MODIFYING CONDENSED TANNIN CONTENT IN PLANTS

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ABSTRACT
Proanthocyanidins (PA) in forage can have a number of benefits for the plant and the grazing ruminant. These polymeric polyphenols can confer resistance to some plant diseases and pests. They can also reduce the parasite load in the animal. Even quite low concentrations of PA in the forage (0.2% dwt) confer bloat-safety. Higher levels (about 1% dwt) are required to achieve substantial protection of forage protein from ruminal degradation. Some plant species have high levels of PA (>5% dwt) which impact detrimentally on palatability and digestibility. It is therefore desirable to be able to genetically modify the PA levels in important pasture species. In *Lotus* spp and *Lespedeza cuneata* it has been possible to exploit natural variation in PA levels to develop new germplasm with reduced PA levels. The cloning of a number of genes of the anthocyanin/PA pathway has now opened up the prospect of genetic engineering for both increased and decreased PA.

KEYWORDS
proanthocyanidins, bloat, by-pass protein, genetics, genetic engineering

INTRODUCTION
Proanthocyanidins (PA, condensed tannins) are plant phenolic polymers based on the condensation and polymerisation of leucoanthocyanidins and catechins, compounds produced at the end of the flavonoid pathway (Fig. 1). There are many types of PA including: procyanidins, based on catechin monomers; prodelphinidins, based on gallocatechin monomers; propelargonidins, based on afzelechin monomers (Porter, 1994). PA molecules also differ in the distribution of condensing linkages and in the molecular weight of the polymer.

PA can have a number of beneficial effects for the grazing animal including bloat-safety and increased ruminal by-pass protein. Some of these polymers can also confer disease and pest resistance on the plant and thereby improve the productivity and sustainability of pasture-based agriculture. However high levels of PA can have detrimental effects for grazing animals, notably decreased palatability and digestibility. It would therefore be useful to improve forage species by modification of PA levels through conventional breeding or genetic engineering.

PHYTOCHEMICAL PROPERTIES OF PA
The actual PA structure depends on the relative proportions of the alternative monomers, such as epicatechin and catechin, and the linkages (eg C4-C8 versus C4-C6). For example the extension and terminal units of sainfoin (*Onobrychis viciifolia*) PA have been shown to be epicatechin, gallocatechin and epigallocatechin, while catechin was found only as a terminal unit (Koupai-Abyazani et al., 1993a). Further, the degree of polymerisation increased to seven, the degree of B-ring hydroxylation and the proportion of epi-catechin-like units also increased as the leaves matured (Koupai-Abyazani et al., 1992; Koupai-Abyazani et al., 1993a). In contrast, the PA present in seed coats of alfalfa consists of epicatechin extension units only with catechin terminal units (Koupai-Abyazani et al., 1993b).

Our view of PA-protein interactions was radically altered by the classic study of Hagerman and Butler (1981). Previously it was thought that the binding of PA to protein was non-specific. These authors demonstrated that binding affinities varied by as much as 4 orders of magnitude. Proline-rich proteins, such as the dominant salivary proteins, had the highest affinities. The increase in salivary protein excretion by mammals in response to high PA food may serve to ameliorate the interactions and inhibition of digestive enzymes (Robbins et al., 1987; Mehansho et al., 1992). With the exception of proline-rich proteins, those with molecular weights less than 20 kD tend to have low affinity for PA. In general the binding is strongest near the pI of the protein.

Other polymers such as polyvinylpyrrolidone (PVP)(Hagerman and Butler, 1981) and polyethylene glycol (PEG)(Barry and Forss, 1983) bind with exceptional avidity to PA.

ASSAYS FOR PA
A number of assays are available for PA and were recently reviewed by Reed (1995). Vanillin/HCl and butanol/HCl are the most widely used for plants (Price et al., 1978; Porter et al., 1986; Hagerman and Butler, 1989); a combination of the two being useful to estimate extractable PA and fibre-bound PA (Terrill et al., 1992a). The vanillin-HCl leaf squash methods fail to detect PA below a concentration of 1% (dwt). Recently we have described an assay utilising DMACA (dimethylaminocinnamaldehyde) which is five times more sensitive than the vanillin-HCl method and is capable of quantifying PA levels down to 0.04 % (dwt). This is near the estimated minimum level of tannin required for bloat safety in pasture legume leaves (0.1-0.5% dwt). When DMACA is used as a histological stain, single PA positive cells can be seen on an otherwise PA free leaf (Li et al., 1996).

Near Infrared Reflectance Spectroscopy (NIRS) is commonly used in the analysis of forage quality traits and has been used in screening procedures for PA in *Sericea lespedeza* (Windham et al., 1988) and *Lotus corniculatus* (Roberts et al., 1993). The standard errors of calibration in these studies were low and it is likely that NIRS will be used extensively in the future to meet the requirement of plant breeders for the rapid measurement of large numbers of samples.

All methods suffer from a lack of suitable standards which results from difficulties in storage due to their high reactivity and also from the different sensitivities of varying PA structures to the assay reagents.

EFFECTS OF PROANTHOCYANIDINS IN FORAGE PLANTS AND ON GRAZING RUMINANTS
Pest and disease resistance. Proanthocyanidins probably play a role in plants in the protection against various diseases and insects. Feeding deterrence against *Schizaphis graminum* and *Aphis craccivora*, was associated with foliar PA in sorghum and groundnut, respectively (Grayer et al., 1992; Harborne and Grayer, 1994). Lepidopteran larvae of the *Pieris*, *Heliothis* and *Pectinophora* genera were strongly inhibited by PA (Harborne and Grayer, 1994). Ridsdill-Smith et al. (1995) showed that catechin had a feeding deterrent effect on redlegged earth mites, *Halotydeus destructor*, and it is therefore possible that PA polymers would also.

The mechanisms of pest and disease resistance are unknown and open to speculation. In some cases it may simply relate to the avid protein binding properties of PA. However Gonzalez-Coloma et al. (1993) showed that PA from four plant sources inhibited feeding

and growth of cabbage looper (*Trichoplusia ni*) larvae to different degrees when added to artificial diet, yet there was no correlation to the protein binding ability of the different PAs. The gut pH of certain types of insects may often not be conducive to PA binding to digestive enzymes or to nutrient uptake mechanisms. For example lepidopteran larval gut pH is highly alkaline, and PA affinity for proteins tends to be greatest near the protein pI. Biological effects other than protein binding may sometimes be responsible, though these remain to be defined. One possibility is the sequestration of metal ions by PA and the subsequent inhibition of metal requiring enzymes. Another possibility is that the PA molecules can be depolymerised in acid conditions, such that some of the generated units are toxic when absorbed (Clausen et al., 1990). Different lepidopteran insects have been shown to differ greatly in their sensitivity to particular PAs (Bernays et al., 1981; Manuwoto and Scriber, 1986).

Cherry, pear, apple and strawberry specifically accumulate catechins and PA in zones surrounding necrotic centres of fungal infections (Feucht et al., 1992). In cotton PA accumulation is correlated with increased resistance to *Rhizoctonia solani*, *Verticillium dahliae*, *Xanthomonas campestris* (bacterial blight) and *Diplodia gossypina* (boll rots) (reviewed in Bell et al., 1992). The inhibition of microorganisms by PA is reviewed by Scalbert (1991). The fungi inhibited include *Botrytis*, *Colletotrichum*, *Aspergillus* and *Trichoderma* species. The bacteria inhibited include *Streptococcus*, *Nitrosomonas*, *Nitrobacter* and *Pseudomonas* species. It has been suggested that PA exerts antimicrobial activity by the chelation of essential metal ions such as Fe. The chelation is mediated by o-diphenol groups in the PA and results in very effective inhibition of fungal metalloenzymes such as peroxidase and laccase (Scalbert, 1991).

Bloat-safety. Frothy bloat is caused by the production of a highly stable protein foam in the rumen during the initial rapid fermentation of fresh legume forage. Legumes containing PA do not cause bloat (Table 1) because the PA precipitates protein in the rumen and thereby reduces the level of soluble proteins below that required for stable foam formation (Majak et al., 1995).

Bloat often results in loss of livestock, and animal productivity may also be reduced considerably by the stress of sub-lethal bloat. The fear of bloat and the required vigilance also has a negative impact on the lifestyle of dairy farmers and cattle ranchers. The annual cost to Australian agriculture may be in excess of $180 million; $100-150 million in Canada (M.Gruber, pers. comm.); $310 million in the U.S.A. (Rumbaugh, 1985); the New Zealand dairy industry estimates its direct losses to exceed $25 million p.a. (Bryant, 1991). The costs include deaths, prevention measures and veterinary expenses. There are substantial benefits to grazing alfalfa in Canada for both dairy and meat production, and freedom from the fear of bloat would substantially speed up the adoption of this technology (Paul McCaughy, pers. comm.). Because of their high nutritive value, bloat-causing legumes such as white clover and alfalfa are used extensively in the dairy and meat industries. Fear of bloat is a major constraint limiting the potential extra value in productivity which could be achieved by grazing these forage legumes. Although it is poorly documented, the production of alfalfa hay, silage or pellets appears to reduce the risk of bloat but also adds to management costs.

We have shown that PA has a dramatic effect on the compressive strength of protein foams produced from legume leaves (Tanner et al., 1995). Although there are differences in the structures and molecular weights of PA from different sources, the effect on foam strength was similar. The decrease in compressive strength of the foams was related to PA concentration as shown in Fig 1. A ratio of PA to protein of 0.1 reduced the foam strength by half. Recently we estimated the threshold effective level of PA to achieve complete bloat-safety to be between 0.1-0.5 % (dwt). This was based on more accurate assays of the levels in various plant species and correlations with their published bloat reputations (Li et al., 1996). Stockdale (1994) attributed the bloat-safety of diets containing mature white clover to the presence of PA in the flowers. Virtually no bloat was observed when the diet contained more than 5% DM as flowers. The PA in the flowers contributed 0.17% PA dwt to the diet. Waghorn and Jones (1989) fed cows a alfalfa/grass diet with and without 10% dock *(Rumex obtusifolius*) included in the herbage. Bloat did not occur when dock was included but did occur when dock was absent. PA was present in the dock and contributed 0.13 to 0.23% of DM eaten by the cows.

Bloat prevention measures including the spraying of oils onto the pasture and the use of feed supplements, such as Teric or molasses, are expensive and labour-intensive (Stockdale, 1991). Poloxalene and monensin-based slow release capsules are relatively expensive (Cameron and Malmo, 1993; Majak et al., 1995). If the important bloat-causing species, such as white clover and alfalfa, could be genetically modified to produce appropriate levels of PA, this would be attractive economically, environmentally and in terms of animal welfare.

Effects on by-pass protein. The presence of PA also increases the efficiency of protein utilisation in ruminants. By protecting protein from rapid microbial degradation in the rumen, the amount of plant protein surviving into the abomasum (so called *by-pass protein*) is increased substantially, giving a nutritional boost. Without PA, much of the rapidly released soluble protein from "soft" legume leaf cells is broken down by the rumen microflora to ammonia which is absorbed and excreted as urea. This represents a major waste of dietary protein. Barry (1989) found 25-33% of the N eaten was absorbed as ammonia in high producing ruminants fed fresh forages *ad libitum*. Likewise McNabb et al. (1993) demonstrated that sheep fed *Lotus uliginosis* (syn. *L. pedunculatus*) lost no methionine or cystine across the rumen, but when the PA was bound to polyethylene glycol (PEG) the sheep lost 30% of the methionine and cystine.

Some legumes such as *Lotus corniculatus*, *Onobrychis viciifolia* and *Trifolium arvense* possess PA and also show increased post-rumen protein availability and reduced protein loss (Reed, 1995). This is thought to be achieved by the complexation of PA with plant protein at rumen pH (6-6.5), protecting it from degradation. Once the complex passes beyond the rumen, *in vitro* studies suggest that the protein is released from the PA at the lower intestinal pH and is available for digestion and absorption by the animal (Jones and Mangan, 1977). Reed (1995) questions the mechanism but not the reality of the positive effect of PA. The benefits of PA-containing forages for ruminant nutrition are expressed through increased flow of nitrogen to the small intestine (Barry and Duncan, 1984) and higher absorption of amino acids such as cysteine (McNabb et al., 1993; Lee at al., 1995).

Live-weight gains, wool growth and milk production respond to increased post-rumen protein supply (Reis & Schinckel, 1963; Ferguson et al., 1967; Barry, 1985; Terrill et al., 1992b; McNabb et al., 1993; Broderick, 1995). Increases in live weight gain of the order of 5-10%, and wool growth in excess of 10% can be expected when protein is protected by PA. The positive effect of PA on the supply of essential amino acids to the duodenum appears to apply for dried feed as well as fresh feed (Thompson et al., 1971; Harrison et al.,

1973; Beever and Siddons, 1985).

We have demonstrated experimentally the protection of protein in ruminal fluids by the presence of PA (Tanner et al., 1994). PA purified from the leaves of forage legumes *Trifolium arvense, Lotus pedunculatus, Lotus corniculatus, Dorycnium rectum, Coronilla varia, Onobrychis viciifolia,* or *Hedysarum coronarium*, were added to soluble alfalfa (*Medicago sativa*) leaf protein and incubated with strained rumen fluid *in vitro*. In the absence of PA, the large subunit (LSU) of ribulose-bisphosphate carboxylase was susceptible to proteolysis by rumen microflora but the small subunit (SSU) resisted breakdown. Purified PA was added to soluble leaf protein, at PA:protein ratios between 1:1 and 1:20. The rate of proteolysis of LSU was significantly reduced at PA:protein ratios of 1:2 and 1:1 ($P<0.001$). The half-life of LSU was extended by approximately 10 fold in both cases. In separate experiments PA isolated from the range of species described, was added to rumen fluid to give PA:protein ratios of 1:5. The addition of PA significantly reduced the rate of proteolysis of LSU, when compared with PA free control. There were only small differences in proteolysis between PA from different species. The inhibitory effect may have been due to PA-protein binding and interference with the action of rumen proteases on susceptible sites within the substrate.

Palatability and digestibility effects. When the level of PA is very high (>5% DM), voluntary feed intake is lowered (Barry and Duncan, 1984; Barry, 1989). In addition, the digestibility of the forage can be adversely affected. This effect might result from sub-optimal activity of rumen microflora caused either by a restricted supply of protein N or by direct effects of PA on specific rumen microorganisms (Jones et al., 1994). This adverse effect is demonstrated in a number of tropical legume species, one being *Desmodium ovalifolium.*

D. ovalifolium is a highly productive forage species and widely adapted in the tropics and sub-tropics. International Centre for Tropical Agriculture (CIAT) studies have indicated that the total PA levels in *D. ovalifolium* are about 5 % DM in young leaves but rise to about 20% in mature leaves. On poor soils the PA levels can be even higher. Genetically reduced levels of PA should improve voluntary intake, rumen ammonia levels and nitrogen retention for this species.

Polyethylene glycol (PEG) avidly binds to PA (Barry and Forss, 1983). This property allows one to neutralise and measure the biological effects which are predominantly due to formation of PA-protein complexes. For example, Barry and Reid (1985) demonstrated that a reduction of available PA from 6.3% to 0.7%, using PEG sprays on *Lotus*, resulted in a 44% increase in voluntary intake of this feed by sheep, as well as a 13% increase in digestibility of structural carbohydrate. Levels of >6% available PA depressed voluntary intake. Similarly, CIAT studies showed that 5% PEG reduces the available PA of young *D. ovalifolium* leaves from 5% to 2% DM (Table 2). This resulted in an increase in voluntary dry matter intake by sheep of 24% and a three fold increase in nitrogen retention (from 1.3 g N per day to 4.3 g per day, Carlos Lascano, personal communication). The high PA levels in *D. ovalifolium* are a major constraint on voluntary intake and hence limit the productivity potential of this legume.

High levels of PA also lower the feeding value of forage as measured by live-weight gain in young animals and milk production in lactating animals (Barry, 1985, 1989; Reed et al., 1990). This effect is the consequence of a large reduction in availability of rumen nitrogen resulting from two processes: the protection of dietary protein by PA to such an extent that none is available for rumen microflora; and the inhibition of extracellular rumen enzymes activity by excess PA that is not already bound to dietary molecules. The reduced ruminal microflora activity results in reduced forage digestibility. There is an optimum level of PA where by-pass protein is maximised but where palatability and digestive problems are avoided.

In summary, with respect to forage quality, we estimate from our own work and the literature that 0.02-0.1% (dwt) PA reduces the incidence of bloat while 0.2-0.5% confers complete bloat-safety. Levels > 0.2% begin to increase rumen by-pass of protein, while allowing release of protein in small intestine. However 2-10% PA further increases by-pass of protein, but reduces release in small intestine. Levels of between 5-10% PA decreases voluntary forage intake and inhibit rumen digestion of soluble and structural carbohydrate.

Effects on ruminant intestinal parasites. Niezen *et al* (1995) found that lambs grazing the PA containing forage, Sulla (*Hedysarum coronarium*), had higher growth rates than lambs grazing alfalfa. In a second experiment lambs with a low intestinal parasite burden were intentionally inoculated with the nematode *Trichostrongylus colubriformis* or drenched to remove all parasites. The drenched lambs had similar liveweight gain and wool growth when grazing either Sulla or alfalfa. In contrast inoculated lambs grazing Sulla had lower faecal egg counts, lower worm burdens at slaughter and higher live weight gain and wool growth than inoculated lambs grazing alfalfa. Parasite-induced anorexia was evident in lambs grazing alfalfa but not in those grazing Sulla, suggesting that PA had a direct effect on suppressing nematodes and their adverse effects.

BIOCHEMISTRY AND GENETICS OF ANTHOCYANIN AND PROANTHOCYANIDIN BIOSYNTHESIS

Biosynthesis, genetics and control during plant development. PA biosynthesis is largely in common with anthocyanin biosynthesis. There have been several major reviews of the genetics, biochemistry and molecular biology of anthocyanin formation in the last five years (Stafford, 1992; Gerats and Martin, 1992; Heller and Forkman, 1994; Forkman, 1994). Elucidation of the enzymology and cloning of the corresponding structural genes involved in anthocyanin synthesis has been well established to 3,4-*cis*-leucocyanidin and is described in Figure 3. Clones for most of the structural genes have also been isolated from more than one species including grapes, snapdragon (*Antirrhinum majus*), alfalfa and *Petunia*.

Beyond leucocyanidin it has been suggested that the flavanoid molecule requires hydroxylation at carbon 2, followed by two dehydration steps to form anthocyanidin. The order of these reactions is not clear, and neither has the cell free enzymology been demonstrated. The maize *A2* and the snapdragon *candica* genes, which probably code for this step (ANS, anthocyanidin synthase, Fig. 3) have been cloned (Menssen et al., 1990; Martin et al., 1991) as have homologues from apple and petunia (Davies, 1993; Weiss et al., 1993). The proposed function of these two genes is supported by the observation that their deduced proteins have a striking homology to the known flavanone 3-hydroxylases (F3H, Fig. 3) and to a lesser extent to 2-oxo-glutarate-dependent-dioxygenases. A rice mutant, N22B, has been reported in which the accumulation of anthocyanins in the pericarp is blocked, but in which PA appears to accumulate in the pericarp (Reddy et al., 1995). It was proposed that the lesion occurs in anthocyanidin synthase, leading to the accumulation of leucocyanidin. The chemical characterisation of the accumulated polymer was not sufficient to establish whether the product was an authentic PA polymer or monomeric leucoanthocyanidin.

Anthocyanidin is stabilised by glycosylation at position 3 by UDP-glucose: flavanoid 3-O-glucosyltransferase (FGT, Fig. 3) activity, coded for by maize *Bronze 1*. It has been suggested that the anthocyanin is then conjugated to glutathione by glutathione S-transferase coded for by maize *Bronze 2* (GST, Fig. 3) (Marrs et al., 1995) and imported into the vacuole by the vacuolar glutathione pump (Martinoia et al., 1993). The imported anthocyanin may then be further modified by acylation with malonic acid.

PA biosynthesis and its relationship to anthocyanin biosynthesis have been reviewed by Stafford (1992) and Heller and Forkman (1994). Kristiansen (1984, 1986) clarified the enzymology of the conversion of dihydroquercetin to catechin in barley. The conversion of the trihydroxy counterpart, dihydromyrecetin, to gallocatechin has been demonstrated for Douglas fir and *Ginkgo biloba* extracts (Stafford and Lester, 1985) and for sainfoin extracts (Singh et al., 1997). There are differences during leaf development between the patterns of activity using dihydroxy and trihydroxy substrates (Singh et al., 1997; Gruber, pers. comm.). These differences correlated with changes in polymer composition during development (Koupai-Abyazani et al., 1993a). Together with other data illustrating the change in the degree of polymerization and content of PA in developing sorghum grain and Douglas fir bark (Stafford et al., 1989; Butler, 1982), these experiments illustrate the dynamic nature of PA biosynthesis and accumulation and its dependence on plant development.

The NADPH-dependent conversion of 3,4-cis-leucocyanidin to catechin has been demonstrated using radio-HPLC in extracts of sainfoin leaves and barley grain testa (Tanner et al., 1992b; Tanner and Kristiansen, 1993). The enzyme performing this reduction is called leucoanthocyanidin reductase (LAR, Fig 3) and appears to require no other cofactors (Tanner et al., 1992b). This enzyme has now been purified to homogeneity and attempts are being made to clone the cDNA (Tanner et al., unpublished).

In soybean seed coat a dominant gene, I, controls the repression of anthocyanidins and proanthocyanidins, and the T gene controls a microsomal 3'-flavanoid hydroxylase (Todd and Vodkin, 1993). The I gene locus consists of a 10kb region containing three chalcone synthase genes, one of which is situated in antisense orientation between two others (Todd and Vodkin, 1996). The molecular analysis of this locus and naturally occurring recessive mutations suggest that the I locus is a natural example where gene duplication has resulted in homology-dependent gene silencing. The mutations delete the extra copies of CHS and restore activity and seed colour accumulation.

Jende-Strid (1991) has localised over 600 PA-free mutants to nine different *ant* genes in barley. Most *ant* genes have been assigned functions but few have been isolated. *Ant* 13, 21, and 25 are regulatory genes which effect anthocyanin and PA biosynthesis. *Ant*17 and *ant* 22 code for subunits of flavanone-3-hydroxylase, and *ant* 18 for dihydroflavanol reductase. The *ant* 17 and *ant* 18 genes have been isolated (Meldgaard, 1992; Kristiansen and Rhode, 1991) and the function of *ant* 18 confirmed by transient gene complementation (Wang et al., 1993). Finally it was initially thought that *ant* 19 encoded leucoanthocyanidin reductase but more recent data have indicated that it is probably involved in controlling nutrient supply to the grain (Meldgaard, 1992; Tanner et al., 1992a; Jende-Strid, 1993).

Three mutations (*ant* 26, 27 & 28) block the synthesis of PA but allow accumulation of catechin in the testa, and anthocyanin in the leaves of the plant, and therefore appear to code for steps unique to the synthesis of PA beyond the synthesis of catechin. The activity coded for by *ant* 26, 27, 28 remains unknown. It is speculated that one or more of these may encode a condensing enzyme required for the formation of PA (Jende-Strid, 1993). However no direct evidence has been cited for the existence of such an enzyme, and the fact remains that PA forms spontaneously from leucoanthocyanidin and catechin at the pH of the vacuole. Further, the structures so formed are similar to those found in plants (Ferreira et al., 1992).

An alternative hypothesis for the function of *ant* 26, 27 and 28 is that they represent vacuolar transporter proteins (Tanner et al., 1992a). Histological studies have suggested that PA is synthesised in the endoplasmic reticulum and transported to the central vacuole (Baur and Walkinshaw, 1974; Parham and Kaustinen, 1977), although direct accumulation in the vacuole appears more likely in sainfoin leaves (Lees et al., 1993; 1995; Singh et al., 1997; Tanner et al., unpublished).

In maize the production of anthocyanins requires expression of one member of each of two families of regulatory genes, *R* and *C1* which are homologous to the mammalian *myc* and *myb* families of transcription factors, respectively (Gerats and Martin, 1992). There are many genes which regulate anthocyanin synthesis in maize. Molecular analysis has shown that the coding regions of the maize regulatory genes *R, Lc, B,* and *Sn* are highly homologous to *R* (myc family) and that *Pl* is highly homologous to *C1* (myb family). Differential expression of anthocyanins reflects the tissue specific expression of the various regulatory genes specified by the promoter regions of these genes. In maize, *R* and *C1* interact together to activate coordinately the first gene of the flavonoid pathway, chalcone synthase, as well as the genes for dihydroflavanol reductase and flavanol-UDP-glycosyl transferase, the last steps of anthocyanin biosynthesis (Fig.3). Enzyme data imply that the intermediate genes coding for chalcone isomerase and flavanone-3-hydroxylase are also regulated in the same way (reviewed by Gerats and Martin, 1992).

In dicots, the molecular genetics are best understood for snapdragon (*Antirrhinum majus*) where the control of anthocyanin biosynthesis differs from barley and maize. In snapdragon, the regulatory genes *delila*, homologous to maize R, and *eluta*, homologous to maize C1, control only the lower part of the biosynthetic pathway including flavanone-3-hydroxylase, dihydroflavanol reductase, anthocyanin synthase and flavanol-UDP-glycosyl transferase (reviewed by Koes et al., 1994). Recently, T-DNA and Tn-tagged mutants of *Lotus japonicus* were shown to ectopically express leaf PAs (Skadhauge, 1996; M.Gruber, pers. comm.). Mutants of this type will facilitate the isolation of PA genes and an understanding of the regulation of PA biosynthesis in legumes.

We have shown that transformation of *Lotus corniculatus* with the maize anthocyanin regulatory gene, *Sn*, can cause the total suppression of PA synthesis in the leaves, apparently by co-suppression of an endogenous regulatory gene required for PA biosynthesis (Damiani et al., 1996; Damiani et al., unpublished). In transgenic plants, in which leaf PA has been down regulated the activities of both dihydroflavanol reductase and leucoanthocyanidin reductase are missing. Conversely, the accumulation of PA and the activity of leucoanthocyanidin reductase is upregulated in roots of the transgenic plants, presumably because the *Sn* transgene complements the lack of expression of the endogenous regulatory gene. This is the first evidence that both dihydroflavanol reductase and leucoanthocyanidin reductase in forage legumes are affected by a single regulatory gene. These findings agree with data from barley. Barley plants carrying a mutation in the A*nt*25 gene do not accumulate PA in the testa/pericarp and also lack both dihydroflavanol reductase

and leucoanthocyanidin reductase in the testa (Tanner, 1992a). It appears that *ant* 25 codes for a gene involved in the regulation of both dihydroflavanol reductase and leucoanthocyanidin reductase.

Cellular and subcellular localization of anthocyanin biosynthesis. Vanillin-HCl or DMACA-HCl are the most useful histochemical stains for PA. Until they are stained, the vacuoles of fresh tannin cells are clear and colourless. The only exception to this is when the cells are oxidised as is thought to occur during the drying of seed coats. No case has been reported of PA and anthocyanin occurring in the same cell. Histochemical staining of PA in sainfoin, *Onobrychis viciifolia*, showed that PA accumulated in the central vacuoles of specific leaf cells in a precise developmental sequence (Lees et al., 1993; Lees et al., 1995; Tanner et al., unpublished). First, a network of PA-containing cells formed on the abaxial surface at early stages of development. This network of cells surrounds the stomata in a configuration that suggests a line of defence to hyphal invasion. Secondly, at the leaf unfolding stage, single cells of the palisade layer next to the abaxial layer begin to accumulate PA. As the vacuoles of these cells continue to fill with PA, the PA content of the abaxial surface network appears to decline. In senescing leaves there appears to be a general decline in PA content of all cells.

A similar cytological profile was observed in *Hedysarum sulfurescens, H. coronarium, H. carnosum* and *Coronilla varia*. By contrast the leaves of *Lotus uliginosis*(syn. *L. pedunculatus*), *L. japonicus*, *L. corniculatus*, *Robinia pseuacacia* and *R. tortuoso* accumulated PA only in the large mesophyll cells (Skadhauge et al., 1996; Tanner et al., unpublished). The leaves of *Lotus angustissimus* and *L. tenuis* only accumulate PA in elongated cells associated with leaf veins, whereas *Trifolium arvense, T. affine* and *T. armenium* only accumulate PA in epidermal leaf cells (Tanner et al., unpublished). PAs were also observed in other tissue types of these species, notably seed, seed pod and flowers. Only *Robinia* had white flowers and accumulated no PA. Li et al. (1996) found a number of legume species to have leaf trichomes which had PA filled central vacuoles. These species included a number, such as alfalfa and white clover, which had no other foliar tannin cells. *Lotus uliginosis* (syn. *L. pedunculatus*) petals had a three dimensional network of tannin cells. In *L. japonicus* flowers, the PA filled central vacuole is distinct from the carotenoid bodies which give the flower its yellow colour. The central vacuole was also the site of PA accumulation in the testa cells of barley and alfalfa seeds (Skadhauge et al., 1996).

PA is highly osmiophilic. When tissues are fixed and treated with osmium prior to embedding and preparation for transmission electron microscopy, the PA filled central vacuoles are highly electron dense and very evident in micrographs. This allows the fine structural details to be confirmed, notably the confinement of PA to the central vacuole (Lees et al., 1993; Li et al., 1996; Tanner, unpublished). Karwatzki et al. (1993) used immunofluorescence techniques to demonstrate that chalcone synthase protein occurred in the leaves of *Kalanchoe* and *Acorus*, but only in the PA containing cells. This links the site of PA synthesis to the site of PA accumulation.

Regulation through environment. PA accumulation is effected by a number of environmental factors. Lees et al. (1994) showed that *Lotus uliginosis* (syn. *L. pedunculatus*) growing under higher temperatures accumulated higher levels of PA. If additional nutrient stress was applied at the higher temperature then PA levels declined, indicating catabolism of PA in the late stages of growth. This is the first report of PA catabolism in any species but has been confirmed by Lees et al. (1995). By contrast *Desmodium ovalifolium* leaves accumulate more PA when grown on acid soils than when grown on neutral soils, and *Lotus uliginosis* (syn. *L. pedunculatus*) leaves accumulate more PA when grown on nutrient deficient conditions (Barry and Forss, 1983).

PA accumulation in tissue cultures of cranberry (*Vaccinium macrocarpon*) was independent of light; however light triggered accumulation of flavonols and anthocyanins, indicating that in this tissue environmental control of the two pathways occurs by separate mechanisms (Madhavi et al., 1995). By contrast in sainfoin, the rate of biosynthesis and accumulation of PA was greatly reduced under low light conditions (Tanner, unpublished). Exposure of Loblolly pine trees to ozone increased the accumulation in needles of PA by 30% and catechin by 80%, compared to untreated trees (Booker et al., 1996). Wounding and giraffe browsing has been shown to trigger increased biosynthesis of PA in a number of plant species (Hay and Brown, 1992; Furstenburg and Van-Hoven, 1994).

GENETIC MODIFICATION OF PROANTHOCYANIDIN

Conventional breeding to reduce PA. The conventional plant breeding approach to the improvement of specific traits like PA starts with screening for variability that is available in germplasm sources. These trials can be designed to provide information on some aspects of gene action underlying the trait and its broad-sense heritability. Early generation crossbreds then can provide additional information on inheritance so that the breeding program can be designed to best exploit the genetic base. This approach is reviewed with respect to species in two legume genera in which breeding for reduced PA content has been attempted.

Lotus

The perennials, *Lotus corniculatus* L. and *L. uliginosus* Schkuhr (syn. *L. pedunculatus*) are used in pastures in North and South America, Australia and New Zealand where they are more productive than other legumes on low-fertility soils. The range of variation that has been reported for PA within cultivars of *L. corniculatus* is 0.13 to 6.0 (%dwt) (Lowther et al., 1987; Kelman and Tanner, 1990; Roberts et al., 1993; Kelman, 1996a). In *L. uliginosus* PA concentrations are generally higher than in *L. corniculatus*, ranging from 2.5 to 10.7% dwt in 10 accessions examined by Kelman and Tanner (1990).

Major gene action with dominance for high PA content has been found in *L. corniculatus* by Ross and Jones (1983) and Dalrymple et al. (1984). In the latter study, the results were consistent with a disomic mode of inheritance, whereas the results of Ross and Jones (1983) fitted a model of tetrasomic inheritance with chromosomal segregation. Tetrasomic inheritance has been demonstrated for cyanogenesis (Dawson, 1941) and self-incompatibility (Bubar and Miri, 1965) indicating that this is the more likely mode of inheritance. Six-parent diallel crosses between high and low PA populations in *L. corniculatus* by Miller (1992) showed that additive genetic variance was greater than dominance genetic variance but qualitative treatment of the data did not detect the presence of a major gene. In *L. uliginosus*, Kelman (1995) also found no evidence of major genes for PA but significant additive gene effects were present in an analysis of F_2 and backcross populations of crosses between New Zealand and Portuguese germplasm sources. In this study, a smaller though significant influence was also present for dominance effects for high PA content. Estimates of narrow-sense heritabilities for PA, based on half sib families and on parent-offspring regression, were close to 0.50 and predictions of response to selection revealed that PA content could be lowered by 10% in a single cycle of selection (Kelman, unpublished). Genotype x environment interaction for PA content in *L. uliginosus* was significant in the studies of Kelman and Blumenthal (1993) and Kelman (1996b) but these genotypic

differences were more a matter of scale than attributable to changes in the ranking of genotypes between environments. More complete documentation of genotype x environment interaction for PA is needed because of the known sensitivity of PA to environmental influences such as water stress, temperature and soil fertility. The results taken together indicate that both major and polygenes influence PA content in the perennial *Lotus* species and that recurrent selection for lowered PA content in *L. uliginosus* would achieve a level that is beneficial to ruminant health and nutrition.

Sericea lespedeza

Sericea lespedeza (*Lespedeza cuneata* (Dumont) G. Don.) is a warm season perennial that is productive on acid and infertile soils and has been used as a forage in the south-eastern United States. Palatability and digestibility of sericea lespedeza are reduced by the high PA content of the plant. Significant variation for PA among half-sib families was reported by Stitt (1943). Bates and Henson (1955) estimated that broad-sense heritability of PA, based on variances of F_2 and parents in two crosses, was moderate (0.43 and 0.34). This estimate may have been influenced by the ferric ammonium citrate method used to measure PA content, as this measures a broader class of phenolic compounds than just PA. In segregating populations of a cross between two inbred lines of sericea lespedeza, Cope and Moll (1969) found that frequency distributions suggested the presence of a major dominant gene for high PA content. Significant additive genetic variance also indicated the presence of polygenes influencing PA content and the broad-sense heritability was high (0.88). Selection for lowered PA content in sericea lespedeza has been successful in the development of two low PA germplasms (Donnelly, 1981; Mosjedis and Donnelly, 1989). The genetic control of PA in sericea lespedeza thus appears similar to that in *Lotus* - the presence of dominant genes, some with large effects on PA, and also the presence of selectable additive genetic variance.

The importance of correlated responses to selection for altered PA content is well illustrated in the development of the 'Lotan' germplasm in sericea lespedeza (Donnelly, 1983). Selection for lower PA was accompanied by increased susceptibility to *Rhizoctonia* foliar blight and cultivar development required concurrent selection for resistance to the disease. Significant negative phenotypic correlations of PA content with *in vitro* digestible dry matter (IVDDM) and crude protein have been found in sericea lespedeza (Peterson et al., 1991) and in *Lotus corniculatus* (Miller and Ehlke, 1994.). In *L. corniculatus* cultivars, erect plant types have higher PA levels than semi-erect types (Lowther et al., 1987), although some prostrate types also have high PA content (Kelman, 1996b). Quantitative genetic analyses are required to estimate the genetic correlations between PA and other herbage quality and agronomic traits and thereby improve progress in breeding for improved overall forage quality. Recently, Miller and Ehlke (1996) have shown that selection for increased PA in *Lotus corniculatus* was correlated with an increase in acid detergent lignin (ADL) and with decreases in crude protein and IVDDM. These relationships may suggest the use of multiple trait selection to incorporate optimal levels of PA into a high quality feed.

Genetic engineering to reduce PA.

Lotus and Tropical legumes

There appears to be little natural variation in the PA content of the high PA tropical legumes. The reductions needed to improve the digestibility and palatability are very considerable. These reductions might best be achieved by the production of transgenic plants where the pathway has been specifically suppressed. There are a number of options for target genes. One of these options is to suppress the transcriptional activators of the phenylpropanoid pathway. These genes encode regulator proteins which control a number of steps of the pathway. We have recently shown that the transformation *of Lotus corniculatus* with the maize *Sn* regulator gene can dramatically reduce the PA content of leaves (Damiani et al., 1996, Damiani et al., unpublished). Evidence was presented showing that this effect was caused by a genetic co-suppression interaction between the transgene and the endogenous regulator gene. However such a dramatic suppression of the phenylpropanoid pathway may have other undesirable effects on the forage, such as the loss of disease resistance mediating phytoalexins. It is advisable therefore to utilise a more PA specific suppression. This would best be achieved by a sense or antisense suppression of the dihydroflavanol (DFR) or preferably leucoanthocyanidin (LAR) genes. Some *Agrobacterium rhizogenes* transformed "hairy root" cultures of *Lotus corniculatus* with an antisense *dfr* gene from snapdragon, had reduced PA content (Carron et al., 1994). This confirms the general feasibility of this approach to reducing PA. There was increased synthesis of less hydroxylated PA molecules and a shift away from prodelphinidin and towards more procyanidin. One inference from such studies is that there may be several enzymes with distinct substrate specificities involved in the synthesis of different stereochemical families of PA.

Genetic engineering to increase PA.

Trifolium and Medicago

A large screening of 59 *Trifolium* taxa found leaf PA in only eight species closely related to *Trifolium arvense* (Fay and Dale, 1993). Extensive screening programs by several labs over many years have failed to find any PA positive *Medicago* species or alfalfa mutants with foliar PA following mutation by gamma, EMS or ethidium bromide (Goplen et al., 1980; Marshall et al., 1979, Marshall et al., 1981; Marshall, pers. comm.; Li et al., 1996). This is so, despite the fact that alfalfa produces PA in the seedcoat, petals and leaf trichomes. The inactivity of the flavonoid pathway in alfalfa leaves can be attributed to low and non-existent levels of chalcone synthase and flavanone 3-hydroxylase mRNA, respectively, but not to dihydroflavanol reductase levels, which are adequate (Charrier et al., 1995).

Genetic engineering of the pathway appears to be the most promising option for producing alfalfa or white clover which synthesize foliar PA. This is so particularly in white clover where variants are available which accumulate anthocyanins in the leaves. Because the pathway to anthocyanin is largely in common to the pathway to PA, only one or two genes may be required to divert intermediates away from anthocyanins toward PA. The first committed step in the synthesis of PA is catalysed by the enzyme leucoanthocyanidin reductase (LAR) (Tanner and Kristiansen, 1993). We have purified the enzyme, obtained amino acid sequence of internal tryptic fragments of the protein and have used these to isolate cDNA clones of the gene from *Lotus* and *Onobrychis* (Tanner et al., unpublished). When we have confirmed the identity of these clones and have full length sequences, it will be fascinating to express them in transgenic anthocyanin-accumulating white clover to test their potential for diversion of flavonoids from anthocyanin biosynthesis to produce PA.

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Table 1
Proanthocyanidins in foliage

	Absent	Present
Bloat-safe	*Dolichos axillaris*	*Onobrychis viciifolia*
	Phaseolus atropurpureus	*Onithopus pinnatus*
	Lotononis bainesii	*Ornithipus compressus*
	Glycine javanica	*Coronilla varia*
	Stylosanthes humilis	*Lotus corniculatus*
	Astragalus cicer	*Lotus pedunculatus*
	Centrosema pubescens	*Lotus purshianus*
		Lotus angustissimus
		Lotus tenuis
		Lespedeza stipulacea
		Desmodium intortum
		Desmodium uncinatum
		Leucaena leucocephala
		Macrotyloma axillare
		Stylosanthes gracilis
		Trifolium dubium
Bloating	*Trifolium hybridum*	
	Trifolium repens	
	Trifolium pratense	
	Dolichos lablab	
	Medicago sativa	

Jones and Lyttleton (1971)
Li et al. (1996)

Table 2
Effect of PEG on utilisation of *D. ovalifolium:*

	effective PA (% dwt)	intake	body weight	N digest.	urine N	faeces N	N retained	Nretained ÷ N intake	rumen NH_4
D.o.	5%	•	•	•	•	•	•	•	•
D.o. + PEG	2%	25%	21%	1.5%	•	¬	2.5x	¬ 2x	¬3x

D.o. is *Desmodium ovalifolium* fresh leaf material
J. Carulla PhD thesis (Uni of Nebraska) data obtained at CIAT following trials in sheep.
• indicates no significant change; indicates increase; ¬ indicates decrease

Figure 1
Tannin decreases foam strength

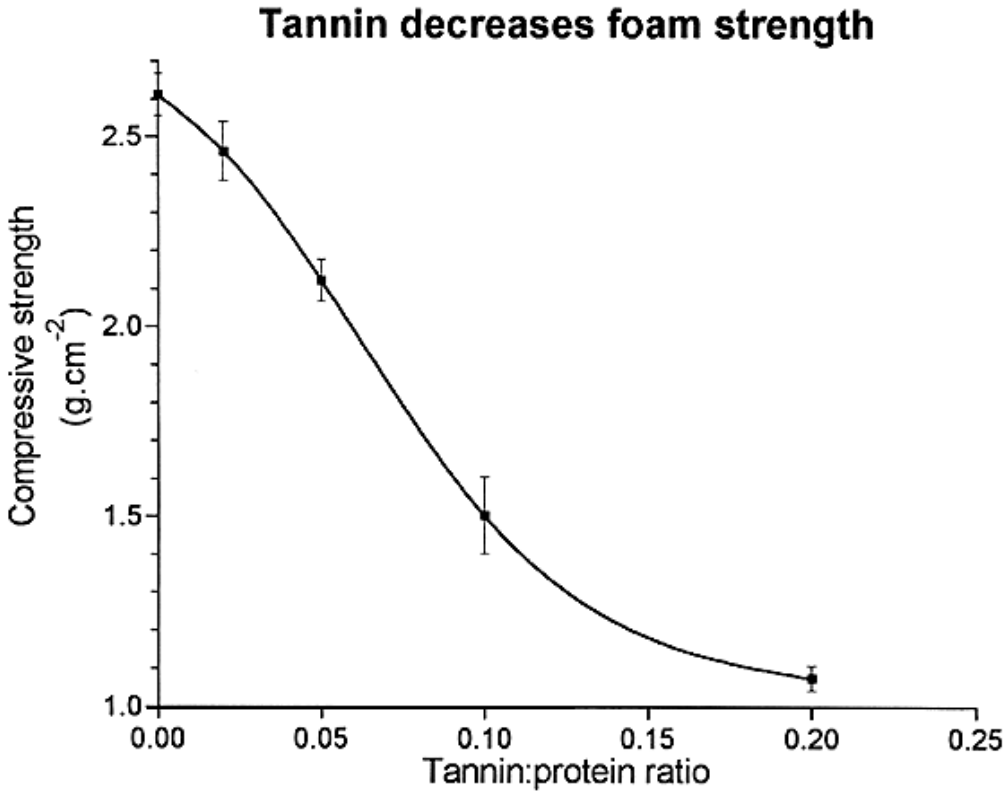


Figure 2
Tannin protects protein

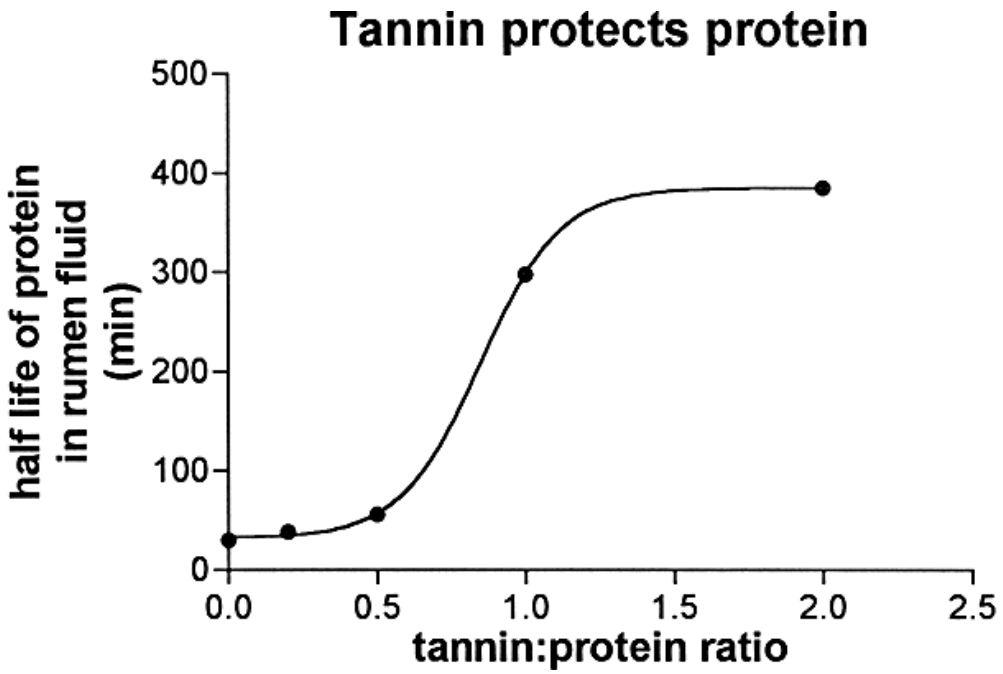
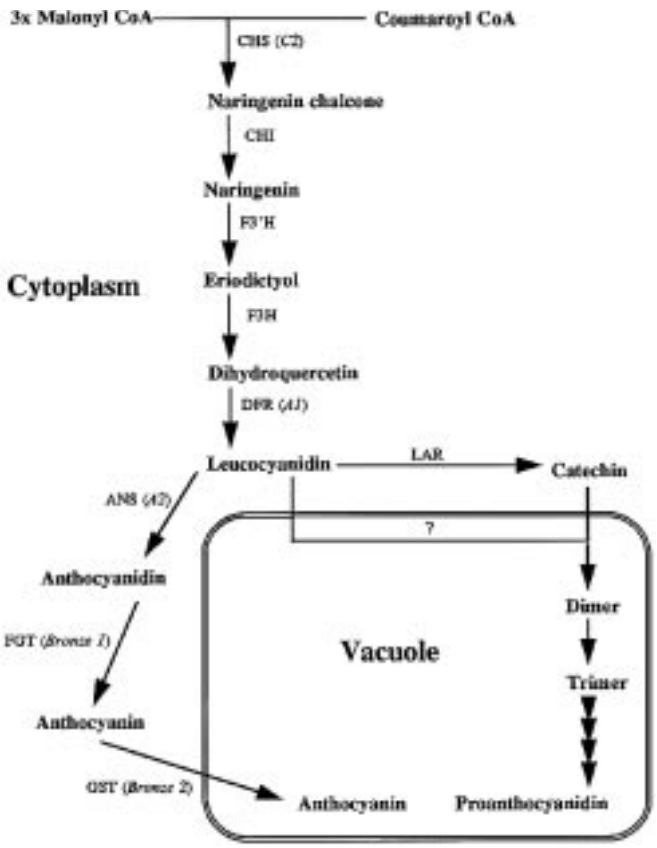


Figure 3
(Pro)anthocyanidin biosynthesis



The intracellular localisation of the intermediates of anthocyanidin and proanthocyanidin biosynthesis are shown with the known enzymes: chalcone synthase (CHS), chalcone isomerase (CHI), flavanone-3'-hydroxylase (F3'H), flavanone-3-hydroxylase (F3H), dihydroflavanol reductase (DFR), leucoanthocyanidin reductase (LAR), the proposed PA condensing enzyme (?), anthocyanin synthase (ANS), flavanol-UDP-glucosyl transferase (FGT), & glutathione-S-transferase (GST). The structural genes which code for these enzymes in maize are shown in brackets.