Foliar expression of candidate genes involved in condensed tannin biosynthesis in white clover (*Trifolium repens*)

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Introduction Bloat disease in cattle and sheep is caused by the rapid microbial degradation of protein-rich fodder in the rumen. This leads to the production of protein foams that trap gases, causing bloat, a condition that is often fatal to livestock and costly to farmers. Condensed tannins (CTs) are phenolic polymers produced by the phenylpropanoid pathway of plants (Figure 1). CTs bind to proteins under acidic to neutral conditions, such as those present in the rumen, slowing their breakdown. A diet with a CT content of between 2% and 4% by dry weight, which is provided by some pasture legumes (e.g. *Lotus corniculatus*), protects livestock against bloat and improves the absorption of amino acids from the diet. White clover (*Trifolium repens* L.), a protein rich legume widely used in temperate regions, has virtually no CTs in leaves, although they are present in flowers.

Materials and methods To test whether the foliar expression of clover candidates for genes involved in CT synthesis enhances CT production in leaves of transgenic white clover plants, a targeted EST discovery program was undertaken and cDNAs encoding white clover homologs of the genes involved in the CT biosynthesis pathway were identified.

Results and conclusions The putative protein encoded by *Tr*CHSh (1599 bp), isolated from cDNA from vegetative stolon tips, shares 84.8% amino acid identity with *Arabidopsis* chalcone synthase (TT4), an early enzyme in the phenylpropanoid pathway. Putative proteins encoded by *Tr*BANa (1309 bp) and *Tr*LARb (1551 bp), both isolated from inflorescence cDNA libraries, share 89.3% and 70.3% amino acid identity with *Medicago truncatulata* anthocyanidin reductase and *Desmodium unicinatum* leucoanthocyanidin reductase, respectively. The latter two enzymes catalyse different steps in the CT-specific branch of the phenylpropanoid pathway.

Transgenic white clover plants ectopically expressing chimeric *Tr*CHSh, *Tr*BANa and *Tr*LARb genes – individually and combined (i.e. *Tr*BANa plus *Tr*LARb; *Tr*BANa plus *Tr*LARb plus *Tr*CHSh) - under the control of constitutive and leaf-prevalent promoters were generated using *Agrobacterium*-mediated transformation. The transgenic nature of the plants recovered was demonstrated by quantitative PCR and Southern hybridisation analysis revealing integration of 1 – 5 transgene copies. Selected transformation events showing elevated levels of *Tr*CHSh, *Tr*BANa and *Tr*LARb transcripts were targeted for biochemical and metabolomic assays to identify the level and composition of CTs in leaves. This research forms part of a molecular breeding approach in white clover deploying exclusively white clover genes and promoters for the development of transgenic white clover cultivars with improved nutritional quality and bloat safety.

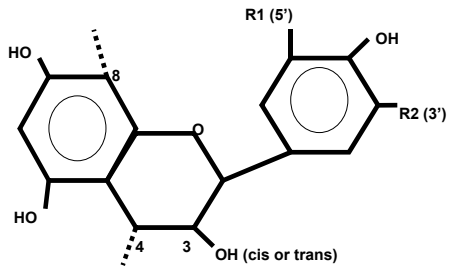

Figure 1 Schematic diagram of a condensed tannin subunit. Subunits are linked by C4 -C8 linkages. Functional groups, shown as R1 and R2, can be either H or OH, depending on precursors entering the CT-specific branch of the phenylpropanoid pathway, where individual enzymes can generate cis or trans conformations at position 3