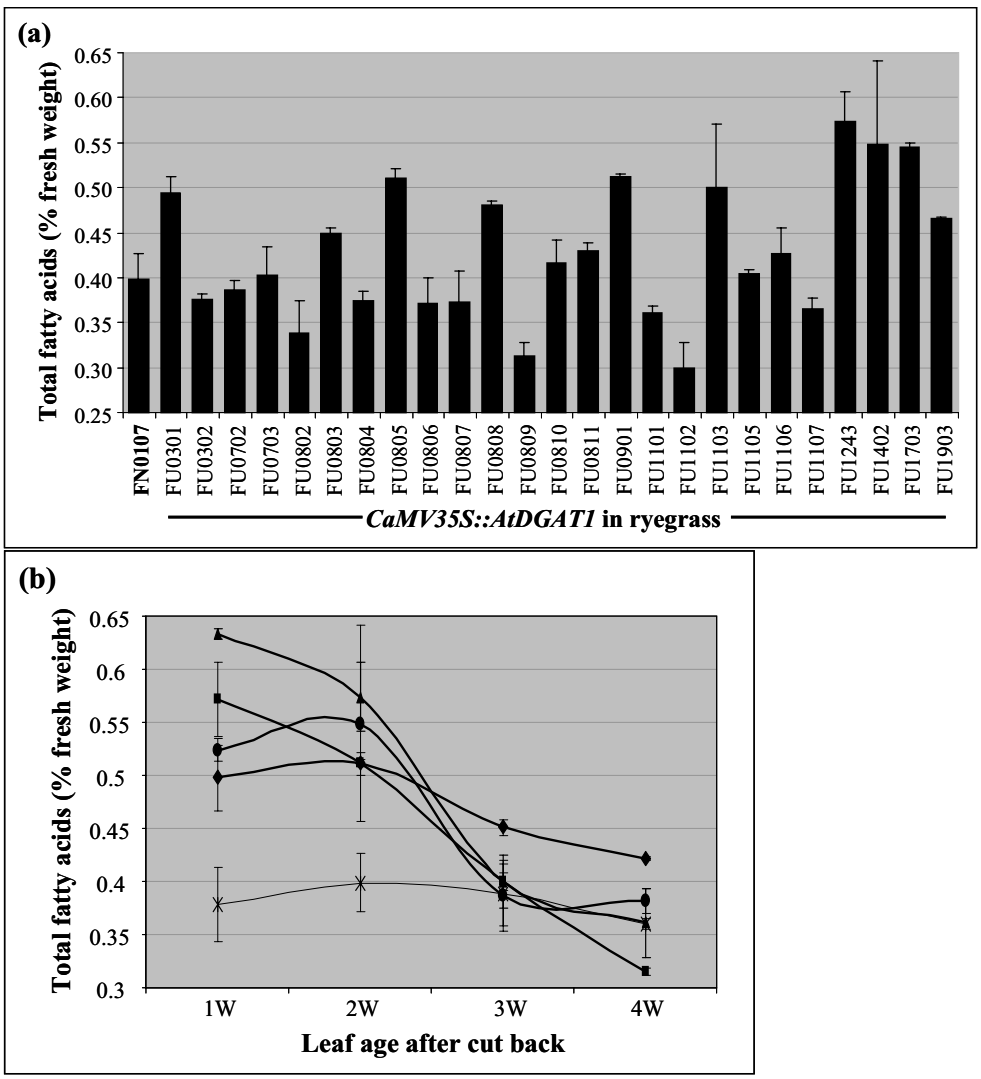
Accumulation of recombinant polyoleosin in the leaves or seeds of Arabidopsis was analysed by SDS-PAGE/immunoblot. Three mature Arabidopsis leaves per plant were ground in 300 µL buffer (50mM Tris-HCl pH8, 5mM EDTA pH8, 0.2% ß-Mercaptoethanol, and 1mM PMSF) and boiled for 10 min with 100 µL of

Table 1 Numbers of transgenic ryegrass lines generated from biolistic transformation of ryegrass *Lolium perenne*.

Constructs	Numbers of lines generated
CaMV35S promoter::AtDGAT1 (original cDNA)	43
Rice actin promoter::AtDGAT1(op)	23
Rice actin promoter::LpDGAT1(op)	25
Rice actin promoter::AtDGAT2(op)	None
Maize UbiQ promoter::AtDGAT1(op)	97
Maize UbiQ promoter::LpDGAT1(op)	4
Maize UbiQ promoter::AtDGAT2(op)	39

CaMV and UbiQ are abbreviated from cauliflower mosaic virus and ubiquitin, respectively.
(op) indicated the translated sequences were optimised for expression in rice.

Figure 1 Analysis of lipid content in transgenic ryegrass by FAMES-GCMS. All 25 FU-lines shown contain the *CaMV35S::AtDGAT1* construct (confirmed by PCR and/or gene blot). FN0107 (shown in bold) is a control ryegrass containing the *CaMV35S::Green fluorescence protein* (GFP) construct. (a) Lipid levels in new leaves that had been allowed to grow for up to 2 weeks (collected all new leaf material 2 weeks after cutting the whole plant and allowing it to re-grow); (b) Lipid levels in different age leaf tissue (collected at 1, 2, 3 and 4 weeks after cutting the whole plant and allowing it to re-grow). x = FN0107 (*35S::GFP*), ♦ = FU0805, ● = FU1402, ■ = FU0901, ▲ = FU1243. Each data point is an average from two independent extraction and FAMES-GCMS analyses.



4x sample loading buffer. Oil bodies from Arabidopsis seeds were extracted and purified for the oil bodies as described by Scott *et al*. (2006).

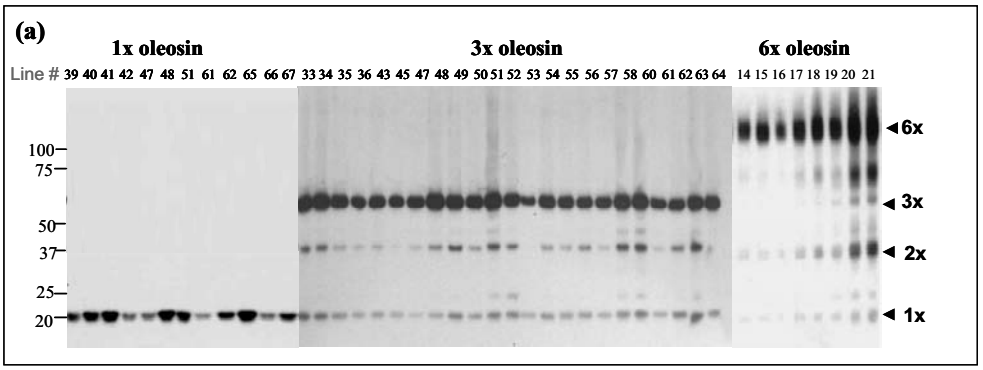
A 30 µL aliquot (leaf extract) or a 10µL aliquot (seed oil body prep.) and the negative control (Arabidopsis plant transformed with pRSh1 binary vector) were loaded onto the 4-10% gradient polyacrylamide gel (BioRad). Proteins were blotted onto nitrocellulose membrane using the iBlot system (Invitrogen); immunoblot development was performed and developed as described by Scott *et al*. (2006).

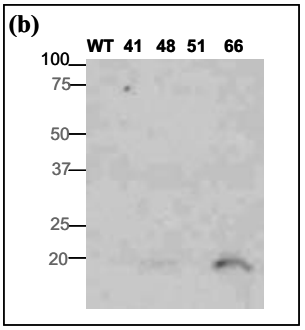
Results and Discussion

Analysis of transgenic ryegrass for higher lipid contents

To date, 231 transgenic ryegrass lines have been generated with various combinations of promoters and DGAT genes (Table 1). These are currently being

Figure 2 SDS-PAGE/immunoblot analysis of oil body extracts from transgenic *Arabidopsis thaliana* (line numbers as indicated). Antibodies against oleosin were raised in rabbit using purified *E. coli* expressing sesame oleosin as described previously in Cookson *et al.* 2007. (a) Total protein extract from seeds of individual lines transformed with single (1x), trimeric (3x), or hexameric (6x) sesame oleosin constructs; (b) Total protein extract from leaves of the Arabidopsis transformed with single (1x) oleosin construct.





screened for lipid content via FAMES-GC/MS. Preliminary data from leaf material shows that our transgenic plants with *CaMV35S::AtDGAT1* (from 25 lines screened) can accumulate up to 40% more lipids in the leaf (Fig. 1a).

Although the DGAT1 transgene was under the control of the *CaMV35S* promoter, the amount of lipid accumulating in the transgenic leaves appears to decrease in older leaf (Fig. 1b). Transgene activity can be regulated by gene silencing, degradation of the recombinant protein or catabolisation of the end product. Given that endogenous DGAT1 and DGAT2 appear to play roles in mature and senescing leaves (Kaup *et al.* 2002; Shockey *et al.* 2006), it is likely that plants possess a number of feedback mechanisms to control their activity. Indeed, Zou *et al.* (2006) recently identified a consensus sequence (X-Leu-X-Lys-X-X-Ser-X-X-X-Val) within *Tropaeolum majus* (garden nasturtium) *DGAT1* (*TmDGAT1*) sequences as a targeting motif typical of members of the SNF1-related protein kinase-1 (SnRK1) with Ser being the residue for phosphorylation. The SnRK1 proteins are a class of Ser/Thr protein kinases that have been increasingly implicated in the global regulation of carbon metabolism in plants, e.g. the inactivation of sucrose phosphate synthase by phosphorylation (Halford & Hardie 1998). Zou *et al.* (2006) went on to demonstrate that the obliteration of a potential SnRK1 phosphorylation site in DGAT1 by single point mutation ($Ser^{197}Ala$ of TmDGAT1) led to the accumulation of significantly higher levels of TAG in the seed. The consensus SnRK1 targeting motif is found in the AtDGAT1 sequence we have used to transform ryegrass but is not present in the LpDGAT1 sequence. The possibility exists that the SnRK1 regulation of AtDGAT1 activity is occurring in our transgenic ryegrass leaves; this is evidenced by the accumulation of a potential AtDGAT1 fragment in transgenic leaves with lower levels of lipid (data not shown).

Accumulation of oleosin/polyoleosin in Arabidopsis

Recently, we have found that oil bodies with an oleosin/polyoleosin outer layer can protect, or at least delay, degradation of oil bodies in the presence of rumen fluid (Cookson *et al.* 2007). We have generated Arabidopsis

plants which show accumulation of single oleosin and polyoleosin (trimeric and hexameric) in oil bodies in the seed (Fig. 2a). These results suggest that sesame oleosin/polyoleosins are correctly targeted to seed oil bodies in Arabidopsis and that the properties afforded by polyoleosin should be conferred on the oil bodies. While the predominant immunoreactive band corresponded with a protein of the expected size, a range of intermediate sized immunoreactive products (not seen in the controls) also occurred in the plants transformed with the trimeric and hexameric polyoleosin constructs. The smallest of these bands corresponded to the size of a single oleosin repeat (Fig. 2a). It is possible that these represent early termination translation products that occur at specific sites after the first oleosin coding sequence.

The fact that the recombinant oleosin could accumulate in the mature leaves of Arabidopsis (Fig. 2b) that had not been transformed with the DGAT1 gene indicates that the combination of both oleosin and DGAT1 expression in the leaves of transgenic plants should lead to the formation of functional oil bodies.

Future prospects

The accumulation of up to 40% additional lipid is an encouraging result especially given that the plants tested were hemizygous and had been transformed with the non-optimised AtDGAT1 cDNA. It is anticipated that our second generation of ryegrass plants which combine monocotyledonous promoters with sequences optimised for rice will yield plants with even higher lipid levels still. The correlation of possible AtDGAT1 break down products and comparatively low lipid levels has led to the building of third generation DGAT1 constructs with the $Ser^{205}Ala$ mutation as described by Zou *et al.* (2006). To confirm the identity of the break down products, we will use purification via anti-DGAT1 antibodies followed by SDS-PAGE separation, trypsin-digestion then Mass Spectrometry. The targeting of recombinant polyoleosin to the oil bodies in the seeds indicates that they are correctly processed by the plant and the physical properties associated with polyoleosin will be able to be exploited via *in planta* expression. We are currently engineering plants to express both DGAT1 and oleosin/polyoleosin in the leaf in order to generate plants which accumulate protected lipids in the form of oil bodies in the leaf.

The pathway to implementation of these plants presents both technical and regulatory challenges. Following the initial screening we will generate clonal material for detailed genetic and phenotypic (under a range of environmental and simulated grazing conditions) analysis. The latter will include metabolic profiling in order to determine the influence of accumulating TAG on other essential components such as protein, water soluble carbohydrate and fibre content. Plants that are selected from this analysis will be introduced into a breeding programme within AgResearch.

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