Opportunities in Molecular Biology: Enhancement of the Nutritional Value of Forages

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Introduction

Pasture plants, while producing a cost-effective source of feed for grazing ruminants, can frequently be less than optimal in meeting the animals' nutrient requirements. Over the past half century, there has been a major effort to improve the quantity and nutritional quality of pasture plants using conventional plant breeding. Although considerable progress has been made in improving the quality and agronomic characteristics of our major pasture plants by this means, breeding can only be applied to plants capable of sexual crosses. This poses severe limitations both in terms of speed of progress and in the number of genes available for transfer (Ulyatt, 1991; Ulyatt *et al*., 1995).

The recent development of novel genetic techniques bypasses the requirement for sexual crosses to transfer genes. This has vastly extended the range of potentially useful genes for transfer and has also provided means of suppressing undesirable traits where the genes responsible can be identified. There is also the potential to manipulate genes expressed at a particular stage of development and where gene expression results in unwanted characteristics, these may be removed by gene inactivation. Genes normally inactivated by the plant may also be expressed at any other stage. As the genes appear to be present but inactive, re-activation should permit accumulation of the desired products. Examples of each of these possibilities will be given in this paper. It is important to note, however, that when biosynthetic pathways in plants are regulated by multiple genes it is much more difficult to apply the techniques of molecular genetics.

In 1997 Smith *et al*. conducted a world-wide survey among prominent agricultural scientists to identify and prioritise the most important traits for the genetic improvement of nutritive value in dairy pasture. For legumes, the most highly ranked trait was considered to be the absence of anti-quality factors. This was followed closely by the need to optimise the ratio of rumen degradable protein to undegradable protein (RDP/UDP), followed by increased dry matter digestibility (DMD) and water-soluble carbohydrates (WSC). For grasses, this assessment ranked an increase in DMD as the most important goal, with increased non-structural carbohydrate (WSC) and improved rate of digestion as the two next most important properties.

By making precise changes at the gene level, genetic manipulation (GM) provides a means of addressing these and other issues in a way that can not be achieved by traditional forage breeding. To date significant progress has been made in the transformation and regeneration of a large number of legume and grass species, as a primary step towards achieving this goal.

Legumes

Condensed tannins

Legumes are beneficial in a pasture-based system because they improve both the intake and quality of the diet. However there are a number of areas where genetic improvement would enhance their nutritive characteristics. The absence of anti-quality factors, especially bloat was considered the most important goal for the genetic improvement of legumes (Smith *et al.*,1997). Bloat occurs when fermentation gases formed in the rumen become trapped in the digesta resulting in a protein-based foam complex that often produces sufficient pressure to kill the animal (Tanner *et al.*, 1997).

Although certain legumes such as white clover (*Trifolium repens*), red clover (*Trifolium pratense*) and lucerne (*Medicago sativa*) are the primary cause of bloat, it is known that the presence of condensed tannins (proanthyocyanidins) in other legume species is responsible for the prevention of bloat (Lees, 1992; Tanner *et al*., 1995). The presence of condensed tannins (CT) in *Lotus corniculatus, Lotus pedunculatus,* sulla (*Hedysarum coronarium*) and sainfoin (*Onobrychis viciifolia*) also provide other benefits, such as increased liveweight gain, wool growth and milk production, and changed milk composition (Marten *et al.*, 1987; Douglas *et al.*, 1995; Waghorn *et al.* 1998). Moreover, CT provide further nutritional benefits by decreasing degradation of protein in the rumen, thereby reducing, loss of dietary nitrogen (N) via ammonia. Unfortunately these CT-containing legumes do not compete well agronomically with major temperate pasture species.

Condensed tannins form strong hydrogen bonds with proteins and other macromolecules in the pH conditions of the rumen, due to the high level of free phenolic hydroxyl groups. This characteristic reduces the formation of foams with plant proteins thus preventing bloating. The CT/protein complex dissociates in the lower pH conditions of the abomasum liberating free protein for digestion in the small intestine (Tanner *et al*., 1994).

Several approaches including screening gene pools and mutagenesis have failed to provide a white clover, lucerne or red clover containing foliar CT (Woodfield *et al.*, 1998). Moreover, attempts using protoplast fusion to transfer this trait from the high-CT legume species, sainfoin to the CT–free legume lucerne yielded only one somatic hybrid containing 0.03% CT in its leaves (Li *et al.*, 1993; 1995).

Molecular biology offers a new approach in addressing this goal. Firstly, much could be gained in identifying genes involved in the CT synthesis pathway from CT-accumulating legumes such as *Lotus* spp., (Bavage and Robbins, 1994). Further research using molecular biology techniques should also provide a better understanding of the genetic regulation of CT biosynthesis, tissue distribution, levels and biochemical structure (Morris and Robbins, 1997). The use of EST (expressed sequence tag) libraries, transposon mutagenesis and linkage maps using restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNA's (RAPDs) may all aid the identification of genes involved in the CT biosynthetic pathway in these plants. Such genes could then be transferred or modified to allow accumulation of specific CTs in the leaves of elite, more persistent pasture plants, such as white clover, lucerne and red clover, all which show persistence to grazing yet do not contain CT in their leaves. Furthermore, because excessive levels of CT (>4-5% DM) are anti-nutritional, accumulation of CT in these plants would also need to be regulated to provide safe levels (1-3% DM).

An alternative approach is to alter the accumulation site of CT in the elite pasture legumes, white clover, red clover and lucerne, whose foliage is essentially devoid of CT. Since these pasture plants accumulate levels of CT in their seed coats, trichomes or flower petals, the genes for CT biosynthesis are therefore present within the plant genomes (Morris and Robbins, 1997; Woodfield *et al.*, 1998). Alteration of promoter control could modify the patterns of tissue-specific distribution, allowing the pathway to express specifically in the vegetative tissue of these legumes. Alternatively, transformation of transcription factor genes, such as *Sn*, *R*, and *B*, has been suggested as a way to trans-activate anthocyanin biosynthesis

in legume foliage currently devoid of CT (Morris and Robbins, 1997). The role of such trans-activating genes has been investigated in a number of model plants (Goff *et al.*, 1990; Grotewold *et al*., 1998). Maize (*Zea mays*) cell lines expressing the maize transcriptional activators C1 and R accumulated two cyanidin derivatives, similar to the anthocyanins produced in differentiated tissues (Grotewold *et al*., 1998). Also, a number of tissue–specific B regulatory proteins transactivated the *bronze1* anthocyanin gene promoter when expressed in intact maize tissues or embryonic callus cells (Goff *et al.*, 1990). The transient expression of the maize anthocyanin regulatory genes has also been reported for some legume species. When the maize *B* and *C* regulatory genes were introduced into white clover and peas (*Pisum sativum*) by microparticle bombardment, anthocyanin biosynthesis was activated in a tissue specific manner (Majnik *et al*., 1998).

For other CT-accumulating legume species, reducing excessive CT levels would greatly enhance nutritional value. Initial experiments aimed at achieving down-regulated CT biosynthesis have produced favourable results. Transformation of *L. corniculatus* with the anti-sense *cDNA dihydroflavonol reductase (DFR)* gene from *Antirrhinum* resulted in reduced CT levels of up to 80% in tissue culture lines. The leaves, roots and stems of regenerated plants showed a 40% reduction in CT levels with no obvious effects on plant biomass (Robbins *et al*., 1994; 1998). In addition, transformation of the full length sense *DFR* gene from *Antirrhinum* also yielded observable reduced CT levels in 30% of the root culture lines, relative to the controls (Bavage *et al*., 1995; 1997). Moreover, the transformation of the maize *Sn* transcription factor, an anthocyanin regulatory gene, into *L. corniculatus* has also resulted in a number of regenerated plants with reduced proanthocyanidin content in leaf tissue (Damiani *et al*., 1999). Hence, the use of different biotechnological strategies; namely antisense and sense suppression plus regulatory genes, have all yielded notable reductions in CT concentrations. This confirms the potential of molecular biological techniques to generate legume species with modified CT levels.

Protein degradability

Another primary issue is the provision of an optimal ratio of rumen degradable protein to undegradable protein (RDP/UDP) in fresh pasture diets. A significant proportion of soluble dietary protein is degraded in the rumen producing large amounts of ammonia. This represents a loss of N, wastage of energy and can lead to insufficient absorbed protein to meet animal requirements. This problem is worse on fresh high nutritive value pasture plants, compared to processed diets, where potentially up to 50% of crude protein can be lost (Ulyatt and McNabb, 1999).

Providing dietary proteins protected from rumen degradation is one of the means currently being explored to correct this imbalance. The provision of such undegradable protein in the diet of ruminants has been reported to provide a number of benefits. Infusion of methionine directly into the abomasum, thus by-passing rumen degradation, has resulted in increases in wool growth in sheep (Reis and Schinckel, 1963; Reis, 1988). The live-weight gain in sheep fed fresh pasture is also positively correlated with the amount of protein absorbed through the small intestine. There is also evidence that the protein content of milk may be increased by supplying elevated levels of essential amino acids to cows (Beever and Thorp, 1997).

Accumulation of undegradable proteins in leaves of pasture plants would improve feed quality by circumventing degradation of essential amino acids within the rumen ecosystem, thus improving ruminant nutrition (Higgins *et al*., 1989). Gene technology has been used for a number of years in an effort to address this problem. Assessment by *in vitro* rumen digestion has routinely allowed identification of certain dietary proteins that are more resistant

rumen degradation than other proteins (Mangan, 1972; Nugent and Mangan, 1981; Spencer *et al* 1988; McNabb *et al*., 1993; Hancock *et al.,* 1994). The majority of these proteins are seed storage proteins from both monocot and dicot species, that are rich in essential, especially sulphur-based amino acids. These proteins, albumins (from pea or sun flower [*Helianthus spp.*]), prolamins (from maize and rice [*Oryza sativa*]) as well as chicken ovalbumin and pumpkin (*Cucurbita pepo*) inhibitors have all been shown to be rumen stable. Many of them have been reported to also remain highly sensitive to rumen intestinal proteases (Hancock *et al*., 1994). Such proteins are candidates to supply elevated levels of essential amino acids for absorption in the ruminant intestine. To test this possibility such rumen-protected proteins have been cloned and transformed into a number of forage legumes including lucerne, white clover, *L. corniculatus*, and subterranean clover (*Trifolium subterraneum*).

The results obtained have been extremely variable. Dicot albumins generally require sequestering in the endoplasmic reticulum for stable protein accumulation. Expression of chicken ovalbumin in lucerne resulted in low (0.001-0.01%) but stable protein accumulation (Schroeder *et al.,* 1991). Similar results were reported using pea albumin 1 (PA1) in white clover with the protein only stable if targeted to the endomembrane system, although in low abundance (<0.001% total cell protein; Ealing *et al.,* 1994). In contrast, the expression of an endomembrane-targeted sunflower albumin in subterranean clover reached levels up to 1.3% in leaf tissue (Khan *et al.,* 1996). For monocot prolamins, the results are less clear-cut. The 10kDa zein from maize was also stable in white clover leaves and protein accumulation increased with increasing leaf age, reaching levels of up to 1.3% of total cell protein (Sharma *et al.,* 1998). In contrast, the 10-kDa oryzin from rice was only stable in tobacco (*Nicotiana tabacum*), with undetectable levels produced in transformed white clover leaves. Further investigation of those transgenic tobacco and clovers accumulating detectable levels of prolamins has revealed that these proteins, previously highly resistant to rumen proteolysis up to 24 hours, have lost this essential characteristic of rumen stability, degrading within 10 minutes (Hancock *et al.,* pers. commn.). Similar instability was also reported when gamma-zein was expressed in tobacco, lucerne and *L. corniculatus*. Although mRNA was detected in all three species, zein protein was detected only in tobacco plants (Bellucci *et al.,*1999).

More recently grass species have also been transformed with a rumen-protected protein, specifically the sunflower albumin SFA8, also directed to the endoplasmic reticulum with an attached KDEL signal. The resultant transgenic tall fescue (*Festuca arundinacea*) plants produced detectable levels of mRNA and a corresponding SFA8 protein to levels up to 0.2% total cell protein (Spangenberg *et al*. 1998).

Major hurdles have been encountered in the majority of these reports. The instability of the expressed protein seems to have been overcome in some cases by specifically directing the proteins to the endomembrane system. The resultant low levels of these proteins (<0.001-0.3%) that accumulated in the forage leaves are also of concern. Such concentrations are well below the nutritionally useful levels of 2-5% of total cell protein. Moreover, the loss of resistance to rumen degradation is another unforeseen challenge to be overcome. Further investigation of these transgenic plants will help identify the causes of such instability, low accumulation levels and the loss of resistance to rumen proteases, which will in turn lead to optimised protein accumulation.

Increased soluble carbohydrate

The last aspect to be addressed for legumes is the need to provide higher levels of soluble carbohydrate in legume plant leaf tissues to enable the rumen bacteria to efficiently capture ammonia released by the digestion of dietary protein. This is also one of the primary goals for improving grass nutritive value (Smith *et al.,* 1997). Such carbohydrates must be

stored efficiently, exhibit restricted use by the plant itself, and be degraded slowly by the rumen microflora. Also, efforts to breed for increased soluble carbohydrates must not compromise other desirable features of pasture plants such as disease resistance. Increasing non-structural carbohydrates should have a major influence on milk production, as they provide a major source of digested energy (Reid, 1974; Reed, 1994).

Alteration of assimilate partitioning has been achieved by over-expressing such genes as the sucrose phosphate synthase in transgenic plants, but this concomitantly caused a reduction in the level of starch, resulting in no net increase in the total soluble carbohydrate content (Galtier *et al*., 1993). More recently Lorberth *et al*. (1998) have reported that the activity of a R1 gene protein decreased starch phosphate levels and resulted in a reduction of starch degradability within the transformed potato plant (*Solanum tuberosum*), as shown by net increases in leaf starch. The partitioning of soluble carbohydrates into sucrose and starch is however under complex physiological and developmental regulation. An alternative approach is to create a novel soluble carbohydrate biosynthetic capacity in leaf cells which is not subject to normal plant regulatory processes.

The addition of fructan, a polymer of fructose, to pasture plants such as white clover is one option being investigated. Such fructans are a group of storage carbohydrates widely distributed in nature and rivalling in abundance the better known polymers based on glucose; namely starch. Chemically fructans contain either ß(2-1) or ß(2-6) bonds, as determined by the synthesising enzyme. Addition of such enzymes would allow the production of a specific class of fructan molecules. The introduction of a single bacterial levansucrase (*sacB* gene from *Bacillus subitilis*) into starch-accumulating plants, tobacco and potato, resulted in these normally fructan-devoid plants accumulating significant levels of fructan as an alternative non-structural storage carbohydrate. Moreover no significant effects on plant growth rate or yield were reported (Ebskamp *et al.,* 1994; Van der Meer *et al.,* 1994; Pilon-Smits *et al.*, 1995). Although similar results have also been reported using the levansucrase (*lsc*) from *Erwinia amylovora* for potato (Röber *et al*., 1996), the expression of this gene in tobacco and white clover has caused significant effects on growth and morphology (Hancock, K.R. and Biggs, D.R., In preparation).

Compared to bacterial fructan synthesis, which requires a single enzyme, the formation of fructans in plants requires two enzymatic steps; encoded by genes for SST and FFT. Transformation of such genes from various monocot and dicot plant species, has similarly resulted in the accumulation of plant fructans in species such as chicory (*Cichorium intybus*), tobacco and potato (Sprenger *et al*., 1997*;*Vijn *et al.,* 1998; Sévenier *et al.,* 1998; Hellwege *et al.,*1997, 2000). Some of these genes are also being transformed into white clover species in an effort to accumulate plant fructans in the leaf tissue so an increase the total available soluble carbohydrate in the diet of ruminants will occur.

Grasses

Increasing the dry matter digestibility, digestion rate and non-structural carbohydrate content were considered the most important goals for breeding grasses by Smith *et al*., (1997). Senescent grass pastures have low nutritional value due to a decline in plant cell wall digestibility caused by lignification and the onset of flowering. Since grasses are the major component of pasture-based systems, minor changes in nutrient accessibility should result in significant gains in nutritive value.

Although incremental improvements, such as an increased rate of particle breakdown in perennial ryegrass (*Lolium perenne*), have been achieved by selection for lower leaf shear strength (Easton, 1989), traditional breeding has found this goal challenging because the link between heredibility and digestibility is low and involves a large number of genes. Molecular

biology provides an alternative avenue that may help attain this goal. Reducing the lignin content and strength or preventing the onset of flowering and leaf senescence by genetic mmanipulation may lead to significant increases in the digestibility of grasses with concomitant increase in particle breakdown, thereby enhancing the intake and nutritive value of pasture forage.

Genetic manipulation of flowering

Most perennial pasture grasses predominantly persist in a non-flowering state, producing vegetative tillers that originate from the shoot meristems. These tillers support growth and persistence under grazing conditions while providing forage leaf tissue for the animals. However, grasses consequently undergo a sharp decline in available leaf forage and a decrease in nutritional value during the onset of flowering (Parsons and Robson, 1980). This results from a significant number of available meristems being committed to follow the alternative developmental program to produce flower meristems with a concurrent decrease in the number of shoot meristems remaining to sustain vegetative growth (Moore *et al.*, 1991).

Conventional breeding has successfully delayed the onset of flowering in a number of cultivars by utilising the inherent natural genetic variation in flowering time that exists in grass species. Another potential means available to help avoid this decline in forage feed supply and quality that occurs during summer would be to delay or abolish the onset of flowering using molecular biology. Current research suggests that genes controlling flower development are greatly conserved over a diverse range of plant species. Thus the information gained using model plants can be used to locate similar genes involved in flowering in grasses and may minimise or abolish flowering to maintain grasses in a vegetative state.

A large array of genes involved in flowering (*constans, Leafy, Apetala1, luminidependans, terminal flower1* and *UFO*), have been isolated from model plants such as *Arabidopsis thaliana* (Lee *et al*., 1994; Putterhill *et al.*, 1995; Levin and Meyerowitz, 1995; Sundas-Larsson *et al.*, 1998; Aukerman *et al*, 1999; Liljegren *et al.*, 1999). Mutations in these *Arabidopsis* genes have diversely modified flower development.

The protein of the *constans* (*CO*) gene promotes flowering by acting as a transcription activator. Mutations in the *CO* gene, which is classed as a photoperiod responsive gene, causes the plant to flower later than wild type *Arabidopsis* when exposed to long photoperiods, whereas plants containing multiple copies flower significantly earlier than flowering control plants (Putterhill *et al.*, 1995).

The *luminidependans* (*LD*) gene from *A. thaliana* is in the autonomous flowering pathway and contrasts to *CO* as it is unregulated by photoperiod length. A mutation in this *LD* gene also confers a late-flowering phenotype indicating that LD also plays an important role as a flowering signal. Moreover, in an *Apetala1* background, the *LD* mutation is able to convert a reproductive shoot apex to a more vegetative state, similar to the phenotype reported for the *leafy* mutant and hence may participate in the regulation of *leafy* (Lee *et al*., 1994; Aukerman *et al.*, 1999).

Research is also beginning to elucidate the complex interactions that exist between various genes involved in floral initiation in *Arabidopsis*. *CO* regulates *leafy* as leafy transcription is rapidly initiated in response to CO expression (Simon *et al.*, 1996). A recent article by Liljegren *et al*. (1999) has reported the regulatory interactions existing between the flowering genes *leafy, Apetala1 and terminal flower1*. *Ieafy* can positively regulate *Apetala1* as the onset of *Apetala1* is delayed in *leafy* mutant. In addition, *Apetala1* is a positive regulator of *leafy* with premature expression of *leafy* occurring in plants where *Apetala1* is being constitutively expressed. Moreover, *Apetala1* down regulates the expression of

terminal flower1, known to prevent both *Apetala1* and *leafy* expression in inflorescence shoot meristems.

One can extrapolate from these results that suppression of similar genes using sense or antisense suppression, such as overviewed for the CT manipulation experiments above, should result in reduced or later flowering in pasture plant grasses. Complete abolition of flowering may also be achievable if multiple gene activity is completely removed by molecular biological techniques.

Producing grass cultivars with minimised or total loss of flowering capacity may alleviate the nutritional problem for grazing animals, but concomitantly could remove the seeding properties required by seed producers which require acceptable seed yields. One option available to address this issue is the use of inducible promoters, such as the ethanol or copper-inducible promoters, that would allow removal of the non-flowering state and allow normal seed production to occur when required (Salter *et al*., 1998; Mett *et al*., 1996).

Manipulation of lignin biosynthesis

Another avenue available to increase access to available nutrients is to modify the lignin content in forage grasses. Brown rib grass mutants with 8-30% reductions in lignin content obtained using conventional plant breeding have already increased digestibility (Campbell and Sederoff, 1996). Similar improvements have been made using brown rib mutants in a number of other monocot species, such as maize, sorghum (*Sorghum vulgare*) and pearl millet (*Pennisetum glaucum*). Such species have mutations within lignin biosynthesis genes which have resulted in reduced lignin contents up to 50% (Cherney *et al*., 1991).

There are significant differences in lignin content within pasture plants; tropical grasses possess a higher lignin content than temperate grasses, and both become more lignified as they mature. With these grasses the vascular tissue, parenchyma and schlerenchyma are all lignified, compared to legumes, such as white clover where only the vascular tissue is heavily lignified (Wilson and Hatfield, 1997). With the more erect growing legumes, such as lucerne, lignification increases with maturity. Since grasses compose the majority of the pasture based ecosystem, the diet of grazing ruminants may therefore contain up to 50% indigestible fibre, which leads to low degradation and digestion rates.

Lignin serves a number of important functions in plants; providing mechanical support, forming xylem tissues for water transport and playing a role in plant defence mechanisms. However, lignin also decreases the availability of existing nutrients within plant tissue. During rumen digestion, plant materials are utilised by microbial activity until the highly resistant lignin is reached. Any digestible nutrients beneath this barrier remain unattainable until this plant fibre barrier has been breached (Chesson, 1993; Wilson and Mertens, 1995). Altering the plant well wall component by modifying the lignin thereby increasing the digestibility of the forage, is a promising option to increase the digestibility of forage tissues.

Standard molecular techniques used in genetic engineering, such as sense, or anti-sense suppression, may make the modification of lignin biosynthesis feasible. This would result in an increase in the nutritional value of the forage by allowing access to a greater portion of the available nutrients (Watson, 1990; Dixon et al., 1996; White, 1997). Research using molecular biology will provide a better understanding of lignin biosynthesis, its structure and chemical properties in pasture grasses. Moreover, modification of lignin must not adversely affect plant physiology, function, decrease yield nor cause the plants to become more susceptible to known pests and diseases (Clark and Wilson 1993). Targeting the

linkages between the lignin and the structural carbohydrates instead of solely decreasing total lignin content may minimise such affects (Chesson *et al.* (1995).

Lignin biosynthesis involves a number of complex metabolic pathways that lead to the formation of three monolignols. These monolignols; namely 4-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, polymerise to form the polymer lignin (Whetten and Sederoff, 1995). Lignin levels, the degree of linkage with structural carbohydrates (cellulose and hemicellulose), as well as the ratio of the monolignols units all affect the degree of digestibility of lignin present in vegetative grass tissue.

Many of the enzymes involved in lignin biosynthesis are from multigene families and are often developmental specific, as in the case for the peroxidase genes, *pxdA*, *C* and *D* from lucerne that show leaf, root and stem-specific expression respectively (Abrahams *et al.*, 1996). It may thus be possible to specifically alter only those genes from a family that affect lignin composition that are specifically expressed as flowering commences. This would allow the production of normal lignin for those functions that should remain unaltered, such as xylem cell walls.

Genes that have been explored so far include *caffeic acid 0-methyltransferase* (*COMT*), *cinnamyl alcohol dehydrogenase* (*CAD*), *4-coumarate:CoA ligase* (*4CL*), *cinnamoyl CoA reductase* (*CCR*), and *peroxidase* (*PER*) which all play pivotal roles in lignin biosynthesis.

The effects of altering the expression levels of some of these genes have been investigated in the model plant tobacco. Antisense suppression of the *CAD* gene in transgenic tobacco resulted in plants with reduced CAD activity. No developmental processes or changes in the concentration of lignin in the cell wall were detected. However, the composition of the cell wall was altered, with higher levels of phenolics resulting in more extractable lignin (Halpin et al., 1994). In addition, antisense suppression of the *COMT* gene yielded similar changes, with down regulation of COMT activity resulting in a reduction in lignin content, changes in lignin structure and an increase in degradability (Sewalt *et al.* 1997). Similar attempts to suppress PER, the enzyme involved in polymerisation of the three monolignol residues to form lignin has not shown any detectable changes in lignin content, composition or degradability (Lagrimini *et al.*, 1997).

The lignin biosynthesising genes *COMT*, *CAD*, and *PER* have been isolated from a number of pasture plants including lucerne, S*tylosanthes humilis*, and perennial ryegrass (Ni *et al*., 1994; McIntyre *et al*., 1996; Spangenberg, 1998).

The recent isolation of three *COMT* cDNA clones from ryegrass will facilitate a greater understanding of lignin biosynthesis in grasses (Heath *et al*., 1998). These clones are being utilised to suppress COMT expression in both transgenic ryegrass and tall fescue. The overall aim of such down regulation is to allow production of grasses with modified lignin content (Spangenberg *et al.*, 1998).

In addition, the transformations of two forage legumes, lucerne and *L. corniculatus*, with an antisense *CAD* gene have also recently been reported. Constitutive expression resulted in depressed *CAD* mRNA levels in the transgenic lucerne plants compared to levels measured in the controls (Brisibe *et al.*, 1998). Moreover, a decrease in CAD activity up to 50% was found in transgenic *L. corniculatus* expressing an antisense *CAD* gene with concomitant lower lignin content and increases in *in vitro* digestiblity (Akashi *et al.*, 1998).

Increased soluble carbohydrate

The possible introduction of fructans, as discussed above for legumes, may not be so easy when applied to grass species where fructans are already produced as their main form of storage carbohydrate and where fructan biosynthesis and hydrolysis is already under

controlled plant regulation. However, transgenic ryegrass with altered fructan metabolism has been reported using bacterial levansucrase genes (Ye, 1997). In addition, native fructan biosynthesis genes from grasses are being isolated and characterised, which will increase our understanding of fructan metabolism and carbohydrate partitioning in grasses (Spangenberg *et al*., 1998). Another option to address the supply of fructan in grasses is to regulate the activity of the enzymes responsible for fructan hydrolysis, such as fructan exohydrolases (FEH; Simpson and Bonnet, 1993; Marx *et al.*, 1997). The nutritive value of grasses would be improved if the mobilisation of foliar fructan could be delayed or minimised.

Furthermore, the accumulation of these fructans in pasture plants may provide the added advantage of enhancing performance under conditions of water stress. Transgenic tobacco and sugar beet (*Beta saccharifera*) accumulating fructan have already shown enhanced drought resistance (Pilon-Smits *et al.*, 1995; 1999). The introduction of such genes will also provide an improved understanding of the physiological and biochemical mechanisms involved.

Conclusion

Pasture is often sub-optimal when it comes to meeting the nutritional requirements of both the rumen microflora and the animal itself. The genetic potential of grazing ruminants for meat, wool or milk production is rarely achieved because of less than optimal nutritive value and intake of the forage. There is potential, by altering the composition of pasture plants, to improve the nutritive value of pasture, thereby increasing the efficiency and quality of pasture based systems. This has largely been achieved by crossing cultivated pasture species by conventional breeding, which has for many years been the mainstay for improving the nutritional quality of pasture grasses and legumes. But in a number of instances conventional plant breeding cannot address the issues, due to lack of a trait in the general plant population, or alternatively has reached its limit to change characteristics. The techniques of molecular biology offer the potential to break through these barriers. Conventional plant breeding is based on gene selection from within the cultivars own gene pool or an interbreeding species. On the other hand molecular biology offers the exciting prospect of introducing precise changes at the gene level through a non-sexual means, from their native genome as well as from a potentially universal gene pool. This will allow us to introduce into pasture plants a range of valuable genes allowing many of these problems to be addressed.

The use of such molecular biological techniques to enhance the nutritive value of pasture plant species, although initially lagging behind the progress made in numerous crop species, is now progressing rapidly. Various techniques allowing the efficient transformation and regeneration of pasture legumes and grasses have improved significantly during the last 10 years and have developed into valuable tools. Although transformation and regeneration have been demonstrated to be very species and even cultivar specific, further refinements should allow the transformation of additional agronomically important species. In addition, future developments in genetic techniques and their applications to model plants and crop species will also provide invaluable information and improve the precision with which genes can be introduced and modified in pasture plants. Moreover, the recent application of molecular markers and EST methodologies to pasture species widens the prospects of identifying genes already present in the pasture plant genomes that may help to address some of the nutritional issues. However, there is a lack of scientific knowledge on forage plant species and a lack of basic information on gene function and plant development in general which needs to be further clarified. One of the first priorities therefore, is to fill the gaps

existing in our knowledge of genetics, physiology and biochemistry of pasture plants, especially of the traits being manipulated.

Improvements in the nutritional value of forages will be realised through the combined efforts of all disciplines of science. Molecular biology is an additional discipline that if integrated with other scientific disciplines and linked to conventional plant breeding will result in the development of new and improved forage grass and legume cultivars that meet the ever-changing requirements for the forage industries.

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