The Hunt for the "Holy Grail": Condensed Tannins in Perennial Forage Legumes

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Abstract
A recent advance using molecular biology has identified a transcription factor or master switch that can 'turn on' the condensed tannin pathway present in white clover, and with the appropriate promoters allows biologically significant levels of condensed tannin expression in leaf tissue. *In vitro* tests have demonstrated that the condensed tannins produced in white clover leaves can bind protein at a pH 6.5, as found in the rumen, and then release them at pH 2.5, the pH in the abomasum, before entering the small intestine for amino acid absorption. Additional tests have demonstrated that these condensed tannins can reduce methane production by up to 25% in the first 6 hours of incubation. The journey to this point and the challenges ahead to deliver white clover cultivars with condensed tannin expression will be described.

Introduction
Forage legumes provide high protein feed for ruminants, either in single species swards such as occurs with lucerne or in mixed species swards containing grasses and clovers. These high legume diets are known to improve animal production (Harris et al. 1997). However, there are downsides when protein is degraded in the rumen resulting in urinary nitrogen losses to the environment (Pacheco and Waghorn 2008), and an increased susceptibility to bloat (Ledgard et al. 1990). It has long been understood that these negative effects can be mitigated by the protein being bound and protected from degradation in the rumen (Waghorn et al. 1987). Condensed tannins (CTs) are known to protect protein resulting in not only reduced nitrogen loss and reduced bloat but also reduced methane production (Pellikaan et al. 2011), reduced internal parasite burden (Villalba et al. 2010), and improved animal productivity (Waghorn 2008)). Unfortunately, the main legumes used in pastoral agriculture in temperate grazed grassland – lucerne, white clover and red clover do not produce sufficient levels of condensed tannin in their leaves to be effective (Woodfield et al. 1998). However, using molecular biology approaches a transcription factor or master switch that can 'turn on' the CT pathway present in white clover and lucerne has been identified. The process of developing this new advancement for white clover and its impact on protein binding and methane production is described.

Methods
Plant materials The plant material used in this study are progeny of genetically engineered white clover containing a transcription factor TaMYB14-1 from *Trifolium arvense* (Hancock et al. 2012, 2014); the production of white clover transgenic plants described in Roldan et al. (2019) and Woodfield et al. (2019). The backcross 2 (BC2) progeny which were pairwise crossed to produce the seed for the field trial described below were grown in a physical containment 2 glasshouse in AgResearch Grasslands Campus in Palmerston North, NZ.

Field trial 2019 – USA
A total of 25 families derived from pair-crosses between 8 genotypes from a BC2 population (as shown in Table 1) where compared with 8 genotypes from white clover cultivar Mainstay, and 2 from *Lotus corniculatus* cultivar Goldie. Each of the 25 families were represented by 8 individuals. These were each characterised as being either homozygous (74 genotypes) or heterozygous (82) for the transcription factor, or not having the transcription factor (nulls) (44). Each plant was transplanted into a 30cm ring set into the ground.

Table 1 – Crossing design for the production of the 25 families derived from pair-crosses between 8 genotypes from a backcross 2 population.

		BC2 Parent 2					
BC2 Parent 1		C	D	E	F	G	H
	A	X	X	X	X	X	X
	B	X	X	X	X	X	X
	C		X	X	X	X	X
	D			X	X	X	X
	E					X	X
	F					X	X

A randomised block design with 2 replicates (4 genotypes per family per replicate) was used with plants arranged in 3 double rows (to remove edge effects); the distance of plants within double row was 30 cm; the

distance of plants between double rows was 75 cm; and the distance between columns was 30 cm. Plants were grown in an insect proof cage at a site in south Wisconsin managed by Great Lakes Agricultural Research Services (GLARS), Delavan, Wisconsin, USA under permit number 19-045-105rm issued by USDA Biotechnology Regulatory Service. Measurements were made of: shoot dry weight (at 5cm from ground), CT levels (measured by extracting CTs from freeze dried leaf material), leaf size, petiole length and stolon diameter.

Quantification ***of soluble and insoluble condensed tannins in white clover leaves***

The total (soluble and insoluble) CTs were determined using freeze-dried leaf samples from the experimental site in GLARS, Wisconsin, USA. The methods for CT extraction and assay are described in detail in Roldan et al. (2019) and Peel and Dixon (2007). Soluble CTs were calculated spectrophotometrically at 640 nm against a standard curve of epigallocatechin (Indofine chemical company, Hillsborough, NJ) after reaction with 0.2% (w/v) 4-Dimethylamino cinnamaldehyde (DMACA) reagent in methanol-3N HCl. Insoluble CTs were calculated by determining absorbance values of the extract at 550 nm, before and after boiling for 1 h. Difference in absorbance values were converted to CT equivalent using a standard curve of purified CTs from white clover leaves. Soluble and insoluble CTS were summed up to obtain the values for total CTs.

Protein binding assay

Purified CTs from the leaves of CTG-T1 progeny (# 1066) was used in the CT- protein binding assay using a protocol modified from Zeller et al. (2015) with bovine serum albumin (BSA) as the test protein. Protein binding experiment was conducted using 50 mM 2-(N-morpholino) ethanesulfonic acid MES buffer, pH 6.5 while for dissociation assay same buffer but lower pH (pH 2.5) was used; experiment was conducted in 3 replicates.

Methane production in vitro

To assess the effect of the condensed tannins in white clover leaves on total gas and methane production, leaf material was used in a small-scale incubation system following the procedure previously described (Muetzel et al. 2014). Three experiments were conducted with 4 substrate treatments (leaves from 2 high CT (HiCT) white clover plants; and wild-type white clover plants flowers and leaves), with 2 replications per treatment using rumen fluid from two different donor cows. There were 2 incubation bottles per replicate, one treated with polyethylene glycol (PEG) 6000 (control) and the other untreated. Each bottle contained 600 ± 15 mg of milled freeze-dried plant materials. Total gas and methane production were recorded after 6h of incubation then percent reduction was determined based on actual production with and without PEG

Statistical analyses

Data were analysed using Minitab statistical software. Grouping information used the Fisher LSD method with 95% confidence limits to determine differences between means.

Results

Condensed tannin expression and plant growth

In the past two generations, of progeny from the original transformants, we have shown evidence of a stable accumulation and enhancement of condensed tannins in white clover leaves (data not shown). This trend continues to this third generation (T3) of progeny, plants which are homozygous to the transcription factor TaMYB14-1 significantly produce elevated soluble and total CTs by up to 2.4- fold and 1.6-fold, respectively, relative to heterozygous individuals (see insert in Figure 1A). In comparison, individuals which did not inherit the TaMYB14-1 transgene produce significantly low (almost nil) CTs in the leaves (Figure 1A).

In terms of plant vigour however, a yield penalty is being observed in individuals with higher CT levels (Figure 1B). There is an overall significant negative correlation ($r = -0.619$) between levels of CTs in leaves and plant growth. Nevertheless, when grouped by zygosity, within each category plant growth was not significantly affected by the CT levels (Figure 1B). Previous observations show that by selective breeding, the yield penalty is being reduced.

Condensed tannin effects on protein binding and methane production

Results of protein binding assay provided evidence that the purified condensed tannins from the leaves of HiCT white clover efficiently bound protein at a pH value which resembles the average ruminal pH. This was noted following incubation of the CT-protein mix in an aqueous MES buffer, pH 6.5, where the CT-protein interaction formed into complex molecules that precipitated into pellet upon centrifugation, leaving

untraceable amount in the supernatant (Figure 2A). Upon resuspension of the pellet in an acidic buffer (pH 2.5), the CT-protein complex dissociated, and the protein was again detected in the supernatant.

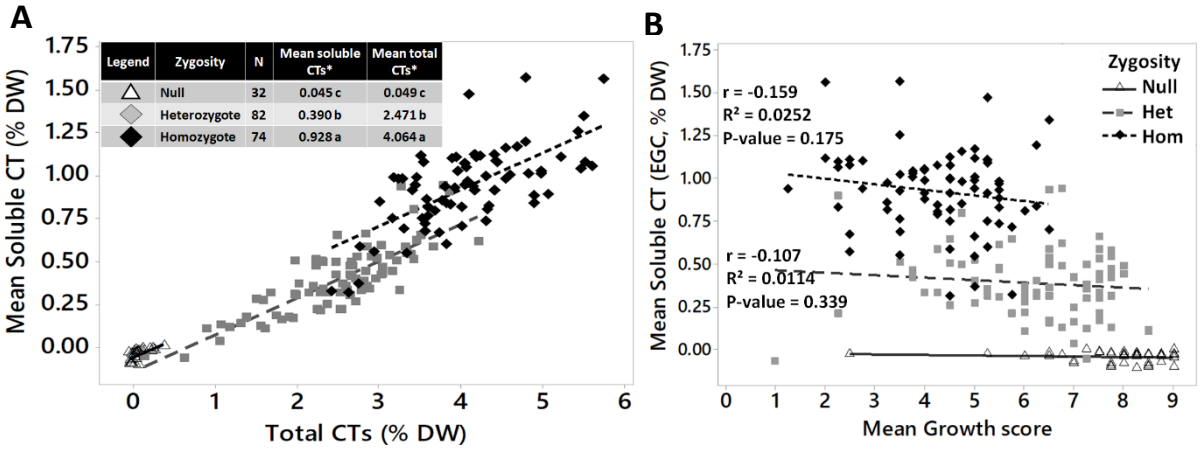

Figure 1. Soluble condensed tannin (CT) in white clover leaves plotted against A) total CTs and B) plant vigour in null, heterozygous and homozygous individuals, as indicated. Each data point in A & B represents the mean of 2 measurements (8 & 12 WAP). In table insert in A, means within each column with different letters are significantly different by LSD (P value < 0.01)

Initial (over first 6 hours) depression of gas and methane production using an automated small-scale incubation system with rumen fluid added averaged about 12.5% and 25% respectively (Table 2) for two HiCT expressing leaf samples. Gas production is a measure of general fermentation (a positive attribute for animal productivity) and a 2-fold reduction in methane over total gases suggests that CTs have a greater impact on methane-producing microbes than the general microbial population.

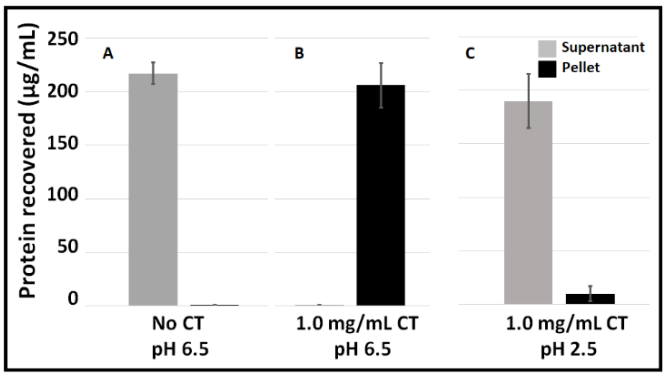

Figure 2. Protein detected in the supernatant or pellet, as indicated, following incubation at pH 6.5 (A-B) and pH 2.5 (C) without (A) and with (B-C) white clover leaf CTs. The error bars indicate error mean square of 3 replicates.

Discussion

For over 70 years plant breeders have aspired to develop white clover capable of expressing biological significant levels of condensed tannins. Forage legumes such as white and red clover improve the nutritional quality of grazed pasture but also cause bloat as these feeds are fermented by rumen microbes. This rapid fermentation also contributes to environmental issues through higher methane emissions and high urinary nitrogen losses. Condensed tannins have been shown to reduce urinary nitrogen and methane production from grazing animals, reduce bloat, reduce internal parasite burden, and to improve animal productivity. This array of environmental, animal health and animal productivity benefits make condensed tannins the holy grail of forage legume breeding. Several forage legumes including birdsfoot trefoil and sainfoin do have good levels of condensed tannins but unfortunately these species fail to persist in intensively grazed pasture systems. Conventional approaches of phenotypic selection and breeding have failed to deliver condensed tannins in legumes that do persist under grazing such as white clover, red clover or lucerne. The transcription factor TaMYB14-1 has provided the opportunity to produce condensed tannins in white clover leaves at levels that will be effective in binding protein in the rumen, resulting in reduced methane production. The challenges to deliver this technology will include increasing yield while maintaining expression of condensed tannin levels, and gaining regulatory approval for its release from containment for general use in grazed pastures.

Table 2. Effects of HiCT clover on total gas and methane production. Substrates were mixed with buffered rumen fluid collected from 6 different donor cows (representing 6 replicates) with and without PEG additive, then incubated in a fully automated small scale incubation system (Muetzel et al 2014). Total gas and methane production was recorded after 6 h incubation and % reduction (- PEG) was calculated relative to control (+ PEG). Means with different letters within each column under % reduction indicate significant difference at P<0.05.

Substrate	Additive	N	Total production (mL/g)		% Reduction	
			Gas	Methane	Gas	Methane
WT WC leaves	- PEG	6	194.07	14.05	0.20c	1.43b
	+ PEG	6	194.53	14.28		
WT WC flowers	- PEG	6	180.60	13.83	20.73a	34.00a
	+ PEG	6	217.87	18.46		
HiCT-1 leaves	- PEG	6	159.62	10.84	16.40ab	26.60a
	+ PEG	6	185.77	13.72		
HiCT-2 leaves	- PEG	6	171.87	11.94	8.73b	23.87a
	+ PEG	6	186.63	14.76		

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