Assessment of Nutritional Characteristics of Virus-Resistant Transgenic White Clover (*Trifolium repens L.*) Grown under Field and Glasshouse Conditions

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Abstract

White clover (*Trifolium repens* L.) is an important pasture legume in temperate areas throughout the world, providing fodder for grazing animals and improving soil fertility via symbiotic nitrogen fixation. However, the persistence and stress tolerance of white clover is affected by a number of viruses including alfalfa mosaic virus. Transgenic white clover plants with ectopic expression of the alfalfa mosaic virus coat protein were resistant to the virus under field and greenhouse conditions. With all genetic modifications of major consequence there is the possibility of unintended effects on forage quality and natural toxicant levels. In this paper we describe the evaluation of a range of parameters related to the nutritive value of white clover herbage to grazing animals and a suite of naturally occuring secondary metabolites that have the potential to be natural toxicants in transgenic white clover plants and wild-type control plants with a similar genetic background. Samples were collected from plants grown under both field and glasshouse conditions. Several commercial cultivars were included for comparison. Although there was plant-to-plant variation, as expected from an obligate outcrossing species, there were no significant differences in the range of this variation between transgenic and wild type plants. Furthermore, no consistent significant differences were found between groups of transgenic and wild type plants from the same generation, when mean nutritional parameters (crude protein, in vitro dry matter digestibility, neutral detergent fibre and water soluble carbohydrates) and natural toxicants (cyanogenic glucosides, phytoestrogens and saponins) were compared.

Keywords: white clover, transgenic, toxicant, *Alfalfa mosaic virus,* biosafety

Introduction

White clover (*Trifolium repens* L.) is the most important perennial forage legume in permanent pastures in temperate regions of the world. White clover is a favoured pasture legume due to its high nutritive value to grazing animals (Caradus *et al.* 1996; Ayres *et al.* 1998; Fulkerson *et al.* 2007) and high productivity in favourable environments. However, the poor persistence of white clover in sub-optimal conditions (Lane *et al.* 2000) has led to declines in the clover content of pastures and the prioritisation of traits related to persistence in breeding programs (Lane *et al.* 1997).

One major factor that limits the productivity and persistence of white clover is its susceptibility to viruses such as alfalfa mosaic virus (AMV) (Barnett and Gibson 1975; Latch and Skipp 1987; Garrett 1991; Norton and Johnstone 1998). For instance, AMV was the most prevalent virus in a survey of white clover cultivars sown at 17 sites throughout Australia with rapid infection of newly established plots common at some sites (Norton and Johnstone 1998).

Due to the prevalence of the virus and a lack of natural resistance for use in breeding, a program was undertaken to develop white clover with viral coat protein mediated resistance to AMV using transgenesis (Panter *et al.* 2011). Field trials of these plants at two locations have shown the transgenic plants to be uninfected by AMV even when aphids and virus source plants were included in the trials and control plants had very high rates of infection (Panter *et al*. 2011). Recent modelling has shown the potential value of AMV resistant white clover to be in the order of $170/ha/yr on Australian dairy farms (Lewis *et al.* 2013).

Biosafety assessment of a GMO is measured in the context of the risk that the unmodified parental organism poses to the receiving environment. To our knowledge, there are no published biochemical data comparing GM white clover germplasm to similar non-GM germplasm, with a view towards biosafety assessment. Therefore, this study aimed to evaluate herbage samples from the ‘Sustain’-type and ‘Mink’-type AMV-resistant transgenic white clover lines developed through crossing a transgenic event into the white clover cultivars Grasslands Sustain and Mink respectively (Emmerling *et al.* 2004; Panter *et al.* 2011), and genetically similar wild-type control lines in terms of nutritive characteristics and secondary metabolite concentrations. Analysis of the data was expected to reveal whether the GM and non-GM control plants were substantially equivalent, or if the genetic modification caused an unintended negative effect on forage quality and therefore posed a biosafety risk.

Natural toxicants that are known to be present in white clover include isoflavonoids, also known as phytoestrogens, as well as saponins and cyanogenic glucosides. Phytoestrogens are plant compounds that have oestrogenic and/or anti-oestrogenic activity and can cause reproductive anomalies in livestock. The isoflavones daidzein, genistein and their methyl ether derivatives (formononetin and biochanin-A, respectively), and the coumestan coumestrol have been demonstrated to be present in *T. repens* throughout the plant, but at very low concentrations (Nykänen-Kurki *et al.* 1993, Saloniemi *et al.* 1995, Vetter 1995, Mazur *et al.* 1998, Wu *et al.* 2003). Saponins are compounds that can affect digestibility and feed intake in ruminants. There is a great diversity of saponin structures, triterpene saponins being the most commonly found type in cultivated crops and legumes. Saponins have not been extensively studied in white clover despite some reports of their presence (Walter *et al.* 1955, Sakamoto *et al.* 1992). Cyanogenesis is the ability of a plant to release hydrogen cyanide (HCN) from damaged leaf tissue due to the hydrolysis of cyanogenic glucosides by a β-glucosidase. White clover is polymorphic for the cyanogenic trait (Kakes and Hakvoort 1994, Pagano and Rosso 2000) and a range of cyanogenic potential has been measured in commercial white clover cultivars (Wheeler and Vickery 1989; Clark *et al.* 1992; Crush and Caradus 1995). This study aimed to assess substantial equivalence between the ‘Sustain’-type and ‘Mink’-type transgenic white clover lines from the H6 transgenic event (Panter *et al.* 2011) and their wild-type parents, in terms of three classes of natural toxicants, namely, cyanogenic glucosides, phytoestrogens and saponins.

Materials and Methods

Plant material

AMV-resistant transgenic white clover plant material was used for assessment of nutritional composition and cyanogenic glucoside, phytoestrogen and saponin content. This transgenic germplasm was derived from the white clover cultivars ‘Grasslands Sustain’ (Caradus *et al.* 1997) and ‘Mink’ (Jahufer *et al.* 2001) and had been developed using a combination of *Agrobacterium*-mediated genetic transformation and subsequent crossing methods. The transgenic germplasm was shown to have durable resistance to AMV (Panter *et al.* 2011).

‘Sustain’-type T_2 generation AMV-resistant transgenic white clover plants from the H6 transformation event and wild-type ‘Grasslands Sustain’ plants were grown under field conditions at the DIR047/2003 breeding nursery located at the Department of Primary Industries (DPI) in Hamilton, Victoria (OGTR 2003).

Twenty eight ‘Sustain’-type AMV-resistant *Synthetic* generation *0* (*Syn0*) transgenic white clover plants from the H6 transformation event and 11 agronomically elite

‘Grasslands Sustain’ wild-type parents, used as progenitors for the *Syn0* lines, were grown under containment glasshouse and field conditions at the DIR047/2003 breeding nursery. In addition, 11 *Syn0* generation ‘Mink’-type AMV-resistant transgenic white clover plants and their 7 agronomically elite ‘Mink’ wild-type parents were grown under PC2 glasshouse conditions.

Plants from five commercially available white clover cultivars were grown from seed under glasshouse conditions. Ten ‘Aber Herald’, 8 ‘Mink’, 14 ‘Grasslands Demand’, 9 ‘Grasslands Nu Siral’, and 8 ‘Grasslands Sustain’ plants were used as additional wild-type control lines.

Analysis of nutritional composition

Herbage of 21 randomly selected T_2-generation ‘Sustain’-type AMV-resistant transgenic white clover plants and 11 wild-type ‘Grasslands Sustain’ parental lines was sampled from field-grown plants. Herbage from all the *Syn0* generation ‘Sustain’-type and ‘Mink’-type AMV-resistant transgenic white clover plants and their wild-type parental lines was sampled from plants grown under field and glasshouse conditions.

At least 15 g of herbage that included leaves, petioles and stolons was sampled from each plant. Samples were oven-dried in paper bags (Thermotec 2000 series, Contherm, Biolab, New Zealand) at 60ºC for at least 24 h. Dried samples were ground individually to a <1 mm particle size using a Cyclotec 1093 sample mill (Tecator, Hoganas, Sweden).

NIRS analysis

Approximately 2 g of each ground sample were packed into a black aluminium sample cup containing a 30 mm diameter quartz window and a paper–polystyrene pressure pad backing. Samples were irradiated individually with near-infrared monochromatic light and the diffuse reflectance from a spectra between 1100 and 2500 nm with a measuring point every 2 nm was collected using a near-infrared spectrophotometer (NIRSystems 5000, Perstorp Analytical, Silver Spring, MD,USA).

Levels of crude protein (CP), acid detergent fibre (ADF), neutral detergent fibre (NDF), water soluble carbohydrates (WSC), *in vitro* organic matter digestibility (IVOMD), *in vitro* dry matter digestibility (IVDMD), digestible organic matter in the dry matter (DOMD), and estimated metabolisable energy (ESTME) were estimated from the raw NIRS data using the methods described by Smith and Flinn (1991).

Statistical analysis

Data obtained by NIRS for each nutritional component was analysed with GenStat software (GenStat Committee 2003) using a two-tailed t-test to compare the nutritional composition of transgenic lines and corresponding wild-type controls, with a 95% confidence level.

Hydrocyanidic acid (HCN) analysis

Leaves from field and glasshouse-grown *Syn0* generation ‘Sustain’-type and ‘Mink’-type AMV-resistant transgenic white clover plants, their wild-type parents and the five commercial cultivars were collected for measurement of cyanogenic potential. Approximately 1 g of young leaves without disease symptoms or insect damage were collected from each plant, only from the two nodes closest to stolon tips. Leaf samples were stored at 4°C prior to analysis.

The procedure used for quantification of hydrocyanidic acid (HCN) from white clover leaves was described by Ayres *et al*. (2001). This method is a semi-quantitative procedure based on a computational colorimetric analysis of a chemical reaction between an alkaline picric acid solution and HCN released from white clover leaf tissue, observed on paper strips. The results are calibrated against HCN standard solutions. Paper strips were scanned at 250 dots per inch (dpi) (Expression 1600, Seiko-Epson, Nagano, Japan). Red, green and blue (RGB) components were calculated for each strip, using a percentage scale (1-100) to score the colour spectral intensity using MVH Image (Measuring Vegetation Health, PC ver.7). A mean score was calculated for each of the CN standards and a b-spline regression was performed for each colour using S-plus ver. 6 for Windows (Insightful, Seattle, USA) in order to obtain a calibration curve with predicted values for levels of CN from 0.00 up to 70.00 μg CN·100 μL^{-1} for each RGB colour (Electonic Supplementary Figure S1).

The concentration of CN in each sample was calculated by comparing RGB values from the samples to those provided by the CN standard solutions. The mean RGB values of duplicate assays performed for each leaf sample and mass data were used to predict the HCN concentration in each sample.

Phytoestrogen and saponin analysis

Leaves with petioles were collected from plants and freeze-dried. Seven randomly selected T_2-generation 'Sustain'-type AMV-resistant transgenic white clover plants and seven wild-type 'Grasslands Sustain' plants grown under field conditions were sampled in September 2006. In December 2007, leaves was sampled from field and glasshouse-grown *Syn0* generation 'Sustain'-type and 'Mink'-type AMV-resistant transgenic white clover plants, their wild-type parents and the five glasshouse-grown commercial cultivars.

Each freeze-dried sample was ground to a fine powder with a mortar and pestle. Bulked samples were prepared for field-grown or glasshouse-grown *Syn0* generation 'Sustain'-type transgenic lines, *Syn0* generation 'Mink'-type transgenic lines, 'Grasslands Sustain' parental lines, 'Mink' parental lines as well as five glasshouse-grown commercial cultivars. Each of the bulked samples was prepared by accurately adding an equal proportion by mass of each plant sample to a total mass of 1.756±4 g. The 28 *Syn0* generation 'Sustain'-type transgenic plants from glasshouse and field growth conditions were represented by two different bulks, each contaning samples from 14 individual plants. Three technical replicates were prepared per bulked sample.

Freeze-dried, ground samples (90±2 mg) were accurately weighed into 15 mL centrifuge tubes and 5 mL of 80% ($v.v^{-1}$) methanol in water was added. The samples were sonicated for 15 min and centrifuged for 10 min. The supernatant (4 mL) from each mixture was removed and extraction was repeated on the pellet. The supernatants from the two extractions were combined and 500 µL of the extract from each sample was transferred to a high performance liquid chromatography (HPLC) vial for analysis of saponins. The remaining extract (7.5 mL) was dried and reconstituted in 2 mL of 80% ($v.v^{-1}$) water in methanol for the first study of phytoestrogens or 2 mL of 60% ($v.v^{-1}$) water in methanol for the second study.

LC-MS analysis

Samples were analysed by liquid chromatography-mass spectrometry (LC-MS) alongside standard compounds to allow identification and quantification of constituents, particularly the phytoestrogens formononetin, coumestrol, daidzein, genistein and biochanin A.

For LC-MS/MS experiments, an Agilent 1100 series high-performance liquid chromatography (HPLC) system (Agilent, Waldbronn, Germany) equipped with a quaternary gradient pump, column heater, autosampler with sample cooler (maintained at 4ºC), and diode array detector, was coupled to a Thermo Scientific LTQ ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). 10 µL of neat extract was injected onto a 150 x 2.1 mm id., 3µ, Thermo BDS Hypersil C18 column (Thermo Fisher Scientific) maintained at 30^{o}C. The mobile phase consisted of two components: A (water with 0.1% v.v^{-1} formic acid) and B (acetonitrile with 0.1% v.v^{-1} formic acid); and was applied to the column with the following gradient: 0-45 min, 96% A to 16% A (0.2 mL.min^{-1}); 46-51 min, 96% A (0.3 mL.min^{-1}).

LC-MS was run in negative electrospray ionization (ESI) mode. For LC-MS/MS experiments, a data-dependent protocol was used in ESI negative mode with a mass range of 220 to 2000 atomic mass units (amu). Dynamic exclusion was engaged with a 20 sec exclusion time. Data were acquired using automated MS^{n} settings with a target of 30,000, a normalised collision energy of 35 and an ion max time of 200 ms. The heated capillary was maintained at 275 ºC and the sheath, auxiliary and sweep gases were set at 5, 0 and 10 units respectively on the first study and 13, 6 and 8 units respectively for the second. Source voltage was set at 3.4 kV with a capillary voltage of –41 V. Prior to data acquisition, the system was tuned using a 250 µg.mL^{-1} mixed standard (formononetin, coumestrol, daidzein, genistein and biochanin A). The mixture was infused via a syringe pump through a T-piece at a rate of 5 µl.min^{-1} with an HPLC flow rate of 0.2 mL.min^{-1} and a solvent composition of 50% A and 50% B.

Standard curves were prepared by serial dilution of coumestrol, formononetin, genistein, daidzein and biochanin A stock solutions, and analysed at the same time as the plant-derived samples. Data were processed using Xcalibur Quan software with peak analysis by mass and retention time. The results were linear over the range examined (0.09-6.50 μg.mL^{-1} for the first study and 0.03-3.26 μg·mL^{-1} for the second study). Saponin analysis was carried out using the same LC-MS conditions as were used detection of phytoestrogens.

Results

In general plants grown under glasshouse conditions generally had higher mean CP and mean ADF levels and lower mean NDF, WSC and ESTME levels than those grown under field conditions (Figure 1). No significant differences (P>0.05) were found between herbage samples from field-grown T_2 generation 'Sustain'-type transgenic white clover plants and wild-type 'Grasslands Sustain' plants in levels of CP, ADF, NDF, WSC, IVOMD, IVDMD, DOMD, and ESTME (Figure 1). Similarly, no significant differences (P>0.05) were found in mean IVDMD, WSC, IVOMD, DOMD and ESTME levels between the *Syn0* generation 'Sustain'-type and 'Mink'-type transgenic lines and their corresponding wild-type parents grown under glasshouse or field conditions (Figure 1). 'Sustain'-type transgenic lines showed higher mean CP (P<0.05) and lower mean NDF (P>0.05) levels than their respective wild-type parents regardless of the growth conditions. *Syn0* generation 'Mink'-type *Syn0* generation transgenic lines grown under glasshouse conditions had a lower mean ADF content (P<0.05) than their wild-type parents.

A b-spline curve was used to model the effect of change in colour intensity of paper strips containing picric acid (Electronic Supplementary Figure S1). An estimate of HCN content was derived for each of the three curves representing red, green and blue intensity values. Red colour intensity was inversely proportional to HCN concentration and did not change when the HCN concentration exceeded 20 mg HCN.100 mL^{-1} of water. Green and blue colour intensity increased in proportion to HCN concentration when the HCN concentration exceeded 20 mg HCN.100 mL^{-1} of water. Cyanogenic potential was measured in *Syn0* generation 'Sustain'-type transgenic plants and their wild-type parents grown under field (Figure 2a) and glasshouse conditions (Figure 2b), *Syn0* generation 'Mink'-type transgenic plants and their wild-type parents grown under glasshouse conditions (Fig 2c) as well as 5 commercial cultivars grown under glasshouse conditions (Fig 2d). No significant differences (*P*>0.05) were found between mean values of *Syn0* generation 'Sustain'-type or 'Mink'-type transgenic plants and their wild-type parents grown under either field or glasshouse conditions (Fig 2d). Although no significant differences were found in mean cyanogenic potential values between *Syn0* generation transgenic plants and their wild-type parents, (Fig 2d**Error! Reference source not found.**), a high level of variation was found between individual genotypes within the lines (Figure 2a-d). However, all *Syn0* generation 'Sustain'-type and 'Mink'-type transgenic plants and their wild-type parents grown under field and PC2 glasshouse conditions showed cyanogenic potential values of less than 400 μg $HCN \cdot g^{-1}$ DM (Figure 2a-d). Some genotypes from the Aber Herald, NuSiral, Demand and Mink commercial cultivars had cyanogenic potential values that were higher than 500 μg $HCN \cdot g^{-1}$ DM (Figure 2d-e). All lines tested in this study represented acyanogenic genotypes.

Phytoestrogens

Only two known phytoestrogens were detected in this study, namely, formononetin and genistein. No significant differences (P<0.05) were found between seven T2 generation 'Sustain-type' transgenic lines and seven wild-type 'Grasslands Sustain' plants grown in the field in terms of mean formononetin (125.9 and 110.4 $\mu g \cdot g^{-1}$ DM, respectively) and genistein (24.3 and 23.6 $\mu g \cdot g^{-1}$ DM, respectively) concentrations, although considerable variation was seen between individual plants (Figure 3).

Likewise, no significant differences (P<0.05) were found between bulked *Syn0* generation 'Sustain'-type transgenic lines and their wildtype parents grown in the field, or between *Syn0* generation 'Mink'-type transgenic lines and their wildtype parents grown in the glasshouse, in terms of formononetin levels (Figure 4). Wild-type 'Grasslands Sustain' parental lines had grown higher formononetin concentration than the corresponding *Syn0* generation transgenic plants ($P>0.05$) when grown under glasshouse conditions. Plants with a 'Mink' genetic background generally had higher phytoestrogen concentrations than those with other genetic backgrounds.

Saponins

Saponins that were previously reported as being present in white clover, namely, soyasaponin I, astragaloside VIII, and the 22-*O*-glucoside and 22-*O*-diglucoside forms of soyasaponin I, were detected at low levels or not at all (Electronic Supplementary Material Table S1). However, five major metabolites, likely to be saponins, but not unambiguously identified at the molecular level, were present at varying levels in all the white clover samples analysed in this study (Electronic Supplementary Material Table S2). The molecular weights of these metabolites were 922, 958, 988,1084 and 1114. Analysis of these components was not quantitative as commercial standards were not available. Therefore, results were expressed as relative amounts based on signal intensities. Peak areas for specific saponins were measured (Fig 5). These peak areas were averaged across the technical replicates and are expressed as values relative to those in one sample (Mink, wild type, glasshouse, data not shown).

The metabolites predicted to be saponins were present in high concentration relative to the phytoestrogens. The high concentration of saponins had a negative effect on linearity and reproducibility of results for the mass spectral analysis (average error approximately 10%). The relative concentrations of the different saponins within each sample were examined for the bulked samples containing herbage from individual T_2 generation transgenic and wild-type white clover plants (Figure 5). No significant differences were found between transgenic and wild-type plants within cultivars in terms of mean saponin concentration (P>0.05).

Discussion

No significant differences were found between the *Syn0* generation transgenic lines containing the *AMV CP* transgene and their wild-type parents in most nutritional parameters estimated by NIRS analysis, namely, WSC, *in* IVDMD, IVOMD, DOMD and ESTME (Figure 1). Some differences were found between the *Syn0* generation transgenic lines and their wild-type parents in CP, NDF and ADF. *Syn0* generation ‘Sustain’-type transgenic white clover plants grown under both field and glasshouse conditions, had significantly higher CP content then their wild-type progenitors. The *Syn0* generation ‘Sustain’-type transgenic population showed lower NDF content than their wild-type parents when grown both under glasshouse and field conditions, and the *Syn0* generation ‘Mink’-type transgenic lines had lower ADF values than their wild-type parents. It was interesting that differences in fibre content were not reflected in significant differences between the transgenic and wild-type plants in digestibility traits (IVDMD, DOMD and IVOMD).

CP is directly related to the content of protein and non-protein nitrogen in the plant, where protein accounts for 75 to 85% of total nitrogen measured (Lyttleton 1973). CP is estimated by multiplying the nitrogen content of dry material with the factor 6.25 (CP=N·6.25) (Lyttleton 1973). Concentration of crude protein in white clover is around 25% of dry matter content (Stypiñski 1993, Harris *et al.* 1998). Fibre is a term used to describe the insoluble fraction of the plant and approximates the cell wall. In forages, fibre refers to the complex of nutrients that are resistant to digestion and can be only partially degraded by ruminants (Moore and Hatfield 1994). ADF measures the least digestible portion of the fibre (cellulose and lignin) in feed and is used to predict energy content and digestibility. NDF measures the total fibre or bulk component (hemicellulose, cellulose and lignin). NDF is used to predict feed intake; an increase in feed NDF results in decreased feed intake. The lowest NDF content is found in leaves and petioles of white clover (Søegaard 1993). CP and fibre content are related to nutritional quality, since protein is important for animal growth and development and fibre has an influence in intake potential, digestibility, gut fill and passage rate (Nelson and Moser 1994, West 1998).

Higher CP content and lower NDF and ADF content in the transgenic lines, in comparison to their wild-type parents, could have been caused by a difference in the ratio between leaves and other vegetative tissues in the samples. Stolons and petioles have a higher concentration of lignified cells and therefore a higher fibre content than leaves, which contain more soluble proteins and many mesophyll cells without secondary cell walls (Hoffman *et al.* 1993, Fulkerson *et al.* 1998).

Leaf size was among the traits used for selection of *Syn0* parents from the T_2 transgenic populations, suggesting that differences in CP, ADF and NDF could reflect the generational advance of agronomically-elite germplasm during the breeding process. This is supported from results obtained from the T_2 generation 'Sustain'-type transgenic population, where no significant differences were found between randomly transgenic selected plants and the 'Sustain'-type wild-type parents for any of the nutritional components tested.

Syn0 generation 'Sustain'-type transgenic lines and their wild-type parents showed a lower CP content when grown in the field (approx. 25 % DM) than the same lines and *Syn0* generation 'Mink'-type transgenic lines and their wild-type parents grown in the glasshouse (approx 32% DM). The exposure of plants to different environmental conditions in the field, as compared to the glasshouse, could account for this observation, since CP content is known to positively correlated with the presence of immature, actively-growing plant tissues (Nelson and Moser, 1994), which could have been promoted under glasshouse conditions, but restricted in the field by abiotic stress. Plants grown in the glasshouse experienced consistent watering and controlled temperatures, but plants in the field were subject to temperature changes each day and seasonal heat and water stress during summer.

WSC content was found to be higher in field-grown plants than plants grown under glasshouse conditions (Figure 1). Variation in WSC and CP tend to be negatively correlated. This trend was seen in this study, where field-grown plants with higher WSC levels had a lower CP content.

Cyanogenic glucosides are the most studied natural toxicants in white clover. Several studies have examined the cyanogenic potential of different white clover cultivars, showing the presence of cyanogenic glucosides in all tested cultivars. The white clover cultivar ‘Grasslands Sustain’ is considered to have a moderate to high HCN content with the frequency of cyanogenic plants ranging from 40 to 85% (Caradus *et al.* 1997, Caradus and Woodfield 1997). This is consistent with the results obtained in this study, where a high proportion (85-90%) of the ‘Sustain’-type transgenic, ‘Grasslands Sustain’ wild-type parental lines and ‘Grasslands Sustain’ genotypes from a commercial seed batch showed cyanogenic potential. In studies where the alkaline picrate assay has been used, concentrations for moderate to high cyanogenic cultivars such as ‘Haifa’, ‘Grassland Kopu’, ‘Grassland Huia’, ‘Grassland Pitau’, and ‘Irrigation’ have been similar to or higher than the ones found in this study. For example, Crush and Caradus (1995) analysed HCN concentration in different white clover cultivars and obtained a mean concentration of 849 μg HCN·g^{-1} DM for the cultivar ‘Grasslands Sustain’. Concentrations of under 400 μg HCN·g^{-1} DM were found in the ‘Sustain’-type transgenic, ‘Grasslands Sustain’ wild-type parental lines and commercial ‘Grasslands Sustain’ genotypes tested in this study. This was consistent with expected results from moderately cyanogenic cultivars such as ‘Mink’ and ‘Grasslands Sustain’

Several studies in white clover have demonstrated that cyanogenic potential shows high variability both between and within cultivars (Daday 1955, Hill *et al.* 1995, Paplauskiene and Sprainaitis 2003).

The results obtained from the commercial cultivars tested in this study support these previous studies. For example plants from ‘Grassland Demand’ and ‘Aber Herald’ cultivars were generally found to have higher cyanogenic potential than plants ‘Grasslands Sustain’ and ‘Mink’ plants, although a high level of genotypic variability was also seen, with some acyanogenic genotypes with values of less than 50 μg $HCN \cdot g^{-1}$ DM.

Some of the variation between genotypes can be explained by incomplete dominance at the *Ac* and *Li* loci, since white clover genotypes that are heterozygous at either locus produce an intermediate amount of the respective cyanogenic glucoside or β-glucosidase (Hughes and Stirling 1982). However, Hayden and Parker (2002) suggested that the variability of the expression of cyanogenesis within genotypes may be influenced by some additional abiotic factors, since cyanogenic potential has been shown to be higher when conditions favour white clover growth (Paplauskiene and Sprainaitis 2003). In this study the mean HCN concentration of glasshouse-grown 'Sustain'-type material was slightly higher (212 μg $HCN \cdot g^{-1}$ DM) than field-grown material (184 μg $HCN \cdot g^{-1}$ DM). However, this difference was not significant ($P>0.05$).

The picric acid test has been one of the most widely used methods for measuring cyanogenic potential of white clover because of its simplicity (Vickery *et al.* 1987, Wheeler and Vickery 1989, Clark *et al.* 1992, Hill *et al.* 1995, Richards and Fletcher 2002). Other studies have quantified cyanogenic glucosides by other procedures such as mercurymetry (Paplauskiene and Sprainaitis 2003), HPLC (Stochmal and Oleszek 1997) or titration with alkaline silver nitrate (Crush and Caradus 1995). Since some clover genotypes are deficient in β-glucosidase (Corkill 1940), some studies have added an exogenous enzyme to promote the release of HCN. However, Crush and Caradus (1995) mentioned that adding an exogenous enzyme might overestimate cyanogenic potential *in vivo,* although it has been shown that the addition of exogenous β-glucosidase had no significant effect on estimates of cyanogenic potential in white clover (Fraser and Nowak 1988, Ayres *et al.* 2001). Furthermore, the amount of β-glucosidase present in clover is not necessarily a critical factor in assessing the potential impact of cyanogenicity on ruminant animals because the rumen microflora of a sheep is capable of rapidly hydrolysing the glucoside (Crush and Caradus 1995).

This study aimed to quantify cyanogenic potential in transgenic and wild-type lines with comparable genetic backgrounds under the same environmental conditions. ‘Sustain’-type and ‘Mink’-type transgenic white clover plants were shown to be substantially equivalent, in terms of cyanogenic potential, to comparable wild-type parental lines when grown under the same conditions. Commercial cultivars that are commonly used in Australia were also analysed following the same procedure. The results showed that the cyanogenic potential of the AMV-resistant ‘Sustain’-type and ‘Mink’-type transgenic lines was within the normal range of Australian commercial white clover cultivars.

Phytoestrogens are known to be present at very low concentrations in white clover, when compared to other agronomically-important *Trifolium* species, such as red clover (*T. pratense*) and subterranean clover (*T. subterraneum*), where concentrations of above 1% of DM have been observed and are associated with reproductive problems in livestock.

The phytoestrogen concentrations found in this study were very low, with concentrations of less than 0.03% DM. Only two isoflavones detected, namely, formononetin and genistein. These results are consistent with other studies in white clover, where isoflavone content was also found at concentrations of less than 0.06% DM, and only formononetin and genistein were identified in white clover leaves (Nykänen-Kurki *et al.* 1993, Saloniemi *et al.* 1995, Wu *et al.* 2003). Coumestrol, which can be induced by foliar diseases, was not detected here although it has been previously been shown to be present in white clover at concentrations of below 9 ppm DM (Francis *et al.* 1967, Adams 1995, Nykänen-

Kurki *et al.* 1993, Saloniemi *et al.* 1995). Wu *et al.* (2003) measured very low concentrations of biochanin-A and daidzein in white clover flowers, but they were also not detected in this study.

Formononetin has previously been identified as the main isoflavone present in some *Trifolium* species, including white clover (Saloniemi *et al.* 1995, Booth *et al.* 2006). It corresponded to 82-94% of the total isoflavone content in all lines tested in this study, which is consistent with previous studies, where it accounted for 90% of total isoflavone content (Nykänen-Kurki *et al.* 1993, Saloniemi *et al.* 1995).

Formononetin is oestrogenically more active than genistein and biochanin-A in ruminants, since the latter two compounds are degraded into *p*-ethylphenol and phenolic acid, which are oestrogenically inactive (Shutt 1976). In contrast, formononetin is degraded to daidzein and then metabolised to equol (Figure 1.6), which is a weak oestrogenic compound (Shutt 1976, Lundh 1995). Several studies have shown that formononetin was responsible for 'clover disease' in Western Australia in the 1940s (Bennetts *et al.* 1946), when ewes showed infertility and reproductive anomalies, and lactation appeared in non-pregnant ewes and in castrated male sheep when grazing subterranean clover. Bennet *et al.* (1976) observed that clovers that have been consistently oestrogenically active in ewes have formononetin values in excess of 0.5% DM. The highly oestrogenic red and subterranean clovers have a formononetin concentration range of between 0.5-1.5% DM (Francis *et al.* 1967, Bennett *et al.* 1976, Vetter 1995, Sivesind and Seguin 2005, Booth *et al.* 2006). However, no concentration higher than 0.08% DM has been detected in white clover (Bennett *et al.* 1976, Nykänen-Kurki *et al.* 1993, Saloniemi *et al.* 1995, Vetter 1995). Formononetin concentrations measured in this and most other white clover studies are well below this value and oestrogenic problems associated with white clover are rare (Adams 1995).

Isoflavone concentrations of between 30-278 μg·g^{-1} DM were seen in analyses of the seven T_2 generation 'Sustain'-type transgenic white clover plants and seven 'Grasslands Sustain' control plants. In contrast, isoflavone concentrations of between 8-50 μg·g^{-1} (less than 0.005% in DM) were measured in the white clover bulks of *Syn 0* generation 'Sustain'-type and 'Mink'-type trangenic plants, their wild-type parents and 5 commercial cultivars. Previous studies have also found a high level of variability in isoflavone concentrations in herbage, namely, leaves (4 to 900 μg·g^{-1} DM), stems (4 to 354 μg·g^{-1} DM), flowers (1 to 213 μg·g^{-1} DM) and mixed stems and leaves (88 to 500 μg·g^{-1} DM) (Carlsen and Fomsgaard 2008). Factors including seasonal conditions, environmental factors, growth stage can affect phytoestrogen concentration in legumes (Saba *et al.* 1974). Since the T_2 generation plants and their controls were collected at a different time of the year from the bulked samples of the *Syn0* generation plants and their controls different phytoestrogen concentrations could have reflected different phenological stages of development. This explanation is supported by Bennett *et al*. (1976), who observed seasonal variation in phytoestrogen concentrations in a white clover cultivar sward (0.08% DM during winter and less than 0.01% DM during spring).

Most of the phytoestrogen variation in subterranean clover can be explained by genotypic variation (Adams 1989). The high genetic variability of white clover due to obligate outcrossing is well known. The variation in phytoestrogen concentrations seen between different white clover genotypes in this study (Figure 5), could have biased the phytoestrogen concentration measured in the bulked samples (Figure 5). However, the mean values from the T_2 generation 'Sustain'-type transgenic and control lines were not significantly different. No significant difference (P>0.05) was found in formononetin levels between bulked samples of *Syn0* generation 'Sustain'-type and 'Mink'-type transgenic plants and their wild-type parents, when grown under the same conditions. However, glasshouse-grown plants had consistently higher formononetin levels than those grown in the field (Figure 5), possibly reflecting glasshouse conditions that promote vegetative growth, which is associated with high isoflavone levels (Adams 1995, Vetter 1995, Seguin *et al.* 2004, Sivesind and Seguin 2005). Overall, the phytoestrogen concentrations measured in this study were similar to or lower than those reported elsewhere, and the concentrations of formononetin and genistein in *Syn0* generation ‘Sustain’-type transgenic lines and the wild-type parental lines grown were lower than those of the tested commercial lines.

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Many different saponins have been identified in white clover plants. Walter *et al.* (1955) separated soyasapogenol B and soyasapogenol A from ‘Ladino’ clover extracts and soyasaponin I, soyasaponin II azukisaponin II and, cloversaponins I to V were subsequently discovered (Sakamoto *et al.* 1992). Oleszek and Stochmal (2002) identified soyasaponin I and astragaloside VIII in white clover seeds. The diversity of saponins is explained by variability of the aglycone structure, which may have a number of different functional groups (-OH, -COOH, -CH_3) and sugar chains that may vary in composition, number, branching patterns and type of substitution (Francis *et al.* 2002).

Five potential saponins were identified in this study with molecular weights (922, 958, 988, 1084 and 1114), that are different to those of the previously reported saponins of white clover (Table S1). However, saponins have been reported in other legumes (Ahmad and Basha 2000) that have the same molecular weights as those observed in our study and are structurally similar to saponins previously identified in white clover (Table 2). Four soyasaponins have molecular weights of 922, 958, 958 and 1084. All of these saponins contain the soyasapogenol *B* 3-*O* aglycone and a β-*D*-glucuronopyranoside sugar moiety, as seen in all previously reported saponins of white clover. According to Suzuki *et al.*(2002) soyasapogenol B (soyasaponin I) and medicagenic acid are both derived from β-amyrin via the same biochemical pathway. This common pathway could lead to the presence of saponins with the medicagenic acid aglycone (i.e. medicagosaponins 9 and A) in white clover, in the same way that soyasapogenols B and E are present in *Medicago. sativa*, *M. truncatula* and *T. repens*. This could explain the presence of a possible medicagosaponin with a molecular weight of 988 as shown in Table S2.

However, the saponins found in this study could be different to the ones shown in Table S2 or any others reported to date. Studies have demonstrated that other legumes such as the model legume *M. truncatula* contain a complex mixture of saponins with at least 5 different triterpene aglycones: soyasapogenol B, soyasapogenol E, medicagenic acid, hederagenin and bayogenin (Suzuki *et al.* 2002, Kapusta *et al.* 2005). Similar observations have been made in lucerne, where more than 20 saponins have been found (Oleszek *et al.* 1990, Nowacka and Oleszek 1994, Oleszek 1998, Bialy *et al.* 1999, Agrell *et al.* 2003). Saponins in white clover have not been widely studied (Carlsen and Fomsgaard 2008) and as is the case for *M. truncatula*, white clover could contain a diversity of saponins that have not yet been reported.

In this study, clover plants growing in the field showed a higher concentration of saponins than glasshouse-grown plants (Figure 6), which was likely to have been due to the different growth conditions. Pecetti *et al.* (2006) studied the influence of environmental conditions on saponin concentrations in lucerne. They found higher saponin concentrations in mid-summer when temperatures were high, and suggested a positive correlation between saponin concentration and maximum temperature.

The relative levels of saponins found in the ‘Sustain’-type and ‘Mink’-type transgenic white clover lines were similar or lower to those found in the wild-type control lines. Although specific saponins could not be identified with a high degree of certainty and absolute quantification was not done, the results suggested that the GM white clover lines did not have higher levels of the potential saponins than their wild-type controls.

Different cultivars and genotypes of white clover are known to show a lot of variation in natural toxicant levels. This variation is due to the high level of genetic diversity associated with obligate outcrossing and the influence of environmental factors (Carlsen and Fomsgaard 2008) including weather conditions, nutrient and disease status and water stress. In this biosafety study, this variation was addressed by the use of non-transgenic control plants with a similar genetic background to the GM germplasm and the comparison of GM and control plants growing under the same environmental conditions, either in the field or in a glasshouse. Analyses of nutritional parameters, cyanogenic potential and phytoestrogen and saponin levels suggested that the AMV-resistant GM germplasm was substantially equivalent to similar non-GM germplasm in terms of nutritional quality and natural toxicant levels. Where variation was seen, factors other than the transgene were likely to have had an important role, including the selection of germplasm on the basis of agronomic traits during the breeding process and differences in environmental conditions.

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References

Adams NR (1995) Detection of the effects of phytoestrogens on sheep and cattle. *Journal of Animal Science* 73:1509-1515.

Agrell J, Oleszek W, Stochmal A, Olsen M, Anderson P (2003) Herbivore-induced responses in alfalfa (*Medicago sativa*). *Journal of Chemical Ecology* 29:303-320

Ahmad VU, Basha A (2000) The spectroscopic data of saponins. The triterpenoid glycosides. Vol. I-III, Boca Raton, Fl. USA, CRC Press.

Ayres JF, Murison RD, Turner AD, Harden S (2001) A rapid semi-quantitative procedure for screening hydrocyanide acid in white clover. *Australian Journal of Experimental Agriculture* 41:515-521.

Ayres JF, Nandra KS, TurnerAD (1998) A study of the nutritive value of white clover (*Trifolium repens* L.) in relation to different stages of phenological maturity in the primary growth phase in spring. *Grass and Forage Science* 53:250-259.

Barnett OW, Gibson PB (1975) Identification and prevalence of white clover viruses and the resistance of *Trifolium* species to these viruses. *Crop Science* 15: 32-37.

Bennett D, Morley FHW, Axelsen A (1976) Bioassay responses of ewes to legume swards. II. Uterine weight results from swards. *Australian Journal of Agricultural Research* 18495-504.

Bennetts HW, Underwood EJ, Shier FL (1946) A specific breeding problem of sheep on subterranean clover pastures in western Australia. *Australian Veterinary Journal* 22:2-12.

Bialy Z, Jurzysta M, Oleszek W, Piacente S, Pizza C (1999) Saponins in alfalfa (*Medicago sativa* L.) root and their structural elucidation. *Journal of Agricultural and Food Chemistry* 47: 3185-3192.

Booth N.L, Overk C.R, Yao P, Totura S, Deng Y, Hedayat AS, Bolton JL, Pauli GF, Farnsworth NR (2006). Seasonal Variation of Red Clover (*Trifolium pratense* L., Fabaceae) Isoflavones and Estrogenic Activity. *Journal of Agricultural and Food Chemistry* 54: 1277-1282.

Caradus J.R, Clifford PTP, Chapman DF, Cousins GR, Williams WM, Miller JE (1997) Breeding and description of 'Grasslands Sustain', a medium-large-leaved white clover (*Trifolium repens* L.) cultivar. *New Zealand Journal of Agricultural Research* 40:1-7.

Caradus JR, Woodfield D, Stewart AV (1996) Overview and vision for white clover. *In: White clover: New Zealand's competitive edge. Proceedings of a joint symposium between Agronomy Society of New Zealand and New Zealand Grassland Association held at Lincoln University, New Zealand*, D. R. Woodfield (*Ed)*. Agronomy Society of New Zealand/ New Zealand Grassland Association; Agronomy Society of New Zealand Special Publication No. 11/ Grassland Reasearch and Practice Series No. 6. Lincoln University, New Zealand, 21-22 November 1995. pp. 1-6.

Caradus JR, Woodfield DR (1997) World checklist of white clover varieties II. *New Zealand Journal of Agricultural Research* 40:115-206.

Carlsen S, Fomsgaard I (2008) Biologically active secondary metabolites in white clover (*Trifolium repens* L.) – a review focusing on contents in the plant, plant–pest interactions and transformation. *Chemoecology* 18: 129-170.

Clark SG, Taylor J, Smith KF (1992) Seed and herbage production and cyanogenic potential of white clover under irrigation at Neuarpurr, Victoria. *In: Proceedings 6th Australian Society of Agronomy Conference*, K. J. Hutchinson and P. J. Vickery *(Eds)*. The Australian Society of Agronomy. Parkville, Victoria. Australia. pp. 288-291.

Corkill L (1940) Cyanogenesis in white clover (*Trifolium repens* L.). I. Cyanogenesis in single plants. *New Zealand Journal of Science and Technology* 23:178B-193B.

Crush J.R, Caradus JR (1995) Cyanogenesis potential and iodine concentration in white clover (*Trifolium repens* L.) cultivars. *New Zealand Journal of Agricultural Research* 38: 309-316.

Daday H (1955) Cyanogenesis strains of white clover (*Trifolium repens* L.). *Grass and Forage Science* 10: 266-274.

Emmerling M, Chu P, Smith K, Kalla R, Spangenberg G (2004) Field evaluation of transgenic white clover with AMV immunity and development of elite transgenic germplasm. *In: Molecular Breeding of Forage and Turf*, A. Hopkins, Y. Wang, R. Mian, M. Sledge and E. Barker *(Eds)*. Kluwer Academic Press, pp. 359-366.

Francis CM, Millington AJ, Bailey ET (1967) The distribution of oestrogenic isoflavones in the genus *Trifolium*. *Australian Journal of Agricultural Research* 18: 47-54.

Francis G, Kerem Z, Makkar HPS, Becker K (2002) The biological action of saponins in animal systems: a review. *British Journal of Nutrition* 88: 587-605.

Fraser J, Nowak J (1988) Studies on variability in white clover: Growth habits and cyanogenic glucosides. *Annals of Botany* 61: 311-318.

Fulkerson W.J, Neal JS, Clark CF, Horadagoda A, Nandra KS, Barchia I (2007) Nutritive value of forage species grown in the warm temperate climate of Australia for dairy cows: Grasses and legumes. *Livestock Science* 107: 253-264.

Fulkerson WJ, Slack K, Hennessy DW, Hough GM (1998) Nutrients in ryegrass (*Lolium* spp.), white clover (*Trifolium repens*) and kikuyu (*Pennisetum clandestinum*) pastures in relation to season and stage of regrowth in a subtropical environment. *Australian Journal of Experimental Agriculture* 38: 227-240.

Garrett RG (1991) Impact of viruses on pasture legume productivity. *In: Proceedings of White Clover Conference*, N. McFarlane (*Ed)*. Department of Agriculture Victoria, Conference proceedings No. 45. Pastoral Research Institute, Hamilton, Victoria, 15-16 October 1991. pp. 50-57.

Harris S, Auldist MJ, Clark DA, Jansen EBL(1998) Effects of white clover content in the diet on herbage intake, milk production and milk composition of New Zealand dairy cows housed indoors. *Journal of Dairy Research* 65:389-400.

Hayden KJ, Parker IM (2002) Plasticity in cyanogenesis of *Trifolium repens* L.: inducibility, fitness costs and variable expression. *Evolutionary Ecology Research* 4:155-168.

Hill MJ, Hockney MJ, Mulcahy CA, Rapp G (1995) The effect of hydrogen cyanide potential (HCNp) and sward morphology on the relative acceptability of sheep of white clover and Caucasian clover herbage. *Grass and Forage Science* 50:1-9.

Hoffman PC, Sievert SJ, Shaver RD, Welch DA, Combs DK (1993) In situ dry matter, protein, and fiber degradation of perennial forages. *Journal of Dairy Science* 76:2632-2643.

Hughes MA, Stirling JD (1982) A study of dominance at the locus controlling cyanoglucoside production in *Trifolium repens* L. *Euphytica* 31:477-483.

Jahufer M ZZ Venkatanagappa S and Lee CK (2001). White clover (Trifolium repens L.) cultivar Mink. *Australian Journal of Experimental Agriculture*, 41:705-706.

Kakes P, Hakvoort HWJ (1994) On the origin of the cyanogenic polymorphism in *Trifolium repens* L. *Journal of Evolutionary Biology* 7:201-215.

Kapusta I, Janda B, Stochmal A, Oleszek W (2005) Determination of saponins in aerial parts of barrel medic (*Medicago truncatula*) by liquid chromatography-electrospray ionization/mass spectrometry. *Journal of Agricultural and Food Chemistry* 53: 7654-7660.

Lane LA, Ayres JF, Lovett JV (1997) A review of the introduction and use of white clover (*Trifolium repens* L.) in Australia - significance for breeding objectives. *Australian Journal of Experimental Agriculture* 37: 831-839.

Lane LA, Ayres JF, Lovett JV (2000) The pastoral significance, adaptive characteristics, and grazing value of white clover (*Trifolium repens* L.) in dryland environments in Australia: a review. *Australian Journal of Experimental Agriculture* 40: 1033-1046.

Latch G, Skipp R (1987) Diseases. *In: White Clover*, M. J. Baker and W. M. Williams *(Eds)*. C. A. B. International, Wallingford, UK. pp. 421-460.

Lewis CD, Malcolm B, Jacobs JL, Spangenberg G, Smith KF (2013) A method to estimate the potential net benefits of trait improvements in pasture species: Transgenic white clover for livestock grazing systems. *AFBM Journal* 10: 30 – 45.

Lundh T (1995) Metabolism of estrogenic isoflavones in domestic animals. *Proceedings of the Society for Experimental Biology and Medicine* 208: 33-39.

Lyttleton JW (1973) Proteins and nucleic acids. *In: Chemistry and biochemistry of herbage*, GD Butler and RW Bailey *(Eds)*. Academic Press, London, UK. pp. 63-103.

Mazur WM, Duke JA, Wähälä K, Rasku S, Adlercreutz H (1998) Isoflavonoids and Lignans in Legumes: Nutritional and Health Aspects in Humans. *Journal of Nutritional Biochemistry* 9:193-200.

Moore KJ, Hatfield RD (1994) Carbohydrates and forage quality. *In: Forage quality, evaluation and utilization*, GC Fahey Jr, M Collins, DR Mertens and LE Moser *(Eds)*. American Society of Agronomy Inc; Crop Science Society of America Inc; Soil Science Society of America Inc, Madison, Wisconsin, USA. pp. 229-280.

Nelson CJ, Moser LE (1994) Plant factors affecting forage quality. *In: Forage quality, evaluation and utilization*, G. C. Fahey Jr., M. Collins, D. R. Mertens and L. E.

Moser *(Eds)*. American Society of Agronomy Inc; Crop Science Society of America Inc; Soil Science Society of America Inc, Madison, Wisconsin, USA. pp. 115-154.

Norton MR, Johnstone GR (1998) Occurrence of alfalfa mosaic, clover yellow vein, subterranean clover red leaf, and white clover mosaic viruses in white clover throughout Australia. *Australian Journal of Agricultural Research* 49:723-728.

Nowacka J, Oleszek WA (1994) Determination of alfalfa (*Medicago sativa*) saponins by High-Performance Liquid Chromatography. *Journal of Agricultural and Food Chemistry* 42:727-730.

Nykänen-Kurki P, Saloniemi H, Kallela K, Saastamoinen I (1993) Phyto-oestrogen content and oestrogenic effect of white clover. *In: White clover in Europe: State of the art*, J Frame (*Ed)*. Food and Agriculture Organisation (FAO). Aarhus, Denmark, 28-31 August 1995.

OGTR (2003) Risk assessment and risk management plan (RARMP) for field evaluation of genetically modified white clover resistant to infection by alfalfa mosaic virus. Office of Gene Technology Regulator (OGTR). Canberra, Australia.

Oleszek W, Price KR, Colquhoun IJ, Jurzysta M, Ploszynski M, Fenwick GR (1990). Isolation and identification of alfalfa (*Medicago sativa* L.) root saponins: their activity in relation to a fungal bioassay. *Journal of Agricultural and Food Chemistry* 38: 1810-1817.

Oleszek WA (1998) Composition and quantitation of saponins in alfalfa (*Medicago sativa* L.) seedlings. *Journal of Agricultural and Food Chemistry* 46:960-962.

Oleszek WA, Stochmal A (2002) Triterpene saponins and flavonoids in the seeds of *Trifolium species*. *Phytochemistry* 62:165-170.

Pagano EM, Rosso BS (2000) Caracterización por cianogénesis de una colección de trébol blanco (*Trifolium repens* L.) en Pergamino, Argentina. *Plant Genetic*

Resources Newsletter 123:41-45.

Panter S, Chu PG, Ludlow E, Garrett R, Kalla R, Jahufer MZZ, de Lucas Arbiza A, Mouradov A, Smith KF, Spangenberg G (2011) Molecular breeding of transgenic white clover (*Trifolium repens* L.) with field resistance to alfalfa mosaic virus through the expression of the AMV coat protein *Transgenic Research* 21:619-632

Paplauskiene V. Sprainaitis A (2003) Variability of cyanogenic glucoside content in white clover plants. *Biologija* 1:85-88.

Pecetti L, Tava A, Romani M, De Benedetto MG, Corsi P (2006) Variety and environment effects on the dynamics of saponins in lucerne (*Medicago sativa* L.). *European Journal of Agronomy* 25: 187-192.

Richards AJ, Fletcher A (2002).The effects of altitude, aspect, grazing and time on the proportion of cyanogenics in neighbouring populations of *Trifolium repens* L. (white clover). *Heredity* 88:432-437.

Saba N, Drane HM, Hebert CN, Holdsworth RJ (1974) Seasonal variation in oestrogenic activity, coumestrol and formononetin content of white clover. *Journal of Agricultural Science* 83:505-510.

Sakamoto S, Kofuji S, Kuroyanagi M, Ueno A, Sekita S (1992) Saponins from *Trifolium repens*. *Phytochemistry* 31: 1773-1777.

Saloniemi H, Wahala K, Nykanen-Kurki P, Kallela K, Saastamoinen I (1995) Phytoestrogen content and estrogenic effect of legume fodder. *Proceedings of the Society for Experimental Biology and Medicine* 208:13-17.

Seguin P, Zheng W, Souleimanov A (2004) Alfalfa phytoestrogen content: impact of plant maturity and herbage components. *Journal of Agronomy and Crop Science* 190: 211-217.

Shutt DA (1976) The effects of plant oestrogens on animal reproduction. *Endeavour*

35:110-113.

Sivesind E, Seguin P (2005) Effects of the environment, cultivar, maturity, and preservation method on red clover isoflavone concentration. *Journal of Agricultural and Food Chemistry* 53:6397-6402.

Smith KF, Flinn PC (1991) Monitoring the performance of a broadbased calibration for measuring the nutritive value of two independent populations of pasture using near infrared reflectance (NIR) spectroscopy. *Australian Journal of Experimental Agriculture* 31**:**205-210

Søegaard K (1993). Nutritive value of white clover. *In: White clover in Europe: State of the art*, J. Frame (*Ed)* Food and Agriculture Organisation

Stochmal A, Oleszek W (1997) Changes of cyanogenic glucosides in white clover (*Trifolium repens* L.) during growing season. *Journal of Agricultural and Food Chemistry* 45: 4333-4336.

Stypiñski P (1993) The effect of white clover on chemical composition and nutritive value of companion grasses in grass/clover mixtures. *In: White clover in Europe: State of the art*, J. Frame (*Ed)*. Food and Agriculture Organisation (FAO). Aarhus, Denmark.

Suzuki H, Achnine L, Xu R, Matsuda SPT, Dixon RA (2002) A genomics approach to the early stages of triterpene saponin biosynthesis in *Medicago truncatula*. *Plant Journal* 32:1033-1048.

Vetter J (1995) Isoflavones in different parts of common *Trifolium* species. *Journal of Agricultural and Food Chemistry* 43: 106-108.

Vickery PJ, Wheeler JL, Mulcahy C (1987) Factors affecting the hydrogen cyanide potentinal of white clover (*Trifolium repens* L.). *Australian Journal of Agricultural Research* 38:1053-1059.

Walter ED, Bickoff BM, Thompson CR, Robinson CH, Djerassi C (1955) Saponins from Ladino clover (*Trifolium repens*). *Journal of the American Chemical Society* 77:4936.

West JW (1998) Factors which influence forage quality and effectiveness in dairy rations. Animal Dairy Science Department. University of Georgia-Coastal Plain Experiment Station.

Wheeler JL, Vickery PJ (1989) Variation in HCN potential among cultivars of white clover (*Trifolium repens*). *Grass and Forage Science* 44:107-109.

Wu Q, Wang M, Simon JE (2003) Determination of isoflavones in red clover and related species by high-performance liquid chromatography combined with ultraviolet and mass spectrometric detection. *Journal of Chromatography A* 1016: 195-209.

Figure Legends

Figure 1. Estimation of nutritional parameters in white clover herbage samples. Dried and ground herbage samples from T_2 and *Syn0* generation 'Sustain'-type AMV-resistant transgenic white clover lines, *Syn0* generation 'Mink'-type AMV-resistant transgenic white clover and their respective wild-type parents were analysed by near-infrared reflectance spectroscopy. CP: crude protein. ADF: acid detergent fibre. NDF: neutral detergent fibre. WSC: water-soluble carbohydrate. IVDMD: *in vitro* dry matter digestibility. IVOMD: *in vitro* organic matter digestibility. DOMD: digestible organic matter in the DM. ESTME: estimated metabolisable energy. Error bars = SD

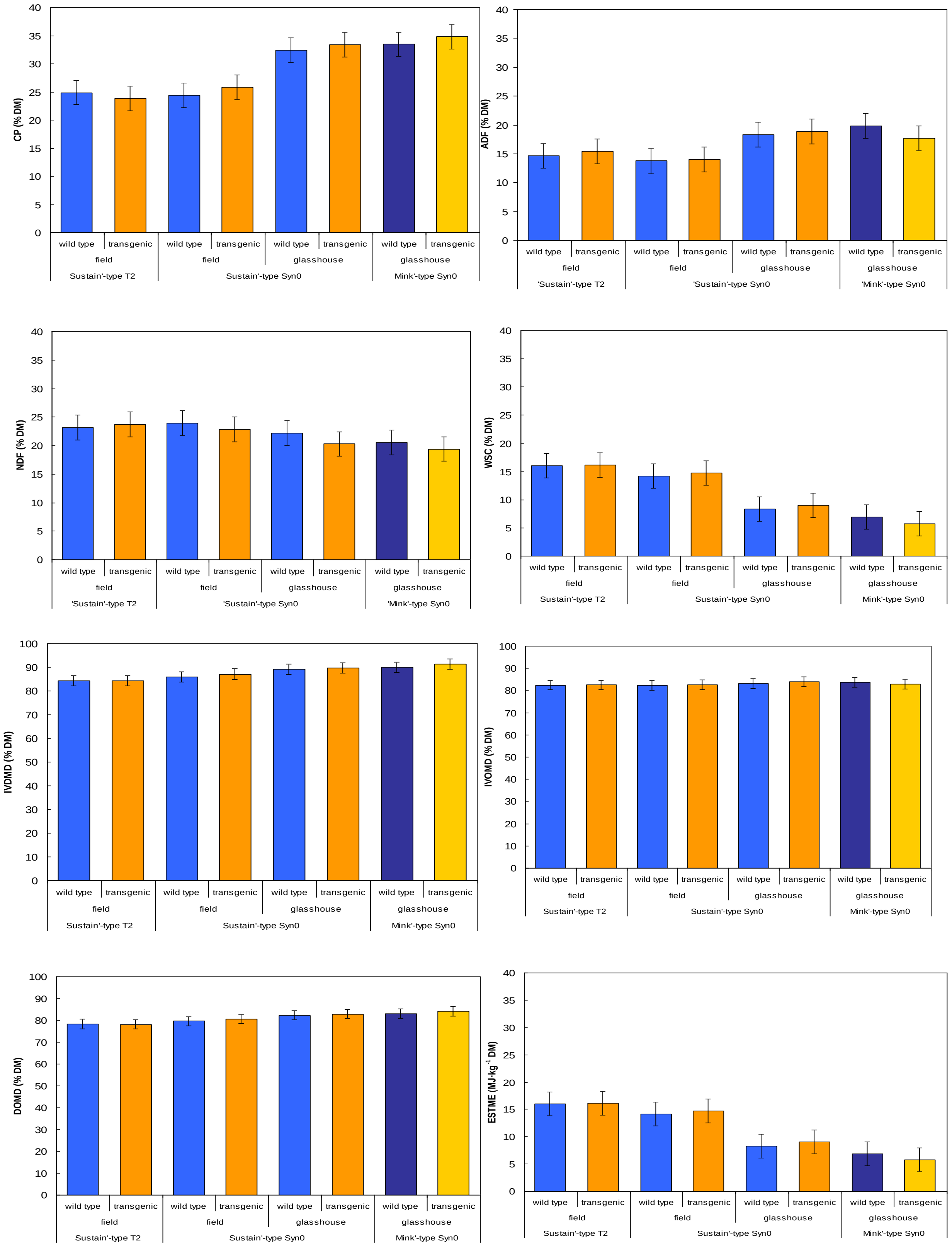
Figure 2. Measurement of cyanogenic potential in leaves of transgenic and wild-type white clover plants A-C. Cyanogenic potential values from individual white clover lines. A. 28 *Syn0* generation 'Sustain'-type transgenic and 11 wild-type 'Grasslands Sustain' parents grown under field conditions. B. 28 *Syn0* generation 'Sustain'-type transgenic and 11 wild-type 'Grasslands Sustain' parents grown under glasshouse conditions. C. 15 *Syn0* generation 'Mink'-type transgenic and 7 wild-type 'Mink' parents grown under glasshouse conditions. D. Mean cyanogenic potential values for *Syn0* generation 'Sustain' type transgenic and wild-type plants (glasshouse and field), *Syn0* generation 'Mink' type transgenic and wild-type plants (glasshouse) and a selection of commercial cultivars (glasshouse). E. Cyanogenic potential in leaves of individual plants from 5 commercial cultivars grown under glasshouse conditions. Herbage samples were analysed using a colorimetric picrate assay and computational processing of RGB images. Error bars = SD

Figure 3. Formononetin and genistein content in leaves of individual T_2 generation 'Sustain'-type transgenic white clover plants and wild-type 'Grasslands Sustain' plants. Phytoestrogens were quantified by LC-MS analysis of leaf extracts, using standard compounds for calibration.

Figure 4. Formononetin and genistein content in bulked white clover samples representing *Syn0* generation 'Sustain'-type and 'Mink'-type trangenic lines, their wild-type parents and 5 commercial cultivars (error bars= analytical error between

technical replicates). Phytoestrogens were identified during LC-MS analysis of leaf extracts, using standard compounds for quantification. Error bars reflect analytical error between technical replicates.

Figure 5. Relative quantification of potential saponins in bulked white clover samples representing *Syn0* generation 'Sustain'-type and 'Mink'-type trangenic lines, their wild-type parents and 5 commercial cultivars. Potential saponins were identified during LC-MS analysis of leaf extracts. Error bars reflect analytical error between technical replicates.


CP (% DM)
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Sustain'-type Syn0
Mink'-type Syn0
ADF (% DM)
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'Sustain'-type Syn0
'Mink'-type Syn0
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wild type
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'Sustain'-type T2
'Sustain'-type Syn0
'Mink'-type Syn0
WSC (% DM)
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glasshouse
Sustain'-type T2
Sustain'-type Syn0
Mink'-type Syn0
IVDMD (% DM)
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40
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60
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80
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wild type
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transgenic
wild type
transgenic
field
field
glasshouse
glasshouse
Sustain'-type T2
Sustain'-type Syn0
Mink'-type Syn0
IVOMD (% DM)
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wild type
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wild type
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glasshouse
glasshouse
Sustain'-type T2
Sustain'-type Syn0
Mink'-type Syn0
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Sustain'-type T2
Sustain'-type Syn0
Mink'-type Syn0
ESTME (MJ·kg⁻¹ DM)
0
5
10
15
20
25
30
35
40
wild type
transgenic
wild type
transgenic
wild type
transgenic
wild type
transgenic
field
field
glasshouse
glasshouse
Sustain'-type T2
Sustain'-type Syn0
Mink'-type Syn0

Figure 1.

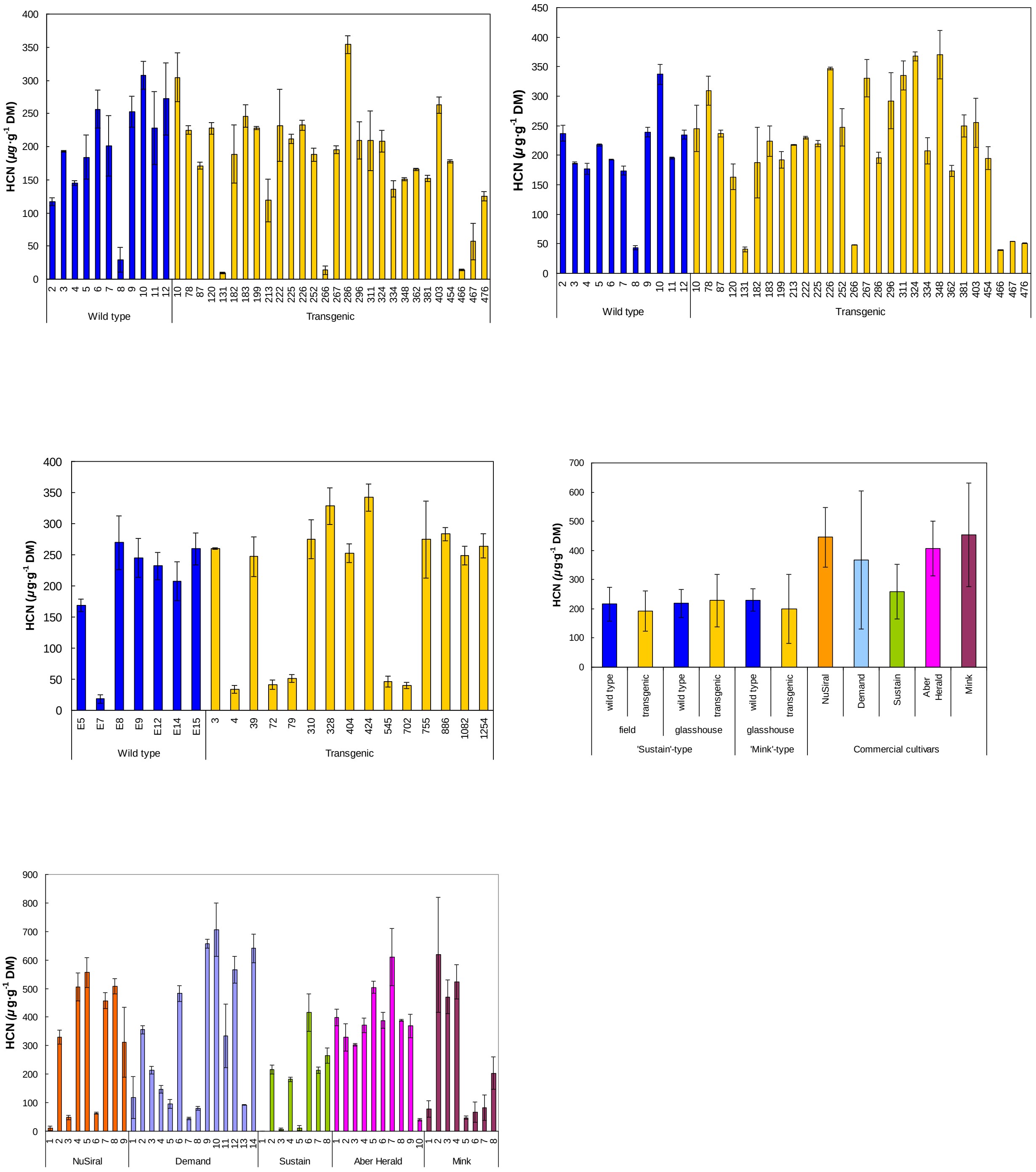
HCN ($\mu g \cdot g^{-1}$ DM)
Wild type
Transgenic
2 3 4 5 6 7 8 9 10 11 12
10 78 87 120 131 182 183 199 213 222 225 226 252 266 267 286 296 311 324 334 348 362 381 403 454 466 467 476
E5 E7 E8 E9 E12 E14 E15
3 4 39 72 79 310 328 404 424 545 702 755 886 1082 1254
wild type
transgenic
field
glasshouse
'Sustain'-type
'Mink'-type
NuSiral
Demand
Sustain
Aber Herald
Mink
Commercial cultivars

Figure 2.

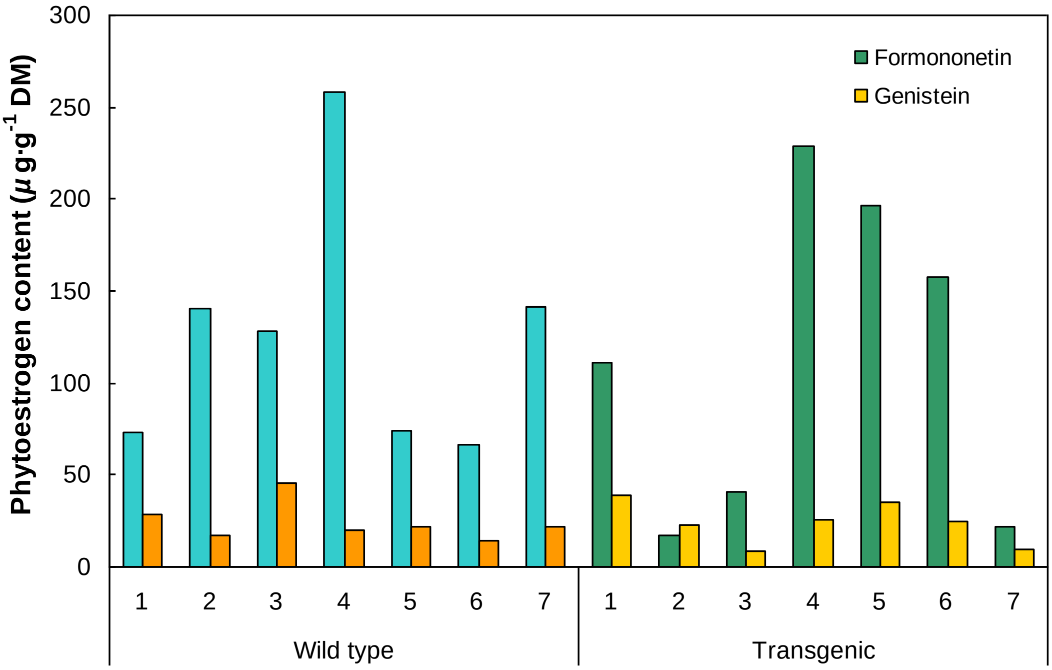
Phytoestrogen content (μg·g^{-1} DM)
300
250
200
150
100
50
0
Formononetin
Genistein
1
2
3
4
5
6
7
1
2
3
4
5
6
7
Wild type
Transgenic

Figure 3.

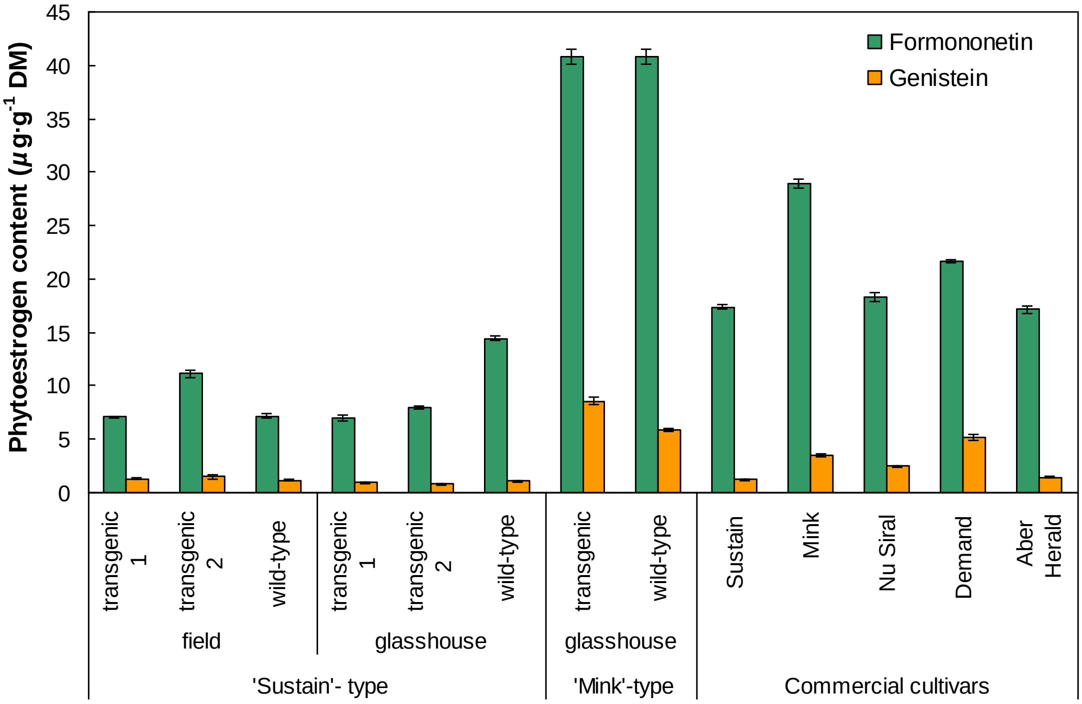
Phytoestrogen content (μg·g^{-1} DM)
45
40
35
30
25
20
15
10
5
0
Formononetin
Genistein
transgenic 1
transgenic 2
wild-type
transgenic 1
transgenic 2
wild-type
transgenic
wild-type
Sustain
Mink
Nu Siral
Demand
Aber Herald
field
glasshouse
glasshouse
'Sustain'- type
'Mink'-type
Commercial cultivars

Figure 4.

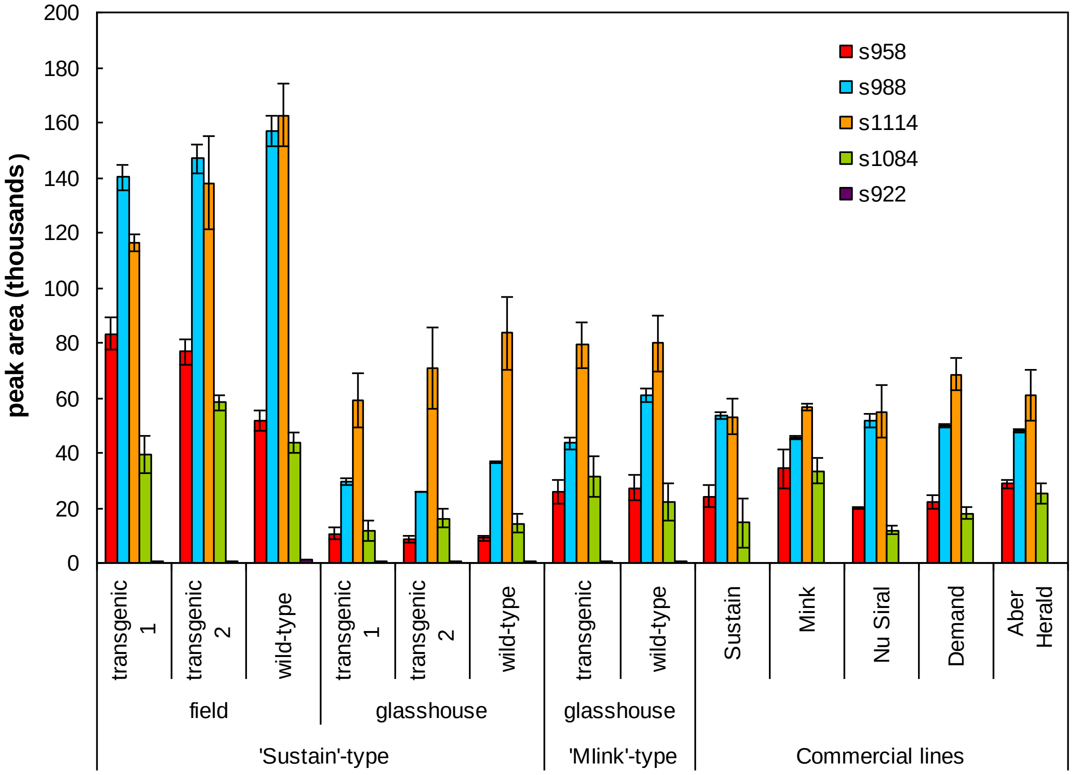
200
180
160
140
120
100
80
60
40
20
0
peak area (thousands)
s958
s988
s1114
s1084
s922
transgenic 1
transgenic 2
wild-type
transgenic 1
transgenic 2
wild-type
transgenic
wild-type
Sustain
Mink
Nu Siral
Demand
Aber Herald
field
glasshouse
glasshouse
'Sustain'-type
'Mlink'-type
Commercial lines

Figure 5.