CHAIRS' SUMMARY PAPER: Tannins: Plant Breeding and Animal Effects

M.Y. Gruber[1] and J. Reed[2]
[1]Agriculture and Agri-Food Canada, 107 Science Place, Saskatoon, Saskatchewan, S7N 0W2, Canada
[2]Dept. of Animal Science, University of Wisconsin, Madison, WI 53706, USA

The invited papers and posters of the tannin session covered two main areas, plant breeding by conventional and genetic engineering techniques, and the biological effects of tannins which impact on their use and manipulation for grassland agriculture. Dr. Garry Waghorn, New Zealand, initiated the session with a lecture on the impact of condensed tannins on animal digestion, focusing particularly on rumen and post-rumen function. He stressed that the reduction of tannins is very important to achieve with tropical plants, since the extremely high levels which exist in these species is antinutritional to all animals. Conversely, the increase or introduction of tannins into plants in temperate climates is important for rumen bloat safety, reduction of sheep dag number and flystrike and an increase in rumen bypass protein. The latter can result in higher milk, meat and wool gains by lowering rumen proteolysis rates (lowering ammonia production) and reducing intestinal parasite levels.

Dr. Waghorn emphasized the need to optimize tannin levels to achieve a net savings from increasing bypass protein, since tannins also reduce the flow of protein to the small intestine and cause a reduction in the fractional absorption of amino acids. The loss in absorption is due to the secretion of excess intestinal mucous. The mucous serves to protect intestinal microvilli from the effect of tannins but causes the site of amino acid absorption to move further along the intestine to a less efficient location. ^{14}C-labelled tannin tracer studies in sheep and chicken have recently proved that tannins are not absorbed by animals, but can be chemically absorbed to other compounds in the digestive tract.

Dr. Phil Larkin, Australia, opened his talk by lecturing on the structure and biochemistry of condensed tannins and their relationship to anthocyanin biosynthesis in plants. Differences in tannin structure do not appear to reduce protein foam strength, an important finding when breeding for bloat safety since polymer structure can change as a function of plant development.

Dr. Larkin described a series of approaches undertaken in his laboratory to genetically manipulate tannins in plants. By using traditional recurrent selection, the genetic diversity of leaf tannin content in *Lotus pedunculatus* (*uliginosis*) was exploited to select plants with low tannin (2.5% down from 10.7%). Protoplast fusion was used to introduce sainfoin DNA into alfalfa. Four alfalfa phenotypes with leaf tannin were recovered, but unfortunately, the trait was unstable.

A series of maize anthocyanin regulatory genes was introduced by plant transformation into forage legumes as a means of genetically engineering tannin content. The maize genes only altered tannin content in tissues which already produced tannin. For example, the *Sn* regulatory gene switched tannins off in birdsfoot trefoil (*Lotus corniculatus*) leaves while increasing levels from very low to high in roots. The *Lotus* transgenic plants were described in more detail in a poster by Paolocci et al. In a second example, the white leaf crescent of white clover was turned into a red crescent in clover transformed with the *B-Peru* regulatory gene, a result of the accumulation of anthocyanins. There was no effect on tannin or anthocyanin accumulation in alfalfa, even by using combinations of these genes.

Dr. Larkin pointed to a strategy used by others in which biosynthetic rather than regulatory genes are introduced. For example, an antisense dihydroflavanol reductase gene has been used to change the composition and content of tannin in *Lotus corniculatus* (Morris and Robbins, 1997) and a chalcone synthase in sainfoin (Gruber et al.). Dr. Larkin's group has isolated gene sequences which are currently being tested for the possibility that they are the leucoanthocyanidin reductase (LAR) gene. The group hopes to use this gene to divert flavonoids from anthocyanin production to produce leaf tannins in the red leaf transgenic clover. Alternatively, an LAR gene could be used in antisense configuration to reduce tannin levels in tropical species.

Several posters focused on nutritional aspects of tannins for animal agriculture. McNabb et al. described *Lotus* prodelphinidin tannin structures (increased B-ring hydoxylation pattern) which improved the ability of rumen fluid to reduce protein degradation compared with procyanidin type (less hydroxylation) while having no effect on protein precipitation. Barahona et al. decribed a reduction in escape protein in sheep when extractable tannin content was lowered using polyethylene glycol (PEG). McMahon et al. were able to overcome the differences in forage degradability between sainfoin and alfalfa by treatment with PEG. PEG binding was also used to determine a marginal value of tannin content which could be achieved in temperate grasses by recurrent selection and which would improve animal performance (Montossi et al.). The tannin content and digestibility of several African browse species were featured by Aganga et al. Effects of drying or freezing tannin-containing forage on fibre digestion and protein metabolism in sheep was highlighted by Terrill et al. One poster also focused on the negative effect of tannins on grasshopper digestion and viability (Soroka et al.).

Several themes emerged from these nutrition posters and formed the basis of discussions on the the diverse methodology used in ecological and nutrition studies. The importance of more standardized analytical methods for tannin and protein should be emphasized here. More specifically, the posters illustrated the differences in interpretation that could arise from using different tannin assay methods or polymer standards, by using non-plant protein in protein binding studies, through alterations in tannin chemical structure which can occur during extraction from plants, and as a result of the many forms of tannin which occur after ingestion, eg. fibre-bound, protein-bound, unbound, or chemically transformed by the digestive tract. There are postharvest changes to tannin structure which can occur when stringent storage conditions are not used for forage. In addition, tannin structure in plants is dynamic and changes as a function of plant tissue and developmental stage, pointing to the need to optimize sample collection.

Diverse analytical methodology will impact strongly when experimental results are used, for example, to optimize pasture performance with new plant species or to justify the genetic manipulation of tannin in plants. A case in point involves tannin structure, which does not appear to affect the strength of plant-based protein foams (Tanner et al., 1995) or protein precipitation ability (bloat-safe traits), but can affect protein degradability (a protein bypass trait) (McNabb et al.). The method of analysis will affect which chemical properties are considered important, and will have an impact on the strategy used to genetically engineer plants to produce more efficient ruminant feed.

During the discussion, *in vitro* assays with polyethylene glycol to measure tannin-protein binding were strongly criticized as a means to determine optimal levels of tannin for plant breeding. However, *in vivo* studies using different plant species to achieve the range of tannin levels required to optimize digestion was seen as less desirable, since different species may also have somewhat different chemistry. Isogenic plant lines appear to be the most sensible option, and several have been isolated by recurrent selection or genetic engineering.

Two posters described new systems for isolating tannin genes from *Lotus japonicus* transposon-tagged mutants (Gruber et al.) and from *Sn*-transformed *Lotus corniculatus* (Paolocci et al.). With new genes from these and other systems on the horizon (Larkin et al., Gruber et al., unpublished), the bottleneck to tannin manipulation will soon be limited to the transformation capability of useful plant species. However, problems with tannin gene expression were also highlighted in several posters, one in which a transgene was eliminated from sainfoin (Gruber et al.) and one in which native genes were cosuppressed in *Lotus corniculatus* (Paolocci et al.). These are general problems requiring attention in other transgenic plant strategies.

A major question not addressed by presentations but raised in discussion was whether tannin condensation is enzyme mediated. To date, it has been impossible to isolate a condensing-step enzyme. As a result, Larkin et al. are banking on the spontaneous formation of polymers from flavan-3-ols in their genetic engineering strategy with LAR. However, defined tannin structural changes which occur as a function of leaf development (Koupai-Abyazani et al., 1993) and plant mutants with post-LAR lesions (Jende-Strid, 1993) point to some type of protein-mediated polymer formation, regardless of whether it is strictly an enzyme. The possibility of flavan-3-ol binding proteins with chaperone-like stabilization/assembly functions was raised as an alternative to a condensing step enzyme.

The question of the initial subcellular site of tannin accumulation in plants is also unclear. Most evidence favours a theory in which endoplasmic reticular vesicles (ER) accumulate tannins and then fuse with other vesicles or with the central vacuole (Stafford, 1990). This evidence stems from single section electron micrographs showing highly osmiophilic ER vesicles close to or fusing with the central vacuole. In contrast, serial sectioning of very young sainfoin leaf tannin cells points to direct deposition of tannin into the central vacuole in this species (Gruber and Lees, unpublished). Hence, there may be two separate mechanisms of accumulation present in plants.

The undefined nature of tannin polymer biochemistry and the lengthy biochemical pathway has made the introduction of tannins into plants very difficult. This type of genetic manipulation ushers in a new and more complex era in the molecular breeding for forage quality. The good news is that the maize anthocyanin regulatory genes function to upregulate both tannin and anthocyanin levels in plants. However, they only upregulate tannins in tissues which synthesize a low level and they do not work in all plants (Larkin et al.).

The function of tannins in plants was not addressed in any of the presentations. The high content of tannin in tropical plants compared with temperate zone plants points to a major early protective role against tissue damage in environments with high light intensity and UV radiation. This is supported by evidence of the dynamic and asymmetric nature of tannin accumulation in different cell types during leaf development, such that light exposed surfaces have the largest tannin content (Lees et al., 1993). Phenolic resonance structure and multiple hydroxyl groups contribute to these flavonoid antioxidant properties. Metal chelation, a property of tannins which depends upon ortho hydroxyl groups present in procyanidin- and prodelphinidin-containing polymers, may have widened the early ecological niche that tannin-containing plants could occupy. The potential application of this feature to land remediation is underscored by the finding that a greater part of the copper accumulated by *Armeria maritima* ssp. halleri growing on copper-rich soil from a mine dump was sequestered and chelated in the vacuole to tannins (Neumann et al., 1995). The third property, protein binding, is dependent on the polymeric nature of tannins and likely arose quite late in flavonoid evolution. Protein binding, protein starvation and enzyme inactivation are defense mechanisms to guard plants against insects, birds and large animals.

The importance of cell and tissue specificity must be emphasized in relation to the function of tannins to plants. For example, tannins were recently shown to provide resistance to aphids when the stylus penetrated the phloem through a surrounding layer of tannin cells. Mechanisms of adaptation of specific animal and insect species to tannin environments are also important considerations. Both tissue specificity and ease with which insects can adapt will influence decisions on the efficacy and method of breeding for tannins.

The difficulty of getting adequate stable funding for molecular tannin breeding and the high associated research costs was acknowledged generally during the discussion. While interested in the outcome of molecular approaches, seed companies are usually reluctant to fund risky research endeavors without the security of a hybrid seed industry and a high return on their investment. The necessity to use simpler, cheaper options to tannin genetic engineering whenever possible and to maximize the returns on research investments was highlighted. Maximizing returns can be achieved by using tannin genes in as broad a context as possible. Molecular breeders should make strong linkages with groups investigating the biological and chemical properties of tannins as a means of finding alternate uses for genes. For example, tannins are being investigated for their potential as human antiviral, antibiotic, antihistaminic and anticancer agents, as well as for effects on specific enzymes. Despite these cautions, the urgency to develop tannins in specifically adapted forage legumes in environments where other species are poorly adapted, eg. New Zealand white clover pastures, was stressed.

REFERENCES

Jende-Strid, B. 1993. Genetic control of flavonoid biosynthesis in barley. Heriditas **119**: 187-204.

Koupai-Abyazani, M.R., McCallum, J., Muir, A.D., Bohm, B.A., Towers, G.H.N. and Gruber, M.Y. 1993. Developmental changes in the composition of proanthocyanidins from leaves of sainfoin (*Onobrychis viciifolia* Scop.) as determined by HPLC analysis. J. Agric. Food Chem. **41**: 1066-1070.

Morris, P. and Robbins, M.P. 1997. Manipulating condensed tannins in forage legumes. In: McKersie, B.D. and Brown, D.C.W. Biotechnology and the improvement of forage legumes. CAB Int'l., New York, pp 147-171.

Neumann, D., Nieden, U.Z., Lichenberger, O. and Leopold, I. 1995. How does *Armaria maritima* tolerate high heavy metal concentrations? J. Plant Physiol. **146**: 704-717. 4. Stafford, H. 1990. Flavonoid metabolism. CRC Press. Boca Raton, Fl. USA.

Tanner, G.J., Moate, P.J., Cavis, L.H., Laby, R.H., Yuguang, L. And Larkin, P.J. 1995. Proanthocyanidins (condensed tannins) destabilize plant protein foams in a dose-dependent way. Aust. J. Agric. Res. **46**: 1101-1109.