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Genetic modification to create novel high quality forages

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ABSTRACT

Genetic improvements in forage quality can lead to improvements in animal performance. We outline two approaches aimed at generating high quality forages that increase the energy available to the ruminant. These include the heterologous expression of fructan biosynthesis genes in the leaf to produce high molecular weight soluble carbohydrate and the modification of the expression pattern of endogenous triacylglyceride biosynthesis genes to produce triacylglycerides in the leaf. *In vitro* experiments demonstrated the potential of the rumen to digest fructans with either β(2-6) or β(2-1) linkages, furthermore both *in vitro* and *in vivo* experiments showed that fructan reduced the concentration of NH_3 in the rumen. We discuss the advantages of raising the lipid content of forage and the ramifications of expressing triacylglcyeride biosynthesis genes in the leaf. The expression of other genes that modify the quality of the fatty acids within triacylgycerol are also discussed briefly.

Keywords: genetic modification, forage quality, carbohydrate, fructans, lipids

INTRODUCTION

Grazed pastures are the major nutritive source for animal production in New Zealand. While the nutritive value of these grazed pastures is variable when compared to concentrate feeds, they are preferred because their low-cost provides New Zealand with a competitive advantage in world trade of agricultural products.

The feeding value of grazed pastures is defined as an animal production response and is quantified by weight gain or milk yield. Nutritive value is a response per unit of feed intake and therefore feeding value is a function of both intake and the efficiency with which the animal utilises the products of digestion (Ulyatt, 1973). The plant factors that influence feeding value include species, cultivar, plus responses to environment and grazing management. Examples of differences in feeding value among species include the lower performance of animals grazing subtropical grasses such as kikuyu in comparison to temperate grasses such as perennial ryegrass and timothy (Buxton & Mertens, 1995). Differences also occur among temperate grass species. The high feeding value of timothy relative to perennial ryegrass is associated with its later flowering, endophyte-free status and slower decline in digestibility as tillers become reproductive (Charlton & Stewart, 2000). The higher feeding value of legumes such as white clover is a major reason for their inclusion in temperate pastures. White clover improves feeding value for young sheep by 50-100% over grasses and by 15-35% over other forage legume species (Ulyatt, 1981). This results from greater intake, higher N content, more rapid particle breakdown, and more efficient use of digested nutrients by the animals fed white clover. Herbs such as chicory have also been introduced over the past decade to improve feeding value.

The impact of plant improvement within species to improve nutritive value is probably more contentious. Traditionally pasture plant improvement has focussed on the development of high yielding, pest and disease resistant and persistent cultivars. While these traits continue to be important for the commercial success of released cultivars, breeding objectives have diversified to include improved protein/energy balance, increased by-pass protein levels, leaf properties affecting intake, and manipulation of compounds that affect animal health, animal welfare, reproductive fertility, animal product flavour and texture (Caradus *et al.*, 2000). Casler & Vogel (1999) reported an average increase of 3.2% in liveweight gain for each 1% increase in digestibility, without negatively affecting forage yield and/or agronomic fitness. Several major genes such as the brown midrib genes from maize and sorghum, and the dwarf gene from pearl millet improve forage quality but have adverse effects on yield and fitness. Increasingly, knowledge and technical advances in molecular biology are providing opportunities to modify some of the more difficult traits influencing feeding value where the heterologous expression of genes that produce high levels of non-structural carbohydrate or increased lipid content in forage crops is an option. This paper reports on efforts to improve the energy content of grazed pastures through manipulation of non-structural carbohydrate and fatty acid content.

MODIFICATION OF NON STRUCTURAL CARBOHYDRATES IN FORAGES

Pasture plants are frequently rich in protein but relatively poor in non-structural carbohydrates. As a result, much of the ingested protein is degraded by rumen micro-organisms, and losses of up to 40% of dietary nitrogen have been recorded (Ulyatt *et al.*, 1988). Protein losses can be prevented by feeding animals carbohydrate supplements. Conventional selection for high non-structural carbohydrate levels was successful in a perennial ryegrass across a range of environments (Smith *et al.*, 1998). Interestingly the selected lines also tended to have higher herbage protein content and higher digestibility. Under appropriate conditions, novel storage carbohydrates can accumulate to significant levels in plant tissues without being broken down.

The feasibility of providing higher levels of non-structural carbohydrates in forages through genetic modification is being investigated by several international

groups (reviewed by Spangenburg *et al.,* 2001). The efficiency of pasture protein utilisation would then be enhanced and the nutritive value of pasture effectively increased. In white clover, for example, the main storage carbohydrate (starch) is stored in the stolons and roots, and is therefore unavailable for animal consumption. Clover leaves are the main component of the plant consumed by the grazing ruminant but they contain low levels of non-structural carbohydrates, such as sucrose and starch.

Increasing the level of sucrose or starch in forage leaf tissues is difficult due to their mobility within the plant. An alternative approach is to engineer fructan synthesis. Fructans, which are not normally accumulated in the ingested fraction of forage plants, are carbohydrate storage compounds synthesised from sucrose-based substrates in a diverse array of species, including mosses, liverworts, algae and bacteria. In addition, fructans share roles in osmoregulation and have been implicated in drought and cold tolerance. The microbial fructans (known as levans) are very large and can contain over 100,000 linked fructose units, however, in dicotyledonous plants fructans (predominantly inulin) are characteristically smaller in size with less than 200 linked fructose molecules.

The ability of the rumen to metabolise fructans was initially investigated *in vitro* by following the disappearance of levan, inulin and fructose (added in equivalent amounts) in rumen fluid. In cow (Fig. 1A) and sheep rumen fluid (Fig. 1B) both the long-chain bacterial levan (DP>100,000) and short-chain plant inulin (DP38) appeared to be readily metabolisable, albeit at a slower rate than that of fructose. The calculated rates of disappearance of each carbohydrate have limited application due to the static *in vitro* experimental system.

The metabolism of all added soluble carbohydrates was associated with a reduction in net ammonia concentration when incubated in rumen fluid from both cows and sheep (Fig. 2). These results indicate that fructans are readily digested by grazing ruminants and that metabolism of supplemental carbohydrates within the rumen is correlated with lower net concentrations of ammonia. Given the limitations of an *in vitro* experiment, the reduction of net ammonia concentration could be due: (1) a reduction in protein degradation or (2) an increase in ammonia capture by rumen microbes, or (3) a combination of both.

A reduction in net ammonia concentration was also observed with fructan supplementation in sheep grazing

FIGURE 1: *In vitro* fermentation of levan (■), inulin (●) and fructose (□) in rumen fluid from either cows (A) or sheep (B). The lines are expressed in terms of % of fructose remaining of total available at T0.
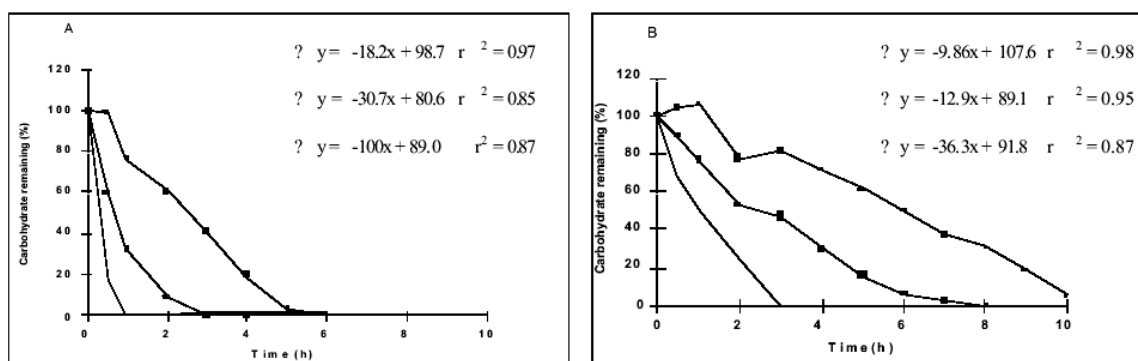

FIGURE 2: The net concentration of ammonia following *in vitro* fermentation of levan (A, D), inulin (B, E) and fructose (C, F) in rumen fluid from either cows (A-C) or sheep (D-F). Symbols are: ammonia in control (▲), ammonia in rumen fluid containing carbohydrate (■), percent carbohydrate remaining (○).
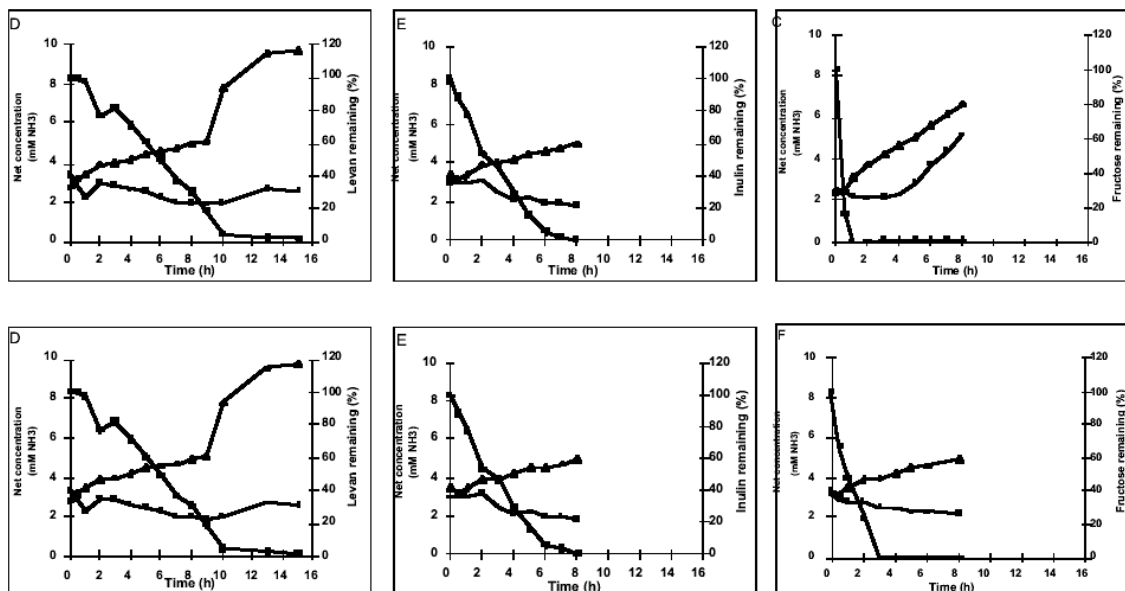
TABLE 1: Effect of *in vivo* supplementation with inulin on ammonia (mM NH_3 ± SE) concentration in the rumen of sheep grazing on either ryegrass or white clover monocultures.

	Control	Inulin
	mM NH_3	
Ryegrass	19.9 ± 3.6	8.1 ± 2.7
White clover	18.7 ± 7.5	11.2 ± 2.6

ad libitum on white clover or perennial ryegrass pastures (Table 1). In this experiment fructan (commercial grade inulin) was continuously pumped into these animals through a rumen fistula. Combined together, the *in vitro* and *in vivo* experiments suggest that reduced net ammonia concentration within the rumen is possible with elevated intake of soluble carbohydrate.

We have transformed white clover with either levansucrase (LSS) from the gram-negative bacterium, *Erwinia amylovora*, which catalyses the production of levan with β(2-6) linkages, or both 1-SST and 1-FFT genes from the dicotyledonous plant Globe artichoke, *Cynara scolymus*, which sequentially catalyse the synthesis of inulin with β(2-1) ructosyl fructose linkages.

Plants transformed with either LSS or co-transformed with 1-SST and 1-FFT produced detectable levels of fructans in comparison to the vector control plants. Levan levels accumulated to 3.9% to 14% dry weight in LSS transformed plants while inulin accumulated to 3-5% dry weight in co transformed 1SST and 1-FFT plants. This data suggests that clover does not have a mechanism capable of degrading β(2-6) levan linkages or β(2-1) bonded inulin. Unexpectedly, the inulin produced in transformed clover had a comparatively high–molecular weight. We are currently investigating the effects of LSS, 1-SST and 1-FFT expression on plant morphology and physiology.

MODIFICATION OF LIPIDS IN FORAGES

Extensive feeding trials with supplemental fats have been undertaken due to the energy density of fats and the high efficiency with which long chain saturated fats are used by ruminants. However, the advantages of supplemental fats must be balanced against their potentially negative effects that include: the inhibition of ruminal microbes by partially soluble fatty acids (e.g., C8-C14 and longer chain unsaturated fatty acids), reduction of total feed consumption and reduction in milk protein concentration (Coppock & Wilks, 1991). In general, supplementation with vegetable oils disturbs ruminal fermentation except when fed with whole seeds (Avila *et al.*, 2000). This may reflect the relatively slow release of triacylglycerol as the ruminant chews the oilseeds. Whatever the cause(s), it appears that the product is relatively inert in the ruminant and is available post ruminally (Coppock & Wilks, 1991).

Currently the lipid profiles of pasture grasses and legumes and the seasonal variation in lipid content are poorly described. Unpublished data (Paul Newton, AgResearch, NZ) suggests that the lipid content of New Zealand pastures varies from 4% to 6% (dry matter basis),

with the lowest content occurring over summer. While the specific contribution of lipids from individual forage tissues is not known, in almost all plants the highest concentration of lipids is found as triacyglycerols in the seeds and pollen (as much as 30% of the total weight). In forage leaves, however, triacylglycerols constitute only about 2.5% of the total lipid content and the bulk of leaf lipids are membrane diacylglycerolipids (Body 1974).

Leaf diacylglycerols are crucial to the maintenance of membrane fluidity, permeability and integrity. Hence the degree to which diacylglycerols can be manipulated may be limited compared to manipulating triacylglycerols that are not an integral part of any cellular organelle. The single committed step in the formation of triacylglycerides is catalysed by acyl CoA:diacylglycerol acyltransferase (DGAT) which was recently cloned from *Arabidopsis thaliana* (Zou *et al.*, 1999) and we are currently cloning DGAT from ryegrass and white clover. When the *A. thaliana* DGAT cDNA was placed under the control of a constitutive promoter in tobacco, triacylglycerol accumulated as oil drops in the cytoplasm of leaf cells; plants were otherwise phenotypically unchanged (Bouvier-Nave *et al.*, 2000). A similar transformation in either ryegrass or white clover should allow triacylglycerol to be accumulated in the leaf without dramatic changes to the overall plant function.

Triacylglyceride production in the leaf would alter the energy content of the forage, in turn this will influence the efficiency of pasture conversion by the ruminant into meat or dairy products. The effect of triacylglycerol production in leaves on the total dry matter intake is unknown since dry matter intake may be reduced by increased lipid content. This issue has not been satisfactorily addressed in supplemental lipid feeding trials to date where the control feed (seed or tallow) often contains an already elevated lipid content compared to forage. An indoor infusion feeding trial is underway to determine whether triacylglyceride will indeed be a limiting factor to pasture dry matter intake. Typically the maximum permissible lipid content of the rumen diet is approximately 10% of the dry matter intake (Garnsworthy, 1997), with the limitations being placed on feeding higher values due to the problems described above. Based on a maximum allowable lipid content of 10% it is possible to speculate how much triglyceride can accumulate to stay below this level and what this would mean in terms of energy content derived from forage triglycerides.

- Assume total lipid content of forage currently varies between 4-6% w/w on a dry matter basis, 5% average (Paul Newton, pers. comm.).
- Assume the maximum allowable lipid content of the diet is 10% (Garnsworthy, 1997). This would mean 5% in the overall lipid content through triacylglycerol biosynthesis is the maximum allowable increase.
- Assume triacylglycerides make up 2.5% of the total lipid content from leaves and stems (Body, 1974), based on an average 5% total lipid content this is equivalent to saying triacylglycerols are 0.125% of forage dry matter. Therefore a rise from 0.125% to 5% DM would require a 40-fold increase in the level

of triacylglycerides. Bouvier-Nave *et al.*, (2000) reported up to a 7-fold increase in the triacylglycerol content of transgenic tobacco depending on the growth conditions.

- Assume only half of the total lipids are absorbable and that purified they provide 37.7 J/g compared to purified carbohydrate and protein which provide 16.7 J/g each. If the lipid, carbohydrate and proteins constitute 5, 70 and 18% respectively then the energy content of a typical grass is 15.7 J/g DM. If we increased the lipid level by 5% and assume that it is all absorbable then the total energy content would rise to 16.8 J/g DM or a 7% rise over the existing level.

It may also be possible to influence the saturation level of the fatty acids forming the triacylglycerol. Plant fatty acid desaturation, and the position of double bonds, is under the control of a suite of fatty acid desaturases which operate sequentially. The desaturases responsible for modifying the majority of plant lipids (C16 and C18) are located both in the chloroplast (FAD6, 7 and 8) and the endoplasmic reticulum (FAD2 and 3). Since DGAT is targeted to the endoplasmic reticulum, then fatty acids within triacylglycerol were desaturated by FAD2 and FAD3. We are in the process of cloning these genes from both ryegrass and white clover.

CONCLUSION

We have outlined potential methods to increase soluble carbohydrates and triacylglycerides in the ingested portion of existing forage crops. Based on preliminary results and projections, we believe that both approaches have merit to develop forages with increased energy available to the ruminant. We also acknowledge the need for rigorous animal feeding studies to accurately determine the efficacy of the proposed genetic modifications.

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REFERENCES

Avilla, C.D.; DePeters, E.J.; Perez-Monti, H.; Taylor, S.J.; Zinn, R.A. 2000: Influences of saturation ration on supplemental dietary fat on digestion and milk yield in dairy cows. *Journal of dairy science 83:* 1505-1519.

Bauman, D.E.; Barbano, D.M.; Dwyer, D.A.; Griinari, J.M. 2000: Technical note: production of butter with enhanced conjugated linoleic acid for use in biomedical studies with animal models. *Journal of dairy science 83:* 2422-2425

Body, D.R. 1974: Neutral lipids of leaves and stems of *Trifolium repens*. *Phytochemistry 13*: 1527-1530

Bouvier-Nave, P.; Benveniste, P.; Oelkers, P.; Sturley, S.L.; Schaller, H. 2000: Nucleotide, protein expression in yeast and tobacco of plant cDNAs encoding acyl CoA:diacylglycerol acyltransferase. *European journal of biochemistry 267:* 85-96

Buxton, D.R.; Mertens, D.R. 1995: Quality-related characteristics of forages. pp 83-96. *In* Forages, Volume II. *Ed.* R.F. Barnes, D. A. Miller and J.C. Nelson. Iowa State University Press, Ames.

Caradus, J.R.; Woodfield, D.R.; Easton, H.S. 2000: Improved grazing value of pasture cultivars for temperate environments. pp5-8. *In* Animal Production for a Consuming World Vol. B. *Ed.* G.M. Stone.

Casler, M.D.; Vogel, K.P. 1999: Accomplishments and impact from breeding for increased forage nutritional quality. *crop science 39:* 1866-1873.

Charlton, J.F.L.; Stewart, A.V. 2000: Timothy–the plant and its use on New Zealand farms. *Proceedings of the New Zealand Grassland Association 62:* 147-153.

Coppock, C.E.; Wilks, D.L. 1991: Supplemental fat in high-energy rations for lactating cows: Effects on intake, digestions, milk yield, and composition. *Journal of animal science 69:* 3826-3837.

Garnsworthy, P. C. 1997: Fats in dairy cow diets. *In* Recent Advances in Animal Nutrition. *Ed.* P.C. Garnsworthy and J. Wiseman. Nottingham, University Press.

Smith, K.F.; Simpson, R.J.; Oram, R.N.; Lowe, K.F.; Kelly, K.B.; Evans, P.M.; Humphreys, M.O. 1998: Seasonal variation in the herbage yield and nutritive value of perennial ryegrass (*Lolium perenne* L.) cultivars with high or normal herbage water-soluble carbohydrate concentrations grown in three contrasting Australian dairy environments. *Australian Journal of experimental agriculture 38*: 821-830

Spangenberg, G.; Kalla, R.; Lidgett, A.; Sawbridge, T.; Ong, E.K.; John, U. 2001: Breeding forage plants in the genome era. pp 1-39. *In* Molecular Breeding of Forage Crops. *Ed.* G. Spangenberg. Kluwer Academic Publishers, The Netherlands.

Ulyatt, M.J. 1973: The feeding value of herbage. pp 131-178 *In* Chemistry and Biochemistry of Herbage Volume 3. *Ed.* G.W. Butler and R.W. Bailey. Academic Press, London.

Ulyatt, M.J. 1981: The feeding value of herbage: can it be improved? *New Zealand journal of agricultural science 15:* 200-205.

Ulyatt, M.J.; Thomson, D.J.; Beever, D.E.; Evans, R.T.; Haines, M.J. 1988: The digestion of perennial ryegrass and white clover by grazing cattle. *British journal of nutrition 60*: 137-149.

Zou J-T.; Wei, Y.; Jako, C.; Kumar, A.; Selvaraj, G.; Taylor, D.C. 1999. The Arabidopsis thaliana TAG1 mutant has a mutation in a diacylglycerol acyltransferase gene. *Plant journal 19*: 645-653.