FOOD STANDARDS
Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

Supporting document 1

Safety assessment – Application A1085

Food derived from Reduced Lignin Lucerne Line KK179

Summary and conclusions

Background

A genetically modified (GM) lucerne line, KK179, has been developed that has reduced biosynthesis of guaiacyl lignin (G lignin), a major subunit of lignin. Lignin is a non-carbohydrate phenolic polymer deposited in plant cell walls, particularly in the vascular tissue, and is a contributor to the quality of forage eaten by grazing animals. The Applicants claim that growers will have the option of being able to harvest KK179 several days later than conventional lucerne without appreciable loss of forage quality typical in conventional lucerne at the same growth stage.

The reduced level of lignin in lucerne KK179 has been achieved through the introduction of a partial *caffeoyl CoA 3-O-methyltransferase* (*CCOMT*) gene sequence derived from lucerne (*Medicago sativa*). The gene transcript acts, via suppression of the endogenous *CCOMT* gene, to reduce the lignin level.

It is not intended that KK179 enter the food supply. However, a food approval is sought in case this inadvertently occurs.

In conducting a safety assessment of food derived from lucerne line KK179, a number of criteria have been addressed including: a characterisation of the transferred genetic material and its origin, function and stability in the lucerne genome; compositional analyses; and evaluation of intended and unintended changes.

This safety assessment report addresses only food safety and nutritional issues associated with the GM line. It therefore does not address:

- environmental risks related to the environmental release of GM plants used in food production
- the safety of animal feed or animals fed with feed derived from GM plants
- the safety *per se* of food derived from the non-GM (conventional) plant.

History of Use

Lucerne is grown primarily for livestock feed and is grown throughout the world (approximately 30 million ha) as forage. It is often harvested for hay, but can also be made into silage and manufactured stock feed (meal and pellets). The main food products from *M. sativa* are alfalfa sprouts, comprising sprouted seeds packed into punnets that are used as a fresh vegetable in salads, sandwiches, soups and stir-fries. Other alfalfa products are widely available in specialised stores, for example alfalfa in the form of dried leaf, health drinks and teas.

Molecular Characterisation

Explants of the lucerne line 'R2336' were transformed via *Agrobacterium*-mediated transformation, the genes of interest being inserted via two separate T-DNAs. T-DNA I contains two *CCOMT* fragments, that when transcribed lead to the production of double-stranded RNAs (dsRNAs) that, via an RNA interference (RNAi) mechanism, suppress endogenous *CCOMT* RNA levels, leading to reduced biosynthesis of G-lignin.

In order to select putative transformants, a T-DNA II was also inserted during the transformation procedure. This contained a *neomycin phosphotransferase II* (*nptII*) coding region that confers resistance to kanamycin. T-DNA II was removed from KK179 by selection.

Comprehensive molecular analyses of lucerne line KK179 indicate there is a single insertion site at which there is a single copy of the T-DNA I. No DNA sequences from T-DNA II or from the backbone of the transformation vector, including antibiotic resistance marker genes, were transferred to the plant. The introduced genetic elements are stably inherited from one generation to the next.

Northern blot analyses were used to compare the RNA levels associated with the endogenous *CCOMT* gene in forage and root tissue of KK179. The data show a clear reduction in the level of *CCOMT* mRNA in KK179 compared to the conventional control and hence that insertion of the *CCOMT* suppression cassette in T-DNA I has resulted in the intended modification.

Compositional Analyses

In order to establish the nutritional adequacy of forage from lucerne line KK179, samples were analysed for 50 analytes comprising nutrients; proximates (ash, fat, moisture, and protein), carbohydrates by calculation, acid detergent fibre, neutral detergent fibre, acid detergent lignin, minerals, amino acids and a number of anti-nutrients and secondary metabolites. In addition, *p*-coumaric acid, ferulic acid, sinapic acid, total polyphenols and free phenylalanine were also analysed to evaluate the effect of *CCOMT* suppression on the lignin pathway and cell wall-associated metabolites.

As expected, the levels of lignin in general, and G lignin in particular, in KK179 were statistically significantly lower than in the control. The overall magnitude of the difference however was small, and the lignin levels were within the reference range obtained for non-GM reference varieties grown at the same time. While the difference in lignin levels between the GM line and the control is of agronomic significance, in that it enables the forage to be harvested at a later date without appreciable loss of forage quality, it is unlikely to have any nutritional significance to humans given the range of natural variation that exists in lucerne.

For the remaining analytes, statistically significant differences were noted in only three analytes (ash, canavanine and ferulic acid). In all cases the differences were typically small

and within the reference range obtained for non-GM reference varieties grown at the same time. Any observed differences are therefore considered to represent the natural variability that exists within lucerne.

Conclusion

No potential public health and safety concerns have been identified in the assessment of lucerne line KK179. On the basis of the data provided in the present Application, and other available information, food derived from lucerne line KK179 is considered to be as safe for human consumption as food derived from conventional lucerne cultivars.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADF	acid detergent fibre
ADL	Acid detergent lignin
AOAC	Association of Analytical Communities
BLOSUM	Blocks Substitution Matrix
bp	base pairs
bw	body weight
CaMV	Cauliflower mosaic virus
CCOMT	caffeoyl CoA 3-O-methyltransferase
DNA	deoxyribonucleic acid
T-DNA	transferred DNA
FARRP	Food Allergy Research and Resource Program
FASTA	Fast Alignment Search Tool - All
FD4	Fall dormancy 4
FSANZ	Food Standards Australia New Zealand
fw	fresh weight
G lignin	guaiacyl lignin
GM	genetically modified
ha	hectare
LOQ	limit of quantitation
MBC	modified backcross
NCBI	National Center for Biotechnology Information
NDF	neutral detergent fibre
nos	nopaline synthase
OECD	Organisation for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulator
ORF	open reading frame
PAL	phenylalanine ammonia lyase
PCR	polymerase chain reaction
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
dsRNA	double stranded RNA
Poly A^+ RNA	polyadenylated mRNA
S.E.	standard error
Ti	tumour-inducing
U.S.	United States of America

1. Introduction

A genetically modified (GM) lucerne line with OECD Unique Identifier MON-00179-5, hereafter referred to as lucerne KK179, has been developed that has reduced biosynthesis of guaiacyl lignin (G lignin), a major subunit of lignin. Lignin is a non-carbohydrate phenolic polymer deposited in plant cell walls, particularly in the vascular tissue, and is a contributor to the quality of forage eaten by grazing animals. Quality decreases as the proportion of cell wall components (cellulose, hemicellulose and lignin) increases. Total lignin levels in KK179 forage are generally similar to lignin levels in conventional lucerne forage harvested several days earlier under similar production conditions. The Applicants claim that growers will have the option of being able to harvest KK179 several days later than conventional lucerne, without appreciable loss of forage quality typical in conventional lucerne at the same growth stage.

The reduced level of lignin in lucerne KK179 has been achieved through the introduction of a partial *caffeoyl CoA 3-O-methyltransferase* (*CCOMT*) gene sequence (fragment) derived from lucerne (*Medicago sativa*). The gene transcript has an inverted repeat and produces double-stranded ribonucleic acid (dsRNA) which, via an RNA interference (RNAi) pathway, suppresses endogenous *CCOMT* RNA levels and results in the reduced biosynthesis of G lignin. This, in turn, reduces the accumulation of total lignin.

The Applicant has advised that lucerne KK179 will be grown and used primarily in northern America and there is no intention to grow the plant line in Australia or New Zealand. The Applicant has anticipated that KK179 would be stacked with two Roundup Ready™ lucerne lines, J101 and J163 (OECD Unique Identifiers MON-00101-8 and MON00163-7 respectively), the food from which has been approved by FSANZ (FSANZ, 2007).

It is not intended that KK179 enter the food supply. However, a food approval is sought in case this inadvertently occurs. In Australia and New Zealand, lucerne that is used for human food is often referred to as alfalfa. Alfalfa would be expected to be consumed in minor quantities and on an occasional basis.

2. History of use

2.1 Host and donor organism

The host organism is a conventional lucerne (*Medicago sativa* L. ssp. *sativa*), belonging to the family Leguminosae (Small, 2011). The commercial cultivar 'R2336' was used as the parental variety for the genetic modification described in this application. 'R2336' is a proprietary cloned line developed by Forage Genetics International; it was selected for regenerability from an elite, high-yielding, fall-dormant breeding population. During development of KK179, 'R2336' transformants were crossed with a non-GM male sterile line designated 'Ms208' (see Section 3.3) before final selection of KK179 from the resulting progeny. Therefore the cross between 'R2336' and 'Ms208' (designated as C_0) is regarded as the near-isogenic line for the purposes of comparative assessment with lucerne KK179

Lucerne is grown primarily for livestock feed but also has a minor place in the food supply (OECD, 2005; Bouton, 2012). With respect to feed, it is grown throughout the world (approximately 30 million ha) as forage[1] and is often harvested for hay, but can also be

[1] The term 'forage' has a number of definitions but is used in this document to refer to the above-ground parts of rooted plants that would be consumed in the field by livestock (Small, 2011). It can be used as a more general term that also encompasses 'fodder' which is defined as feed that is first harvested by humans before being fed to animals.

made into silage and manufactured stock feed (meal and pellets). The major lucerne-producing regions are North America with 11.9 million ha (41%), Europe with 7.12 million ha (25%), South America with 7 million ha (23%), Asia 2.23 million ha (8%), Africa (2%) and Oceania (1%). The leading countries in terms of area of lucerne production (in million ha) are the US (9), Argentina (6.9), Canada (2), Russia (1.8), Italy (1.3) and China (1.3) (Yueago and Cash, 2009).

Lucerne was first introduced into Australia in the early 19th century. A second wave of introductions occurred in the 1980s following devastation of the crop by exotic insect pests. Currently Australia has about 3.5 million ha of lucerne under crop in both irrigated and dryland situations in all states, and produces around one million tonnes of hay (DPIPWE Tas, 2011).

The main food product from *M. sativa* is alfalfa sprouts, comprising sprouted seeds packed into punnets that are used as a fresh vegetable in salads, sandwiches, soups and stir-fries. Consumption would be expected to be in minor quantities on an occasional basis (OECD, 2005). Alfalfa sprouts are not permitted to be imported to Australia or New Zealand. Within Australia, the seed used for making sprouts is largely grown in Australia and accounts for approximately 4% of total lucerne seed production (FSANZ, 2011). It is possible a proportion of imported seed may also be used for sprouts but since there is no discrimination in end use of seed imported as 'seed for sowing' (Table 1A) it is difficult to know how much is used for pasture and how much may be used by the sprout industry. In New Zealand, production of lucerne for livestock and alfalfa sprouts relies on seed imported from large breeding programmes in the US, Australia and Europe (Table 1B); approximately 20% of this imported seed is used for sprouting.

Table 1: Importation (kg) to A) Australia, between 2008 – 2012, and B) New Zealand in 2005 of lucerne seed for sowing/sprouting (by country of origin)

A)

Country of origin	2008	2009	2010	2011	2012
Australia (Re-imports)	37,500	58,711	73,322		11,750
United States of America	19,594	2,214	1,615	6,260	4,406
New Zealand			200		200
Netherlands				250	
Total	**57,094**	**60,925**	**75,137**	**6,510**	**16,356**

Source: ABS imported food data

B)

	Seed for Sowing		Seed for Sprouting	
	1 July '04- 30 June '05		1 July '04- 30 June '05	
Exporting country	Weight (kg)	Proportion (%)	Weight (kg)	Proportion (%)
United States of America	25 000	66.5%	2 720	29.5%
Australia	10 410	27.5%	6 500	70.5%
Italy	2 200	6%		
TOTAL	37 610		9 220	

Source: Ministry for Primary Industries - http://www.biosecurity.govt.nz/related/related_faqs/ihs/search?page=2&expand=2475

Alfalfa products in the form of dried leaf, protein supplement, tablets, capsules, extracts, health drinks, tonics and teas are widely available in specialised stores (Bora and Sharma, 2011; Mielmann, 2013). Some of these products may be regulated as foods (e.g. dried leaf, health drinks and teas), while other products would be regulated as therapeutic goods and dietary supplements (e.g. protein supplements, tablets, capsules and extracts). Another possible food product that can be derived from the lucerne plant is bee pollen (Krell, 1996).

2.2 Other organisms

Genetic elements from several other organisms have been used in the genetic modification of lucerne KK179 (refer to Table 2). These non-coding sequences are used to drive, enhance or terminate expression of the inserted DNA fragment. None of the sources of these genetic elements is associated with toxic or allergenic responses in humans. The genetic elements derived from the plant pathogen *Agrobacterium tumefaciens* are not pathogenic in themselves and do not cause pathogenic symptoms in lucerne KK179.

It is noted that an antibiotic resistance gene (*nptII*) derived from *E. coli* was also used in the initial transformation but that this gene (and its regulatory elements) was segregated out during subsequent selection of line KK179 (see discussion in Section 3.1).

3. Molecular characterisation

Molecular characterisation is necessary to provide an understanding of the genetic material introduced into the host genome and helps to frame the subsequent parts of the safety assessment. The molecular characterisation addresses three main aspects:

- the transformation method together with a detailed description of the DNA sequences introduced to the host genome
- a characterisation of the inserted DNA including any rearrangements that may have occurred as a consequence of the transformation
- the genetic stability of the inserted DNA and any accompanying expressed traits.

Studies submitted:

2011. Molecular Characterization of Reduced Lignin Alfalfa KK179. **MSL0023299.** Monsanto Company (unpublished).
2011. Stability of the DNA Insert in KK179 Across Multiple Generations. **MSL0023312.** Monsanto Company (unpublished)
2012. Bioinformatics Evaluation of the Transfer DNA Insert in KK179 Utilizing the AD_2012, TOX_2012 and PRT_2012 Databases. **MSL0024048**. Monsanto Company (unpublished)
2012. Bioinformatics Evaluation of DNA Sequences Flanking the 5' and 3' Junctions of Inserted DNA in KK179: Assessment of Putative Polypeptides. **MSL0023975**. Monsanto Company (unpublished)

2011. Analysis of the Endogenous CCOMT RNA Level in Alfalfa KK179. **MSL0023329**. Monsanto Company (unpublished).
2011. Heritability of the KK179 Insert in the MBC2, MBC3, and Syn1 Populations. **RPN-2010-0705**. Monsanto Company (unpublished)
2011. Lignin Analysis of Forage from Multiple Generations of KK179 Alfalfa. **RAR-2011-0129**. Monsanto Company (unpublished).

3.1 Method used in the genetic modification

Explants of lucerne 'R2336' were transformed via *Agrobacterium*-mediated transformation. The genes of interest were inserted via two separate T-DNAs (each with their own Right and Left Border) into plasmid PV-MSPQ12633 (refer to Figure 1).The border sequences were isolated from the tumour-inducing (Ti) plasmid of *Agrobacterium tumefaciens* and normally delimit the DNA sequence (T-DNA) transferred into the plant (Zambryski, 1988). During the transformation procedure a portion of each border is expected to be integrated into the host genome (Tzfira *et al*., 2004).

Basically, leaf explants were co-cultivated with the *Agrobacterium tumefaciens* containing the binary vector PV-MSPQ12633. Putative transformants were selected on a medium containing the antibiotics kanamycin and timentin and surviving leaf pieces were then regenerated via somatic embryogenesis (Schenk and Hildebrandt, 1972; Walker and Sato, 1981). Rooted plants (T_0) were transferred to the greenhouse for growth and further assessment.

The T_0 plants were crossed to a non-GM male sterile line (Ms208) to produce F_1 plants in which the unlinked insertions of T-DNA I and T-DNA II were segregated. Subsequent to this, plants that were positive for T-DNA I (containing the *CCOMT* fragment) and negative for T-DNA II (containing the antibiotic resistance gene *nptII*) were identified by polymerase chain reaction (PCR). KK179, an individual F_1 plant, was selected as the lead event (P_0) based on its superior characteristics and absence of T-DNA II.

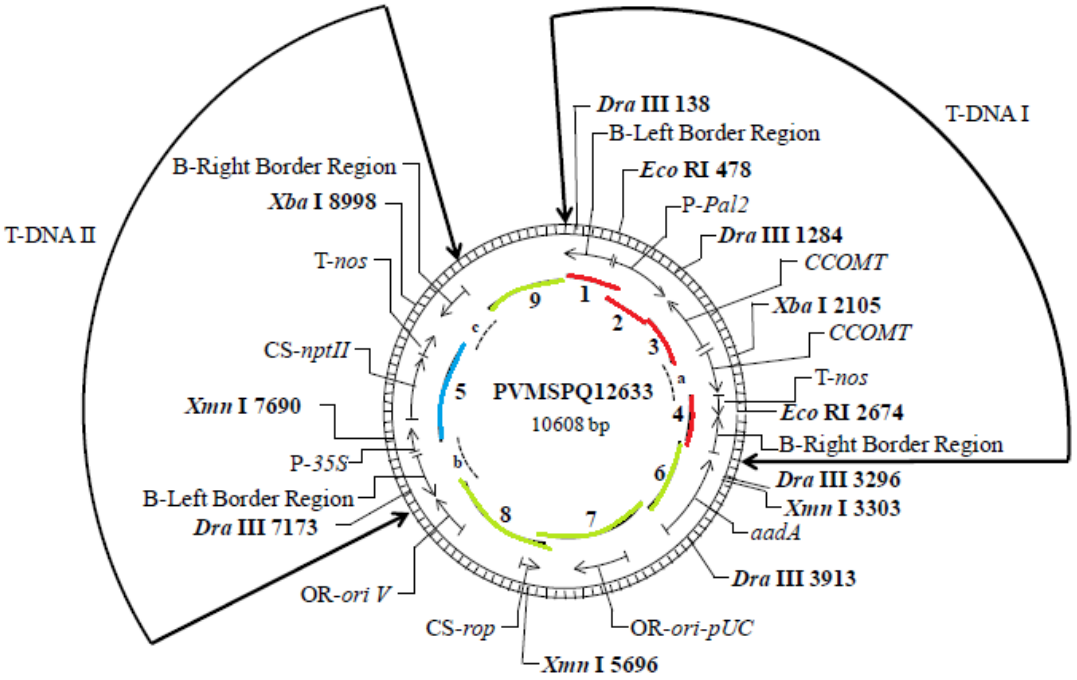

Figure 1: Vector map of plasmid PV-MSPQ12633

3.2 Description of the introduced genetic material

Information on the genetic elements in the two T-DNA inserts is summarised in Table 2.

Table 2: Description of the genetic elements contained in the two T-DNAs of PV-MSPQ12633

Genetic element	bp location on pSYN15954	Size (bp)	Source	Orient.	Description & Function	References
T-DNA I						
Left Border	1 - 442	442	*Agrobacterium tumefaciens*	Anti-clockwise	• Required for the transfer of the T-DNA into the plant cell	Barker *et al* (1983); Zambryski *et al.(1982)*
Intervening sequence	443 - 490	48			• Cloning sequence	
Pal2 promoter	491 - 1567	1077	*Paseolus vulgaris* (bean)	Clockwise	• Drives transcription of the *COMMT* fragment within vascular tissue and thus allows it to mirror the pattern of lignin deposition.	Cramer *et al*. (1462/id/d}
Intervening sequence	1568 - 1584	17			Cloning sequence	
CCOMT	1585 - 2103	519	*Medicago sativa* (lucerne)	Anti-clockwise	• Partial coding sequence of the *CCOMT* gene • Together with the inverted (clockwise) repeat, suppresses expression of the endogenous *CCOMT* gene	Inoue *et al* (1998)
Intervening sequence	2104 - 2110	7			• Cloning sequence	
CCOMT	2111 - 2410	300	*Medicago sativa* (lucerne)	Clockwise	• Partial coding sequence of the *CCOMT* gene • Together with the inverted (anti-clockwise) repeat, suppresses expression of the endogenous *CCOMT* gene	Inoue *et al* (1998)
Intervening sequence	2411 - 2418	6			• Cloning sequence	
nos terminator	2419 - 2671	253	*Agrobacterium tumefaciens*	Clockwise	• 3 UTR sequence of the *nopaline synthase (nos)* gene • Transcriptional terminator	Depicket *et al.* (1982); Fraley et al. (1983)
Intervening sequence	2672 - 2727	56			• Cloning sequence	
Right Border	2728 - 3084	357	*Agrobacterium tumefaciens*	Anti-clockwise	• Required for the transfer of the T-DNA into the plant cell	Zambryski *et al.* (1982)
Vector Backbone (3951 bp)						
T-DNA II						
Left Border	7036 - 7477	442	*Agrobacterium tumefaciens*	Anti-clockwise	• Required for the transfer of the T-DNA into the plant cell	Barker *et al* (1983); Zambryski *et al.(1982)*
Intervening sequence	7478 - 7527	50			• Cloning sequence	
35S promoter	7528 - 7851	324	Cauliflower mosaic virus	Clockwise	• Drives constitutive expression of the *nptII* gene	Odell *et al.* (1985)
Intervening sequence	7852 - 7884	33			• Cloning sequence	
nptII	7885 - 8679	795	*Esherichia coli*	Clockwise	• Coding sequence of the *neo* gene from transposon Tn*5,* encoding neomycin transferase II • Confers resistance to neomycin and kanamycin – for selection purposes.	Fraley *et al.* (1983)

Genetic element	bp location on pSYN15954	Size (bp)	Source	Orient.	Description & Function	References
Intervening sequence	8680 - 8710	31			• Cloning sequence	
nos terminator	3663 - 3915	253	*Agrobacterium tumefaciens*	Clockwise	• 3 UTR sequence of the *nopaline synthase (nos)* gene • Transcriptional terminator	Depicket *et al.* (1982); Fraley *et al.* (1983)
Intervening sequence	8964 - 9048	85			• Cloning sequence	
Right Border	9049 - 9405	357	*Agrobacterium tumefaciens*	Anti-clockwise	• Required for the transfer of the T-DNA into the plant cell	Zambryski *et al.* (1982)
Vector Backbone (1,203 bp)						

3.2.1 *T-DNA I*

Background on lignin biosynthesis

Lignin is a feature of plants adapted to a terrestrial way of life (Vanholme et al., 2010; Weng and Chapple, 2010). It is a complex cross-linked polymer that is deposited in plant secondary walls (i.e. walls laid down in cells that have stopped expanding and started differentiating); its function is to cement together and anchor the cellulose fibres in the wall and hence to provide structural support, especially to the vascular system and aerial parts. After cellulose, lignins are the most abundant organic polymers known (Buchanan *et al*., 2000).

The main building blocks of lignin are the hydroxycinnamyl alcohols (monolignols) of which there are three main types – guaiacyl (G), syringyl (S) and *p*-hydroxyphenyl (H). G lignin is produced predominantly via caffeoyl CoA. The *CCOMT* gene encodes an enzyme (CCOMT) that methylates caffeoyl CoA to produce feruloyl CoA in the pathway that leads to the production of G lignin monomers (Guo *et al*., 2001; Vanholme *et al*., 2010; Zhou *et al*., 2011) (see Figure 2). G lignin occurs in all vascular plants (i.e. angiosperms, gymnosperms, ferns, lycophytes). In lucerne, G and S lignin make up approximately 95% of the total lignin.

Figure 2 shows that CCOMT is also normally required for the production of S lignin. However, if CCOMT is knocked out or reduced (as would occur in KK179), then another enzyme, cinnamoyl CoA reductase 2 (CCR2) is upregulated and allows continued synthesis of S lignin (Zhou *et al*., 2011).

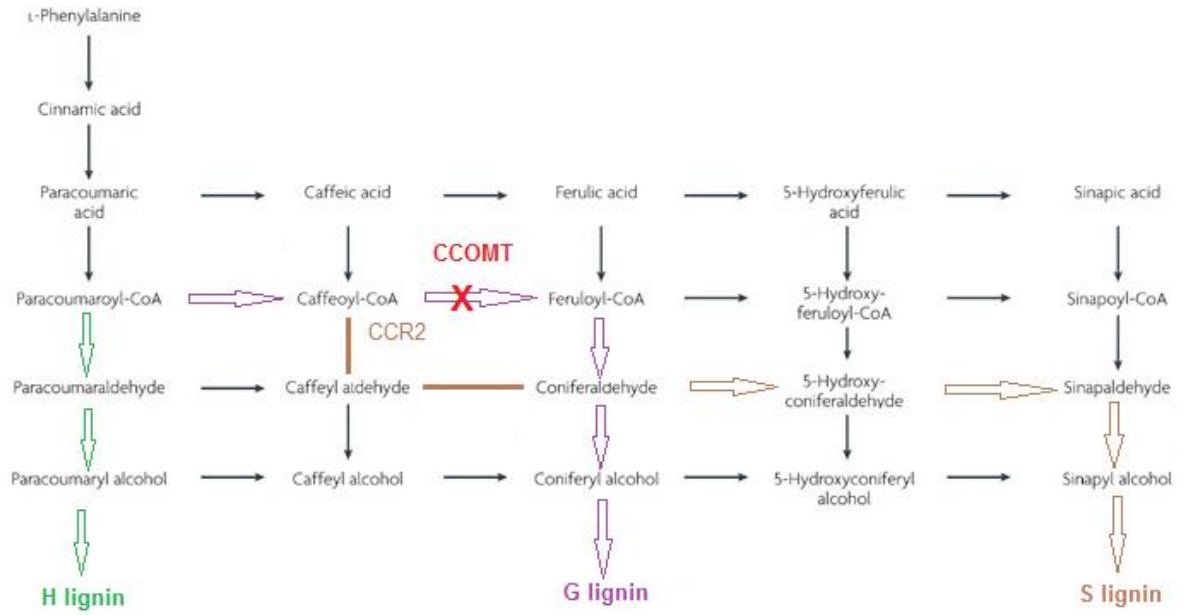

Figure 2: Simplified diagram of the lignin biosynthetic pathways indicating where the CCOMT enzyme acts and where it would be blocked in KK179. Major pathway for each monolignol is represented by large coloured arrows. Adapted from Sticklen (2008).

The CCOMT expression cassette

The suppression cassette in lucerne KK179 contains two *CCOMT* partial fragments, one in the clockwise orientation and the other in the anti-clockwise (inverted) orientation. The fragment was isolated and cloned from lucerne. When transcribed the two fragments, via an inverted repeat, lead to the production of double-stranded RNA (dsRNA) in the form of a structure known as a hairpin that, via RNA interference (RNAi), suppresses endogenous *CCOMT* RNA levels, leading to the reduced biosynthesis of G-lignin (see e.g. Guo *et al*., 2001). RNAi is a naturally-occurring RNA-based mechanism that is used by eukaryotes, including plants, to modulate endogenous gene expression as well as destroy foreign RNA including viral RNA (Parrott *et al*., 2010). In plants RNAi plays a fundamental role in all aspects of growth and development (Bonnet *et al*., 2006).

In RNAi the dsRNA hairpin that is formed is cleaved into small dsRNAs, approximately 21-24 nucleotides long, via an endogenously occurring protein known as Dicer (Hammond, 2005). These mature small dsRNA duplexes contain an interfering antisense strand (the guide strand), which is complementary to the target mRNA sequence, and a passenger strand. The guide strand is incorporated into a multiprotein complex known as the RNA-induced silencing complex (RISC) leading, in the case of KK179, to the targeted destruction of the mRNA transcribed from the endogenous *CCOMT* gene. The result is that production of CCOMT is suppressed.

The *CCOMT* coding region in T-DNA I is driven by a *phenylalanine ammonia lyase* (*Pal2*) promoter from the common bean (*Phaseolus vulgaris*). PAL is a key regulatory enzyme in plant metabolism and is particularly associated with lignin biosynthesis (Cramer *et al*., 1989). Therefore use of the *Pal2* promoter ensures transcription of the *CCOMT* fragment in a pattern of expression that is similar to the pattern of lignin deposition within vascular tissue. The coding region is terminated by a sequence from the 3' end of the *nopaline synthase (nos)* gene from the soil bacterium *Agrobacterium tumefaciens.*

3.2.2 T-DNA II

In order to be able to select putative transformants, a second T-DNA was inserted during the transformation procedure. This contained the *neomycin phosphotransferase II* (*nptII*) coding region derived from transposon Tn5 of the bacterium *Escherichia coli*. The gene confers resistance to kanamycin. It was under the regulation of the constitutive Cauliflower mosaic virus (CaMV) 35S promoter and the *nos* terminator. As discussed in Section 3.1, T-DNA II was removed from KK179 by selection.

3.3 Breeding to obtain lucerne line KK179

A breeding programme was undertaken for the purposes of:

- obtaining generations suitable for analysing the molecular and genetic characteristics of lucerne KK179
- ensuring that the KK179 event is incorporated into elite proprietary breeding line(s) for commercialisation..

The breeding pedigree for the various generations is given in Figure 3.

Initial transformants (T_0 plants) were crossed to a non-GM male sterile line (Ms208) to produce F_1 plants. Following selection for plants with desirable characteristics and not containing T-DNA II, a single plant (P_0) designated KK179 was obtained. Lucerne is an autoploid, self-incompatible outcrossing species which cannot be self-pollinated to produce pure isogenic lines because of severe inbreeding depression (see discussion and references in Katepa-Mupondwa *et al*., 2002). Subsequent generations were therefore developed by traditional lucerne breeding techniques. The P_0 plant was hand-crossed with each of 10 elite lucerne genotypes with a fall dormancy 4 (FD4)[2] phenotype. The FD4 plants were used as the female seed parents and this breeding step is known as a modified backcross (MBC). The resulting progeny are designated MBC1 and were hand crossed with the same 10 elite FD4 genotypes to produce MBC2. Eighty MBC2 plants shown to be positive (by endpoint TaqMan PCR) for the *CCOMT* suppression cassette were then crossed amongst themselves in a breeding step known as a polycross to produce the Syn1 generation, the KK179 preferred population for entry into commercial variety development. Pollen from 20 MBC2 plants shown to be positive for the *CCOMT* suppression cassette was used to pollinate an FD4 population to produce MBC3 seed. Syn1 Adv was a subsequent synthetic population produced by crossing the Syn1 population in a polycross.

An identical breeding process was followed using a C_0 (the cross between ‘R2336’ and ‘Ms208’) plant (see Section 2.1) instead of the T_0 plants in order to produce populations that could be used as conventional comparators.

[2] Fall dormancy refers to the adaptation of lucerne to environments, including shortening photoperiods and declining temperatures in late summer and autumn. FD is usually divided into three types: dormant (FD 1 - 3 classes), semi or intermediate dormant (FD 4 -6 classes), and non-dormant (FD 7 - 9 classes). In autumn, dormant varieties grow very slowly or cease to grow and favour the synthesis and accumulation of soluble sugars, enabling the crops to survive over a hard winter (Small, 2011)

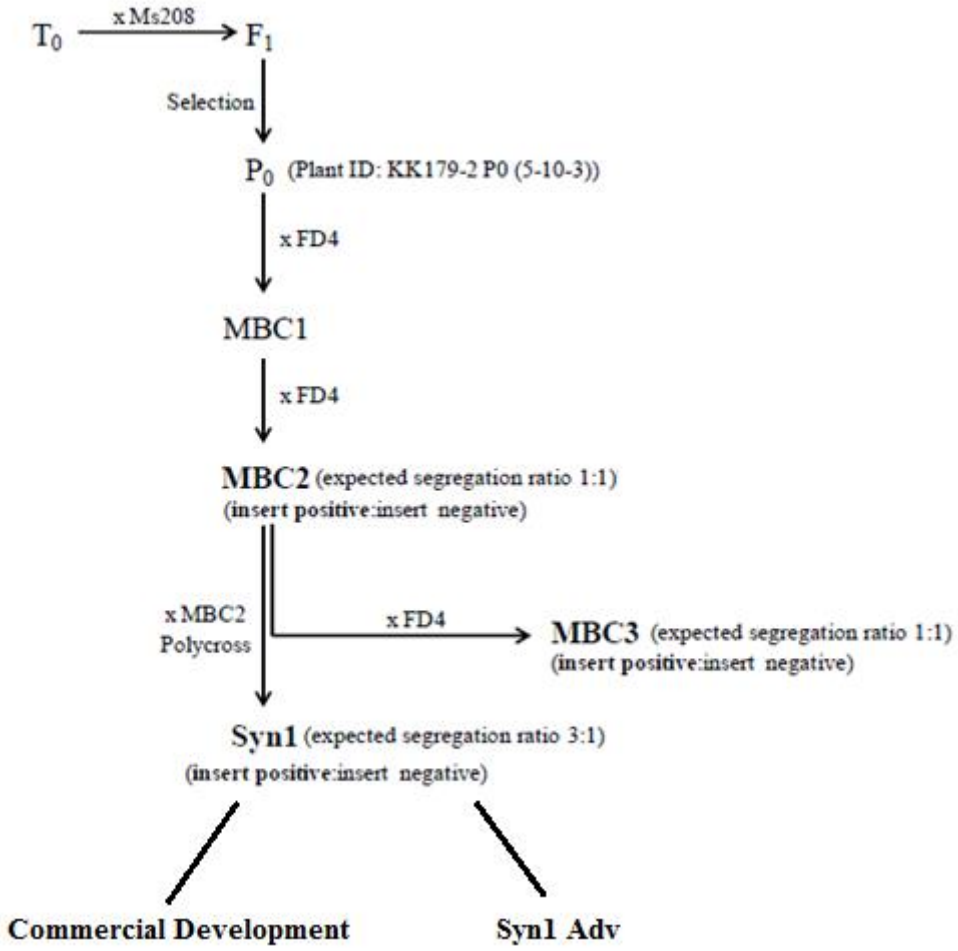

Figure 3: Breeding strategy for plants containing event KK179

Table 3 indicates the generations that were used in the various studies characterising lucerne KK179.

Table 3: KK179 generations used for various analyses

Analysis	KK179 Generation used	Control(s) used
Molecular characterisation	P_0	R2336; Ms208; C_0
Mendelian inheritance	MBC2, MBC3, Syn1	
Genetic stability	P_o; MBC1; MBC2; Syn1	
Phenotypic stability	MBC1, Syn1, Syn1 Adv	C_0-derived equivalents of the 3 generations
Northern blot analysis	Syn1	C_0 Syn1
Compositional analyses	Syn1	C_0 Syn1

3.4 Characterisation of the genetic material in the plant

A range of analyses was undertaken to characterise the genetic modification in lucerne line KK179. These included: DNA sequence, determination of insert copy number and integrity; Open Reading Frame (ORF) analysis of inserted DNA as well as flanking and junction regions and Northern blot analysis to determine whether the level of transcription of the endogenous *CCOMT* gene had been down-regulated as predicted.

3.4.1 Insert characterisation

Genomic DNA was obtained from verified leaf tissue of P_o generation lucerne KK179 and analysed using Southern blotting to determine copy number, insertion site(s), presence/absence of plasmid backbone and presence/absence of T-DNA II. PCR and DNA sequence analysis were used to provide the DNA sequence of the insert and flanking regions and to demonstrate the intactness and organisation of the insertion site.

Transgene copy number, insertion site, T-DNA II presence/absence and plasmid backbone analysis

Copy number, and insertion site of T-DNA I were evaluated by digesting the DNA from P_0 with two sets of restriction enzymes designed to cleave once within the inserted DNA and once within each flanking region. If T-DNA I sequences are present as one copy at a single integration site in KK179, then a specific banding pattern would be predicted. The resulting DNA fragments were separated and transferred to a membrane for sequential hybridisation with five radiolabelled probes (see red lines labelled 1 – 4 in the centre of Figure 1) that represented functional elements within T-DNA I.

The presence/absence of T-DNA II was assessed by using a single T-DNA II probe (see blue line labelled 5 in Figure 1). This probe contained sequences homologous to the nos 3' UTR sequence present in the T-DNA I. Thus, the presence of T-DNA II sequences would be indicated by the appearance of hybridization bands in the Southern blot additional to the band generated by the T-DNA I homology.

The presence/absence of plasmid backbone was assessed by using four backbone probes (see green lines in the centre of Figure 1). The presence of backbone sequences would be indicated by the appearance of hybridization bands on the Southern blot.

A positive control (DNA from 'C_0' spiked with either digested PV-MSPQ12633 DNA and/or probe template(s)) was also included in the Southern blot analyses. C_0 DNA digested with appropriate restriction enzymes was always used as a negative control and R2336- and Ms208-digested DNA were used in blots using Probe 3. Probe 3 covers the *CCOMT* region of PV-MSPQ12633 which therefore contains sequences identical to the endogenous *CCOMT* present in the lucerne genome. Thus, it would be expected that the random segregation of endogenous *CCOMT* in different non-GM lucerne genomes would lead to different banding patterns when probed with Probe 3. The KK179 event was generated by crossing transformed 'R2336' with non-GM 'Ms208' and in order to show all endogenous *CCOMT* alleles, both non-GM parents were included in addition to C_0 as negative controls. A hybridisation band that appeared in KK179 as well as in one or both of 'R2336' and 'Ms208' would indicate endogenous *CCOMT* not associated with the transformation event.

The Southern blot analyses indicated that there is a single insert of the *CCOMT* suppression cassette in event KK179 and that the arrangement of the genetic material is the same as that in the T-DNA I of the PV-MSPQ12633 plasmid (refer to Figure 1). No T-DNA II or plasmid backbone sequences are present in KK179.

Sequence analysis

Five overlapping PCR fragments spanning the insert and adjacent flanking DNA sequences in event KK179 (Figure 4) were amplified and purified before being sequenced using BigDye terminator chemistry (Applied Biosystems, Foster City, CA). The sequences were then aligned to obtain a consensus sequence which was compared to the PV-MSPQ12633 sequence. As a control, PCR using the same ten pairs of primers was also performed on genomic DNA from 'R2336'.

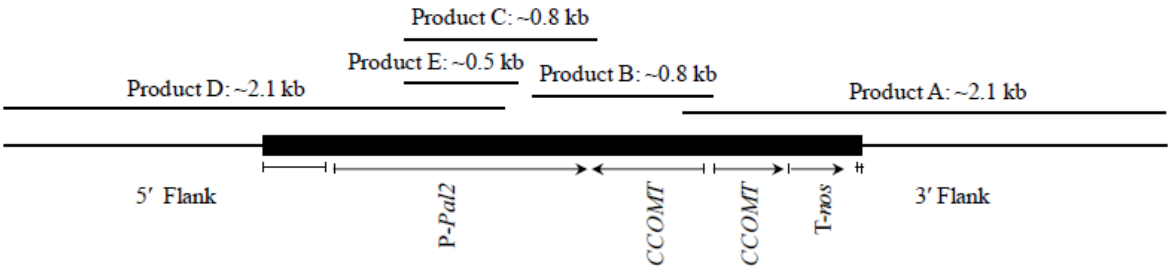

Figure 4: Schematic location and predicted sizes of the five PCR products amplified from KK179

As expected, no PCR products were obtained for 'R2336' DNA. The reactions using KK179 DNA produced band sizes as predicted, thereby confirming the organisation of the insert was the same as that for T-DNA I. The sequencing analysis showed that the insert is 2,582 bp in length and aligns to the T-DNA I sequence in PV-MSPQ12633 beginning at base 168 in the Left Border region and ending at base 2,749 in the Right Border region (refer to Table 2).

Analysis of the flanking regions provided sequences of 1047 bp at the 5' end and 1256 bp at the 3' end.

Integrity of insertion site

PCR and sequence analyses were performed on genomic DNA from KK179 (P_0) and 'R2336'. Two primers were used – a forward primer specific to the 5' flanking region of the insert and a reverse primer specific to the 3' flanking region. PCR of both 'R2336' and KK179 generated a PCR product of approx.. 0.7 kb. The product from 'R2336' was sequenced and then a consensus sequence was generated, by compiling multiple sequencing reactions performed on the verified PCR product, and then aligned to the 5' and 3' flanking sequences of the KK179 insert.

The alignment showed that base 742 to base 1047 of the 5' flanking region of the KK179 insert are identical with base 1 to base 306 of 'R2336' except for one base, and that base 3630 to base 3855 of the KK179 insert are identical with base 409 to base 634 of 'R2336' except for one base. This suggests that 102 bases (between base 307 – base 408) of the 'R2336' genome were deleted as a result of the T-DNA integration.

3.4.2 Novel open reading frame (ORF) analysis

An *in silico* analysis of the flanking regions was done to determine whether any novel ORFs had been created in KK179. Each analysis comprised a search of six-frame translations between stop codons (TGA, TAG, TAA) for sequences coding for eight amino acids or greater. Five ORFs in the 5' flanking region and five in the 3' flanking region were identified. A discussion of the bioinformatic analysis of these ORFs is given in Section 4.1.

For the DNA in the insert, the DNA sequences in the sense and anti-sense strands were translated to yield 6 reading frames and all sequences were then translated using DNAStar, EditSeq (Version 8.0.2). The resultant amino acid sequences were used for bioinformatic analyses described in Section 4.1.

3.5 The expression of endogenous *CCOMT* in KK179

Northern blot analyses were used to compare the RNA levels associated with the endogenous *CCOMT* gene in forage and root tissue of KK179. Since the intention of the genetic modification is to reduce expression of this gene by RNAi, it would be expected that the *CCOMT* mRNA levels would be reduced in KK179.

Four replicates of forage and root tissue from verified plants (Syn1 generation) of KK179 and C_0 Syn1 were harvested and total RNA was extracted. From this, PolyA$^+$ RNA (i.e. polyadenylated mRNA) was extracted and run on Northern blots probed with a radiolabelled *CCOMT* probe amplified from C_0 genomic DNA. An actin probe was also used for the purpose of demonstrating the quality and relative amount of each PolyA$^+$ sample. PolyA+ RNA from the forage tissue of the conventional control produced a strong hybridization signal at the expected molecular weight of ~1.1 kb for the *CCOMT* transcript, whereas no detectable hybridisation signal was produced from the polyA+ RNA isolated from the forage tissue of KK179. Similar results were obtained for the PolyA+ RNA from root tissue except that the KK179 sample did produce a positive hybridisation signal, albeit at a much reduced level. These data show a clear reduction in the level of *CCOMT* mRNA in KK179 compared to the conventional control and hence that insertion of the *CCOMT* suppression cassette has resulted in the intended modification.

3.6 Stability of the genetic change

The concept of stability encompasses both the genetic and phenotypic stability of the introduced trait over a number of generations. Genetic stability refers to maintenance of the modification over successive generations, as produced in the initial transformation event. It is best assessed by molecular techniques, such as Southern analysis or PCR, using probes and primers that cover the entire insert and flanking regions. Phenotypic stability refers to the expressed trait remaining unchanged over successive generations. It is often quantified by a trait inheritance analysis to determine Mendelian heritability via assay techniques (chemical, molecular, visual).

Genetic stability was assessed by Southern blot analyses of genomic DNA isolated from verified leaf tissue from P_0, MBC1, MBC2 and Syn1 generations (refer to Figure 3) of KK179 and from C_0. Two radiolabelled probes covering the T-DNA I in plasmid PV-MSPQ12633 were utilized following digestion with two restriction enzymes. Any instability associated with the insert would be detected as novel bands on the Southern blot. The analyses showed that the hybridization bands specific to the insert were identical for DNA from the P_0, MBC1, MBC2 and Syn1 generations of KK179, and hence that the insert is stably inherited from one generation to the next. No hybridization bands were obtained for DNA from C_0.

Mendelian inheritance was assessed using leaf tissue from verified plants of the MBC2, MBC3 and Syn1 generations (refer to Figure 3). Genomic DNA was isolated from leaf discs and endpoint TaqMan PCR analysis was done using primers and probes specific to the KK179 insert. A chi-square (X^2) analysis of the segregation data over each of the generations was used to test the hypothesis that the insert was inherited according to Mendelian principles i.e the segregation was approximately 1:1 (presence:absence) in the MBC2 and MBC3 generations and approximately 3:1 in the Syn1 generation. The X^2 values

obtained confirmed that the hypothesis was correct and also supported the conclusion that the KK179 insert has been stably integrated into a single locus in the KK179 genome.

Phenotypic stability was also indirectly assessed by measuring the total lignin content (Acid Detergent Lignin = ADL) of the forage of KK179 over three generations (MBC1, Syn1 and Syn1-Adv). The forage was grown on replicated plots at two sites in the U.S. and was analysed for total lignin (acid detergent lignin). Non-GM controls (of each generation) were also grown and analysed. For each generation, the mean %dw of lignin in the KK179 forage was significantly ($P<0.05$) less than in the control forage (Table 4).

Table 4: Levels of acid detergent lignin in various generations of KK179 and non-GM control plants

Generation	ADL in Control (A) (%dw)	ADL in KK179 (B) (%dw)	A vs B (P-value)
MBC1	5.31 ± 0.36	4.37 ± 0.36	0.002
Syn1	4.64 ± 0.36	4.02 ± 0.36	0.034
Syn1-Adv	4.62 ± 0.36	3.91 ± 0.36	0.016

3.7 Antibiotic resistance marker genes

No antibiotic marker genes are present in lucerne KK179. Plasmid backbone analysis and T-DNA II analysis (refer to Section 3.4.1) show that

- no plasmid backbone has been integrated into the lucerne genome during transformation, i.e. the *aadA* gene, which was used as a bacterial selectable marker gene, is not present in lucerne KK179
- the *nptII* gene.incorporated in T-DNA II during the initial transformation procedure has been segregated out of KK179 by traditional breeding.

3.8 Conclusion

Comprehensive molecular analyses of lucerne line KK179 indicate there is a single insertion site containing a single copy of the T-DNA I (containing the *CCOMT* suppression cassette) from plasmid PV-MSPQ12633. No DNA sequences from T-DNA II or from the backbone of the transformation vector, including antibiotic resistance marker genes, were transferred to the plant. As expected from the nature of the genetic modification, there has been a reduction in the mRNA produced from the endogenous *CCOMT* gene. The introduced genetic elements are stably inherited from one generation to the next.

4. Characterisation of novel substances

The *CCOMT* partial sequence introduced into line KK179 is derived from an endogenous gene already present in lucerne and therefore its presence in the plant is not novel. It is designed to give rise to a non-coding dsRNA. Translation of this dsRNA is considered unlikely because the hairpin secondary structure prevents engagement of the 40S ribosomal subunit necessary to initiate translation at the 5’ end of the RNA, and/or it prevents unwinding of the duplex such that the 40S subunit is unable to advance along it (Kozak, 1989). As discussed in Section 3.2.1, such dsRNA is also cleaved into small dsRNAs which themselves would have limited potential for translation. Therefore, no novel proteins are produced as a consequence of the genetic modification.

Any small dsRNAs produced in line KK179 do not present a safety concern. Small RNAs in general are abundantly present in the human diet from both plant and animal sources (Ivashuta *et al*., 2009; Carthew and Sontheimer, 2009), and small RNAs have been identified that are associated specifically with the endogenous regulation of lignin biosynthesis (see e.g. Ong and Wickneswari, 2012).

Given the absence of any novel protein, and the lack of safety concerns associated with the production of small dsRNAs, the remainder of the characterisation focusses on the potential toxicity/allergenicity of any new ORFs created by the insertion of new genetic material into the plant genome, which may potentially or theoretically give rise to novel proteins.

4.1 Potential allergenicity/toxicity of any novel ORFs created by the transformation procedure

Studies submitted:

2012. Bioinformatics Evaluation of the Transfer DNA Insert in KK179 Utilizing the AD_2012, TOX_2012 and PRT_2012 Databases. **MSL0024048**. Monsanto Company (unpublished)
2012. Bioinformatics Evaluation of DNA Sequences Flanking the 5' and 3' Junctions of Inserted DNA in KK179: Assessment of Putative Polypeptides. **MSL0023975**. Monsanto Company (unpublished)

As described in Section 3.4.2, translated sequences in the insert and flanking regions were obtained. In the case of the insert, all possible sequences in the six reading frames were used in the bioinformatic analysis, while for the flanking regions, ten sequences of 8 amino acids or greater (i.e. ORFs) were identified for bioinformatic analysis.

Assessment of potential allergenicity

To evaluate the similarity to known allergens of proteins that might potentially be produced from translation of the sequences, an epitope search was carried out to identify any short sequences of amino acids that might represent an isolated shared allergenic epitope. This search compared the sequences with 1,603 sequences in the Allergen, Gliadin and Glutenin sequence database (designated AD_2012), residing in the FARRP (Food Allergy Research and Resource Program) dataset (Version 10) within AllergenOnline (University of Nebraska; http:www.allergenonline.org/). The FASTA algorithm (Pearson and Lipman, 1988), version 3.4t 26 (July 7, 2006) was used to search the database using the BLOSUM50 scoring matrix (Henikoff and Henikoff, 1992). The BLOSUM series of matrices tabulate the frequency with which different substitutions occur in conserved blocks of protein sequences and are effective in identifying distant relationships. In addition, each sequence was used as a query for an eight amino acid sliding window search (Metcalfe *et al*., 1996) of the AD_2012 database.

For the insert, no alignments with any of the query sequences generated an E-score[3] of $\leq 1e^{-5}$, and no alignment met or exceeded the Codex Alimentarius (Codex, 2003) FASTA alignment threshold for potential allergenicity. A single potential immunologically relevant sequence of eight contiguous amino acids was detected in an eight amino acid sliding window search. The alignment was to a region in wheat (*Triticum aestivum*) dehydrin and was shown, in KK179, to be due to a sequence in the *CCOMT* fragment. As discussed in the introductory paragraph to Section 4, the RNA transcribed from the *CCOMT* fragment will fold

[3] Comparisons between highly homologous proteins yield E-values approaching zero, indicating the very low probability that such matches would occur by chance. A larger E-value indicates a lower degree of similarity. For FASTA searches, hits with E-values of 10^{-6} or less imply homology but any conclusions reached need to be tempered by an investigation of the biology behind the putative homology (Baxevanis, 2005). In this application an E-value of 10^{-5} or less was set as the high cut-off value for alignment significance.

back on itself to form a hairpin structure which is subsequently further processed, making translation unlikely. Furthermore, the CCOMT fragment is derived from a gene that is already naturally present and expressed in lucerne. The identified sequence similarity with a wheat sequence is therefore of no significance.

For the 10 ORFs in the flanking regions, no alignments with any of the query sequences generated an E-score of ≤$1e^{-5}$, no alignment met or exceeded the Codex Alimentarius (Codex, 2003) FASTA alignment threshold for potential allergenicity, and no alignments of eight or more consecutive identical amino acids were found between any query sequence and the sequences in the AAD_2012 database.

Assessment of potential toxicity

The KK179 sequences were also compared with 24,731,719 sequences present in the GenBank protein database (http://www.ncbi.nlm.nih.gov/genbank/), release 187.0 (designated PRT_2012), which contains 12,866 toxin proteins, using the FASTA algorithm. No significant similarities of either the KK179 insert sequences or flanking region ORF sequences to any toxin sequences in the database were found.

Conclusion

It is concluded that, in the unlikely event transcription and translation of frames 1 – 6 of the inserted T-DNA I sequences or flanking region ORFs could occur, the encoded polypeptides do not share any significant similarity with known allergens or toxins.

5. Compositional analysis

The main purpose of compositional analysis is to determine if any unexpected changes in composition have occurred to the food and to establish its nutritional adequacy. Compositional analysis can also be important for evaluating the intended effect where there has been a deliberate change to the composition of food.

The classic approach to the compositional analysis of GM food is a targeted one; rather than analysing every single constituent, which would be impractical, the aim is to analyse only those constituents most relevant to the safety of the food or that may have an impact on the whole diet. Important analytes therefore include the key nutrients, toxicants and anti-nutrients for the food in question. The key nutrients and anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates or enzyme inhibitors as anti-nutrients) or minor constituents (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in an organism, such as compounds whose toxic potency and level may be significant to health (eg solanine in potatoes).

5.1 Key components of lucerne

Main indicators of alfalfa quality for livestock feeding (OECD, 2005) include the proximates, acid detergent fibre, neutral detergent fibre, lignin, and minerals and also saponins, condensed tannins, oestrogen agonists and antagonists and cyanogenic glycosides. OECD (2005) suggests that a minimum compositional analysis where alfalfa is likely to be sold for food use would be the analysis of fresh forage or sprouted alfalfa seed for crude protein, fat, ash, fibre, lignin, amino acids, minerals with the addition of vitamin C, beta-carotene, folate and phytoestrogens to provide a basis for assessment of potential unintended effects with relevance to human food use. Analyses for key components were done on fresh forage (see Section 5.2).

Studies submitted:

2012. Composition Analyses of Forage from KK179 Alfalfa Grown in the United States during the 2011 Growing Season. **MSL0023847**. Monsanto Company (unpublished)
2012. Amended Report for MSL0023982: Composition of Lignin of Forage from KK179 Alfalfa Grown in the United States during the 2011 Growing Season. **MSL0024403**. Monsanto Company (unpublished).
2012. Analyses of Lignin in Forage from KK179 Alfalfa Grown in the United States during the 2011 Growing Season. **MSL0024120**. Monsanto Company (unpublished)
2012. Analyses of Saponin Levels of Forage from KK179 Alfalfa Grown in the United States during the 2011 Growing Season. **MSL0023980**. Monsanto Company (unpublished)

5.2 Study design, conduct and analysis

The test (PCR-verified KK179, seed of Syn1 generation), and control (PCR-verified C_0 Syn1) lines were grown under agronomic conditions pertinent to their geographic regions at six field sites across North America[4] during the 2011 growing season. The sites were representative of where lucerne is commercially grown. Fourteen different non-GM lucerne lines (PCR-verified) were also grown under the same conditions in order to generate a reference range for each analyte. All lines were planted in a randomised complete block design, with four replicated plots at each of the six sites.

Forage from the lines was harvested (first cut) approximately 6 cm above the soil surface when the plants were between 1% and 10% bloom (Ball, 1998), a stage between growth stages 4 and 5 as described by Mueller & Teuber (2007) and recognised as the ideal stage at which to harvest forage for maximum yield and minimal loss of quality. It was immediately frozen and then shipped to a laboratory for grinding to powder before frozen storage. Methods of composition analysis were based on internationally recognised procedures (e.g. those of the Association of Analytical Communities - AOAC), methods specified by the manufacturer of the equipment used for analysis, or other published methods.

For each analyte 'descriptive statistics' were generated i.e. a mean (least square mean) and standard error (S.E.) averaged over all sites (combined-site analysis). The values thus calculated are presented in Tables 5 – 9.

The analytes were analysed using a mixed model analysis of variance. Data were transformed into Statistical Analysis Software[5] (SAS) data sets and analysed using SAS® software (SAS MIXED). The four replicated sites were analysed both separately and combined across all sites (combined-site analysis). In assessing the significance of any difference between means, a P-value of 0.05 was used (i.e. a P-value of ≥0.05 was not significant).

Any statistically significant differences between KK179 and the C_0 Syn1 control have been compared to the 95% tolerance interval (i.e. 95% confidence that the interval contains 99% of the values expressed in the commercial lines) compiled from the results of the fourteen commercial reference lines combined across all sites, to assess whether the differences are likely to be biologically meaningful. Additionally, the results for KK179 and the C_0 Syn1 have been compared to a combined literature range for each analyte, compiled from published

[4] The six sites were: Tulare County, CA; Jefferson County, IA; Clinton County, IL; Pawnee County, KS, Armstrong County, TX and Walworth County, WI.
[5] SAS website - http://www.sas.com/technologies/analytics/statistics/stat/index.html

literature[6]. Any mean value for a lucerne KK179 analyte that fell within the combined literature range was considered to be within the normal variability of commercial lucerne cultivars even if the mean value was statistically different from the C_0 Syn1 control. It is noted, however, that information in the published literature is limited and is unlikely to provide a broad reflection of the natural diversity that occurs within lucerne. Therefore, even if means fall outside the published range, this is not necessarily a concern.

5.3 Forage composition

Forage samples were analysed for nutrients; proximates (ash, fat, moisture, and protein), carbohydrates by calculation, acid detergent fibre (ADF), neutral detergent fibre (NDF), acid detergent lignin (ADL), minerals (Ca, Cu, Fe, Mg, Mn, P, K, Na, and Zn), and amino acids (essential and non-essential). Antinutrient and secondary metabolites included daidzein, glycitein, genistein, coumesterol, formononetin, biochanin A, and canavanine. In addition to the OECD (OECD, 2005) recommended analytes, *p*-coumaric acid, ferulic acid, sinapic acid, total polyphenols and free phenylalanine were also analysed to evaluate the potential effect of *CCOMT* suppression on lignin pathway and cell wall-associated metabolites.

5.3.1 Proximates and fibre

Results of the proximate and fibre analysis are shown in Table 5. With regard to lignin level, two separate analyses using two different methods (designated ADL1 and ADL2 in Table 5) were done on plants from the same trial. The major differences between the analyses were in sample fineness of grind and method automation. It is noted that in the ADL1 lignin, there is no statistically significant difference between KK179 and the control (although the actual numerical value is lower in KK179), while in the ADL2 lignin, the level is statistically significantly lower in KK179 compared to the control. It is accepted, considering also the results provided both in Section 3.6 (Table 4) and in Table 6 that there has been a genuine reduction in the level of lignin in line KK179 compared with the control, even though the reduced level is higher than the level at the lower end of both the reference range and the combined literature range. It is noted that there would be a limit to the amount of reduction in lignin that would be agronomically acceptable since too big a reduction would result in the lodging of plants. It is further noted that the predominant purpose of the genetic modification in KK179 is to provide an agronomic benefit rather than a nutritional change *per se*. It has long been understood that dietary lignin is not appreciably metabolized by animals (Crampton and Maynard, 1938). The amount deposited in cell walls increases with increasing plant maturity thereby reducing forage quality. Therefore when a crop is used for forage there will be a trade-off between maximising yield and minimising lignin content. For line KK179, a reduction in lignin means that plants could be harvested later than standard lines to get the same forage quality but higher yield.

The only other significant difference occurred in a comparison of the ash in C_0 Syn1 and KK179 where the latter had a lower mean value. However, this mean was well within both the reference range and the range reported in the literature.

[6] References included: Smith (1969); Jung & Fahey Jr. (1983); Natelson & Bratton (1984); Cherney *et al.* (1989); Bourquin *et al.* (2013); Rosenthal & Nkomo (2000); OECD (2005); McCann et al (2013); Dairyland Laboratories (2012).

Table 5: Mean percentage ± S.E. of proximates and fibre in forage from C_0 Syn1 and KK179

Analyte	'C_0 Syn' (A)	KK179 (B)	A vs B (P-value)	Reference range	Combined literature range
Protein (%dw)	21.02 ± 1.35	20.83 ± 1.36	NS	14.52 – 30.07	14.91 – 28.34
Fat (%dw)	2.28 ± 0.17	2.28 ± 0.17	NS	0.53 – 4.21	1.3 – 3.24
Ash (%dw)	10.79 ± 0.52	10.38 ± 0.53[5]	0.034	7.54 – 13.23	5.8 – 15.3
Moisture (%fw)	78.15 ±1.54	78.26 ± 1.54	NS	66.10 – 85.30	7.74 – 83.5
Carbohydrate (%dw)[1]	65.97 ± 1.70	66.55 ± 1.71	NS	54.35 – 74.91	56.63 – 74.8
ADF[2] (%dw)	27.02 ± 2.44	27.03 ± 2.45	NS	7.07 – 39.11	21.26 – 42.59
NDF[3] (%dw)	34.46 ± 2.63	33.95 ± 2.64	NS	18.97 – 49.82	26.5 – 53.56
ADL1[6] (%dw)	6.54 ± 0.59	6.22 ± 0.60	NS	3.38 – 9.67	2.31 – 13.71
ADL 2 (%dw)	6.93 ± 0.64	5.39 ± 0.64	0.004	1.70 – 10.03	2.31 – 13.71

[1]Carbohydrate calculated as 100% - (protein %dw + fat %dw + ash %dw)
[2]ADF = acid detergent fibre
[3]NDF = neutral detergent fibre
[4]ADL = acid detergent lignin
[5]Mauve shading represents a KK179 mean with a significantly lower value than the C_0 Syn1 mean.
[6]See text above for explanation of ADL1 and ADL2

Lignin components

Section 3.2.1 describes the three main components that go to make up lignin in lucerne.

An analysis of these individual components of lignin in KK179 and the C_0 Syn1 control was done using data collected at the same time from the same sites and with the same 14 reference varieties. Forage samples were analysed for levels of p-hydroxyphenyl (H) lignin, caffeyl (C) lignin, guaiacyl (G) lignin, 5-hydroxyguaiacyl lignin, and syringyl (S) lignin units. The C lignin and 5-hydroxyguaiacyl lignin unit components were below the limit of quantitation for all samples and were excluded from the statistical analysis. The results for the remaining lignin components are given in Table 6. These results considered the analytes in terms of a) the amount of each analyte present per cell wall residue (CWR); b) the proportion of each analyte in the total (H + G + S) lignin; and c) the ratio of S:G lignin.
As expected from the intention of the genetic modification, the mean level of G lignin in KK179 was significantly lower than the mean in C_0 Syn1 and the proportion of G lignin expressed in the total (H + S + G) lignin was significantly lower in KK179 compared to C_0 Syn1. To counteract this lower proportion of G lignin in the total, there was a corresponding increase in the proportions of H and S lignin and in the S:G ratio in KK179.

The results support the conclusion that suppression of the endogenous *CCOMT* gene, as a result of the genetic modification, acts to decrease the amount of G lignin.

Table 6: Summary of forage lignin unit content in C_0 Syn1 and KK179

Lignin Component	'C_0 Syn' (A)	KK179 (B)	A vs B (P-value)	Reference range	Combined literature range
µmole/g CWR					
Guaiacyl lignin	83.72 ± 9.40	68.10 ± 9.48	0.027	25.34 – 153.11	NA
Hydroxyphenyl lignin	3.88 ± 0.43	5.05 ± 0.45	NS	0.29 – 8.26	NA
Syringyl lignin	50.41 ± 8.78	55.96 ± 8.83[5]	NS	5.64 – 110.93	NA
% Total (H + G + S)					
Guaiacyl lignin	61.69 ±1.87	53.69 ± 1.87	<0.001	50.02 – 76.69	NA
Hydroxyphenyl lignin	3.07 ± 0.54	4.22 ± 0.54	0.001	0.18 – 6.23	NA
Syringyl lignin	35.24 ± 2.35	42.09 ± 2.35	<0.001	17.07 – 46.14	NA
Ratio					
S:G	0.58 ± 0.060	0.80 ± 0.060	<0.001	0.22 – 0.92	NA

5.3.2 Amino acids

Levels of 18 amino acids were measured. Since asparagine and glutamine are converted to aspartate and glutamate respectively during the analysis, levels for aspartate include both aspartate and asparagine, while glutamate levels include both glutamate and glutamine.

Results of the analysis are given in Table 7. There was no significant difference between C_0 Syn1 and KK179 for any of the amino acids and all means fell within both the reference range and literature range.

Table 7: Mean percentage dry weight (dw) ± S.E., relative to total dry weight, of amino acids in forage from C_0 Syn1 and KK179

Amino Acid	'C_0 Syn' (A) %dw	KK179 (B) %dw[1]	A vs B (P-value)	Reference range %dw	Combined literature range %dw
Alanine	1.13 ± 0.074	1.11 ± 0.074	NS	0.80 – 1.66	0.70 – 1.59
Arginine	1.01 ± 0.065	0.99 ± 0.065	NS	0.70 – 1.44	0.62 – 1.54
Aspartate	2.74 ± 0.28	2.77 ± 0.28	NS	1.96 – 5.15	1.40 – 3.52
Cysteine	0.21 ± 0.011	021 ± 0.012	NS	0.16 – 0.31	0.18 – 0.35
Glutamate	1.91 ± 0.12	1.85 ± 0.12	NS	1.31 – 2.80	1.20 – 3.03
Glycine	0.97 ± 0.055	0.95 ± 0.055	NS	0.70 – 1.33	0.60 – 1.47
Histidine	0.44 ± 0.020	0.43 ± 0.020	NS	0.34 – 0.61	0.28 – 0.74
Isoleucine	0.88 ± 0.053	0.86 ± 0.053	NS	0.63 – 1.27	0.50 – 1.26
Leucine	1.47 ± 0.089	1.43 ± 0.089	NS	1.03 – 2.05	0.90 – 2.25
Lysine	1.17 ± 0.067	1.14 ± 0.067	NS	0.82 – 1.73	0.59 – 1.81

Amino Acid	'C_0 Syn' (A) %dw	KK179 (B) %dw[1]	A vs B (P-value)	Reference range %dw	Combined literature range %dw
Methionine	0.24 ± 0.024	0.25 ± 0.024	NS	0.14 – 0.45	0.18 – 0.48
Phenylalanine	1.00 ± 0.061	0.98 ± 0.061	NS	0.71 – 1.39	0.72 – 1.59
Proline	0.92 ± 0.053	0.89 ± 0.054	NS	0.65 – 1.25	0.70 – 1.34
Serine	0.88 ± 0.044	0.87 ± 0.044	NS	0.66 – 1.25	0.60 – 1.36
Threonine	0.88 ± 0.050	0.86 ± 0.050	NS	0.63 – 1.23	0.60 – 1.15
Tryptophan	0.37 ± 0.020	0.37 ± 0.020	NS	0.25 – 0.50	0.16 – 0.35
Tyrosine	0.71 ± 0.042	0.71 ± 0.042	NS	0.52 – 1.01	0.50 – 1.16
Valine	1.07 ± 0.061	1.05 ± 0.061	NS	0.79 – 1.55	0.60 – 1.55

5.3.3 Minerals

Levels of 9 minerals were measured. The means for these are given in Table 8 and show there was no significant difference between C_0 Syn1 and KK179 for any of the minerals. All means fell within both the reference range and literature range.

Table 8: Mean values ± S.E. for mineral levels in forage from C0 Syn1 and KK179

Mineral	'C_0 Syn' (A)	KK179 (B)	A vs B (P-value)	Reference range	Combined literature range
Calcium (%dw)	1.72 ± 0.16	1.68 ± 0.16	NS	0.95 – 2.07	0.90 – 1.96
Copper (mg/kg dw)	8.34 ± 0.85	8.86 ± 0.85	NS	4.54 – 19.67	3.43 – 14.72
Iron (mg/kg dw)	315.74 ± 30.93	272.00 ± 31.45	NS	105.45 – 691.43	0.2 - 4749
Magnesium (%dw)	0.23 ± 0.023	0.22 ± 0.023	NS	0.11 – 0.34	0.11 – 0.45
Manganese (mg/kg dw)	52.45 ± 6.27	52.56 ± 6.30	NS	23.24 – 98.04	15.91 – 109.5
Phosphorus (%dw)	0.28 ± 0.019	0.29 ± 0.019	NS	0.18 – 0.43	0.22 – 0.46
Potassium (%dw)	2.41 ± 0.051	2.35 ± 0.052	NS	1.85 – 3.35	1.39 – 4.31
Sodium (%dw)	0.077 ± 0.024	0.089 ± 0.024	NS	0.016 – 0.20	0.017 – 0.51
Zinc (mg/kg dw)	26.81 ± 2.09	27.83 ± 2.11	NS	17.08 – 47.48	15.2 – 43.62

5.3.4 Phytoestrogens

Phytoestrogens are naturally-occurring plant compounds that are structurally and/or functionally similar to mammalian oestrogens and their active metabolites. Most are phenolic compounds of which the isoflavones and coumestans are the most widely researched groups (Patisaul and Jefferson, 2010). They are ubiquitous in the plant kingdom but are found particularly in soy and other legumes (Kurzer and Xu, 1997; Setchell, 1998).

Levels of daidzein, glycitein, genistein, formononetin and biochanin A (isoflavones), and coumestrol (coumestan) were measured in forage from KK179, C_0 Syn1 and the fourteen reference varieties. For all phytoestrogens except coumestrol, all levels across all sites in all lines were below the Limit of Quantitation (LOQ). For coumestrol approximately 80% of levels across all sites and all lines were below the LOQ. Statistical analysis of the phytoestrogens was therefore not meaningful.

5.3.5 Other analytes

A number of other analytes were included in the compositional analyses. The OECD (OECD, 2005) suggests that saponins and canavanine also be considered in a forage compositional analysis. Saponins are found in many plants, including edible species, but particularly legumes (Deshpande, 2002). In animal forage they have implications for bloating (OECD, 2005). In the human diet, their significance in either a beneficial or adverse role is unclear (Deshpande, 2002). Saponin analysis included measurement of levels of total saponins, and individual saponin compounds, specifically, bayogenin, hederagenin, medicagenic acid, soyasapogenol B, soyasapogenol E, and zanhic acid.

Canavanine is a potentially toxic structural analogue of L-arginine that is a stored by many legumes including alfalfa (Rosenthal and Nkomo, 2000).

Perturbations in the lignin biosynthetic pathway (see Figure 2) may produce effects that go beyond alterations in lignin amount, composition, and cell wall structure and have the potential to affect the expression level of other lignin pathway genes (Vanholme *et al*., 2010). Analyses were therefore also done of the following potentially affected analytes: p-coumaric acid, ferulic acid, sinapic acid, total polyphenols and free phenylalanine.

Too many measurements of sinapic acid were below the LOQ to allow a statistical analysis of this analyte to be carried out.The results for the remaining analytes are given in Table 9.

There were no significant differences between KK179 means and the control means for any of the saponins. The mean canavanine level was significantly lower in KK179 than in C_0 Syn1 but was within the reference range. Both the KK179 and C_0 Syn1 means were below the literature range; it is noted, however, that this range was compiled from only one reference (Natelson and Bratton, 1984). It is further noted that the leaves of alfalfa forage contain much lower levels of canavanine than seeds and sprouts. Typical levels of canavanine in sprouts is around 20,000 ppm (Rosenthal and Nkomo, 2000).

The ferulic acid mean was significantly higher in KK179 compared to the control but was still within both the reference range and literature range

Table 9: Mean levels ± S.E. of other analytes considered in forage from C0 Syn1 and KK179

Analyte	'C_0 Syn' (A)	KK179 (B)	A vs B (P-value)	Reference range	Combined literature range
Total bayogenin (response units /µg)	5.67 ± 0.76	5.10 ± 0.76	NS	1.46 – 11.28	NA
Total hederagenin (response units /µg)	3.47 ± 0.35	2.94 ± 0.35	NS	0.90 – 10.31	NA
Total medicagenic acid (response units /µg)	23.39 ± 2.44	21.88 ± 2.44	NS	2.04 – 48.33	NA
Total soyasapogenol B (response units /µg)	24.53 ± 3.02	22.17 ± 3.02	NS	9.22 – 43.87	NA
Total soyasapogenol E (response units /µg)	3.08 ± 0.54	2.77 ± 0.54	NS	0.91 – 7.53	NA

Analyte	'C_0 Syn' (A)	KK179 (B)	A vs B (P-value)	Reference range	Combined literature range
Total Zanhic acid (response units /µg)	5.16 ± 0.58	4.59 ± 0.58	NS	1.75 – 13.20	NA
Total saponins (response units /µg)	65.58 ± 4.94	59.30 ± 4.94	NS	17.38 – 103.19	NA
Canavanine (ppm dw)	57.24 ± 13.51	40.30 ± 13.53[1]	0.013	11.47 – 151.33	600 – 1,200
Ferulic acid (ppm dw)	1485.81 ± 58.83	1596.41 ± 59.57[1]	0.008	1103.32 – 1906.86	627 - 2840
Free phenylalanine (ppm dw)	283.70 ± 28.69	266.99 ± 28.84	NS	133.05 – 579.05	NA
Total polyphenols (mg/g dw)	7.99 ± 0.34	8.19 ± 0.34	NS	6.17 – 11.17	NA
p-Coumaric acid (ppm dw)	623.54 ± 37.34	639.50 ± 37.62	NS	326.19 – 945.58	398 - 1860

[1] Mauve shading represents a KK179 mean that was significantly lower than the C_0 Syn1 mean; orange shading represents a significantly higher mean value for KK179 compared with C_0 Syn1.

5.3.6 Summary of analysis of key components

A total of 50 analytes were analysed. In addition to the intended difference in lignin generally, and G lignin in particular, statistically significant differences in the three analyte levels found in forage of lucerne KK179 and C_0 Syn1 are summarised in Table 10.:

Table 10: Summary of analyte means found in forage from KK179 that are significantly (P<0.05) different from those found in forage of the control line C_0 Syn1

Analyte	'C_0 Syn'	KK179[1]	% difference	KK179 within Reference range?	KK179 within Combined literature range?
Ash (%dw)	10.79	10.38[1]	3%	yes	yes
ADL 2 (%dw)	6.93	5.39	23%	yes	yes
Guaiacyl lignin (µmole/g CWR)	83.72	68.10	19%	yes	NA
Guaiacyl lignin (% Total H+G+S)	61.69	53.69	13%	yes	NA
Canavanine (ppm dw)	57.24	40.30[1]	29%	yes	No – but neither was the control
Ferulic acid (ppm dw)	1485	1596[1]	7%	yes	yes

[1] Mauve shading represents a KK179 mean that was significantly lower than the C_0 Syn1 mean; orange shading represents a significantly higher mean value for KK179 compared with C_0 Syn1.

5.4 Conclusion

As expected, the level of lignin in general, and G lignin in particular, in KK179 were statistically significantly lower than in the control. However the levels were within the reference range obtained for non-GM reference varieties grown at the same time. While the difference in lignin levels between the GM line and the control is of agronomic significance, in that it enables the forage to be harvested at a later date without appreciable loss of forage quality, it is unlikely to have any nutritional significance to humans given the range of natural variation that exists in lucerne

For the remaining analytes, statistically significant differences were noted in only three analytes (ash, canavanine and ferulic acid). In all cases the differences were typically small and within the reference range obtained for non-GM reference varieties grown at the same time. Any observed differences are therefore considered to represent the natural variability that exists within lucerne.

7. Nutritional impact

In assessing the safety of a GM food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and well-being. In most cases, this can be achieved through an understanding of the genetic modification and its consequences, together with an extensive compositional analysis of the food.

If the compositional analysis indicates biologically significant changes to the levels of certain nutrients in the GM food, additional nutritional assessment should be undertaken to assess the consequences of the changes and determine whether nutrient intakes are likely to be altered by the introduction of such foods into the food supply.

Where a GM food has been shown to be compositionally equivalent to conventional varieties, as is the case for lucerne line KK179, the evidence to date indicates that feeding studies using target livestock species will add little value to the safety assessment and generally are not warranted (OECD, 2003; EFSA, 2008). Lucerne KK179 is the result of a genetic modification to silence the expression of an endogenous gene, with the intention of altering an agronomic characteristic. The extensive compositional analyses of forage, that have been undertaken to demonstrate the nutritional adequacy of line KK179, indicate it is equivalent in composition to conventional lucerne cultivars.

The Applicant did, however supply a lamb feeding study which has been evaluated by FSANZ.

Study submitted:

2012. Alfalfa hay from KK179 is wholesome when fed to growing lambs. **MSL0023898**. Monsanto Company (unpublished).

The analysis did not show any significant difference in the measured parameters (which included growth performance, blood chemistries and necropsy data) and general health between lambs fed a diet containing KK179 hay and those fed a diet containing hay from a conventional control (C_0-Syn Adv). This was consistent with the findings from the compositional analysis.

References[7]

Ball, S.T. (1998) Alfalfa growth stages. Cooperative Extension Service, College of Agriculture & Home Economics, New Mexico State University.

Barker, R.F., Idler, K.B., Thompson D.V. and Kemp, J.D. (1983) Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti plasmid pTi15955. *Plant Molecular Biology* 2:335-350.

[7] All website references were current as at 1 October 2013

Baxevanis, A.D. (2005) Assessing Pairwise Sequence Similarity: BLAST and FASTA. In: Baxevanis, A.D. and Ouellette, B.F.F. eds. *Bioinformatics: A Practical Guide to the Analysis of Genes and Proteins*. Chapter 11. John Wiley & Sons, Inc., pp. 295-324.

Bonnet, E., Van der Peer, Y. and Rouzé, P. (2006) The small RNA world of plants. *New Phytologist* 171:451-468.

Bora, K.S. and Sharma, A. (2011) Phytochemical and pharmacological potential of *Medicago sativa*: A review. *Pharmaceutical Biology* 49(2):211-220.

Bourquin, L.D., Garleb, K.A., Merchen, N.R. and Fahey Jr, G.C. (2013) Effects of intake and forage level on site and extent of digestion of plant cell wall monomeric components by sheep. *Journal of Animal Science* 68(2):2479-2495.

Bouton, J.H. (2012) Breeding lucerne for persistence. *Crop and Pasture Science* 63:95-106.

Buchanan, B.B., Gruissem, W. and Jones, R.L. (2000) *Biochemistry and molecular biology of plants*. American Society of Plant Physiologists, Rockville, Marlyland.

Carthew, R.W. and Sontheimer, E.J. (2009) Origins and mechanisms of miRNAs and siRNAs. *Cell* 136:642-655.

Cherney, J.H., Anliker, K.S., Albrecht, K.A. and Wood, K.V. (1989) Soluble phenolic monomers in forage crops. *Journal of Agricultural and Food Chemistry* 37:345-350.

Codex (2003) *Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants*. Report No. CAC/GL 45-2003, Codex Alimentarius. http://www.codexalimentarius.net/web/standard_list.do?lang=en.

Cramer, C.L., Edwards, K., Dron, M., Liang, X., Dildene, S.L., Bolwell, G.P., Dixon, R.A., Lamb, C.J. and Schuch, W. (1989) Phenylalanine ammonia-lyase gene organization and structure. *Plant Molecular Biology* 12(4):367-383.

Crampton, E.W. and Maynard, L.A. (1938) The relation of cellulose and lignin content to the nutritive value of animal feeds. *The Journal of Nutrition* 15(4):383-395.

Dairyland Laboratories Inc. (2012) Forage summaries 2012. https://www.dairylandlabs.net/pages/interpretations/forage_2012.php.

Depicker, A., Stachel, S., Dhaese, P., Zambryski, P. and Goodman, H.M. (1982) Nopaline synthase: transcript mapping and DNA sequence. *Journal of Molecular and Applied Genetics* 1(6):561-573.

Deshpande, S.S. (2002) Toxicants and antinutrients in plant foods: Section 10.14 - Saponins. In: *Handbook of food toxicology*. Chapter 10. Marcel Dekker, New York, pp. 321-386.

DPIPWE Tas. (2011) Lucerne market profile. Tasmanian Government Department of Primary Industries, Parks, Water & Environment, www.dpiw.tas.gov.au/internnsf/Attachments/.../Lucerne%20Profile.pdf.

EFSA. (2008) Safety and nutritional assessment of GM plants and derived food and feed: The role of animal feeding trials. *Food and Chemical Toxicology* 46:S1-S70, doi:10.1016/j.fct.2008.02.008.

Fraley, R.T., Rogers, S.G., Horsch, R.B., Sanders, P.R., Flick, J.S., Adams, S.P., Bittner, M.L., Brand, L.A., Fink, C.L., Fry, J.S., Galluppi, G.R., Goldberg, S.B., Hoffmann, N.L. and Woo, S.C. (1983) Expression of bacterial genes in plant cells. *Proceedings of the National Academy of Sciences* 80:4803-4807.

FSANZ (2007) *Application A575: Food derived from glyphosate-tolerant lucerne J101 and J163*. Report prepared by Food Standards Australia New Zealand. http://www.foodstandards.gov.au/foodstandards/applications/applicationa575foodd3166.cfm.

FSANZ (2011) *Proposal P1004 - Primary Production & Processing Standard for Seed Sprouts*. Report prepared by Food Standards Australia New Zealand. http://www.foodstandards.gov.au/code/proposals/pages/proposalp1004primary4361.aspx.

Guo, D., Chen, F., Inoue, K., Blount, J.W. and Dixon, R.A. (2001) Downregulation of caffeic acid 3-O-methyltransferase and caffeoyl CoA 3-O-methyltransferase in transgenic alfalfa:Impacts on lignin structure and implications for the biosynthesis of G and S lignin. *The Plant Cell* 13:73-88.

Hammond, S.M. (2005) Dicing and slicing: The core machinery of the RNA interference pathway. *FEBS Letters* 579:5822-5829.

Henikoff, S. and Henikoff, J.G. (1992) Amino acid substitution matrices from protein blocks. *Proceedings of the National Academy of Sciences* 89:10915-10919.

Inoue, K., Sewalt, V.J.H., Ballance, G.M., Ni, W., Stürzer, C. and Dixon, R.A. (1998) Developmental expression and substrate specificities of alfalfa caffeic acid 3-*O*-methyltransferase and caffeoyl coenzyme A 3-O-methyltransferase in relation to lignification. *Plant Physiology* 117:761-770.

Ivashuta, S.I., Petrick, J.S., Heisel, S.E., Zhang, Y., Guo, L., Reynolds, T.L., Rice, J.F., Allen, E. and Roberts, J.K. (2009) Endogenous small RNAs in grain: Semi-quantification and sequence homology to human and animal genes. *Food and Chemical Toxicology* 47:353-360.

Jung, H.G. and Fahey Jr, G.C. (1983) Nutritional implications of phenolic monomers and lignin: a review. *Journal of Animal Science* 57:206-219.

Katepa-Mupondwa, F.M., Christie, B.R. and Michaels, T.E. (2002) An improved breeding strategy for autotetraploid alfalfa (*Medicago sativa* L.). *Euphytica* 123:139-146.

Kozak, M. (1989) Circumstances and mechanisms of inhibition of translation by secondary structure in eucaryotic mRNAs. *Molecular and Cellular Biology* 9(11):5134-5142.

Krell, R. (1996) *Value-Added Products from BeeKeeping*. Chapter 3: Pollen, FAO Agricultural Services Bulletin No. 124, Food and Agriculture Organization of the United Nations, available online at http://www.fao.org/docrep/w0076e/w0076e00.htm#con.

Kurzer, M.S. and Xu, X. (1997) Dietary phytoestrogens. *Annual Reviews of Nutrition* 17:353-381.

McCann, M.C., Rogan, G.J., Fitzpatrick, S., Trujillo, W.A., Sorbet, R., Hartnell, G.F., Riodan, S.G. and Nemeth, M.A. (2013) Glyphosate-tolerant alfalfa is compositionally equivalent to conventional alfalfa (*Medicago sativa* L.). *Journal of Agricultural and Food Chemistry* 54:7187-7192.

Metcalfe, D.D., Astwood, J.D., Townsend, R., Sampson, H.A., Taylor, S.L. and Fuchs, R.L. (1996) Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Critical Reviews in Food Science and Nutrition* 36 Suppl:S165-S186.

Mielmann, A. (2013) The utilisation of lucerne (*Medicago sativa*): a review. *British Food Journal* 115(4):590-600.

Mueller, S.C. and Teuber, L.R. (2007) Alfalfa growth and development. In: Summers, G.C. and Putnam, D.H. eds. *Irrigated alfalfa management for Mediterranean and desert zones*. Chapter 3. University of California, Division of Agriculture and Natural Resources Publication 8289.

Natelson, S. and Bratton, G.R. (1984) Canavanine assay of some alfalfa varieties (*Medicago sativa*) by fluorescence: practical procedure for canavanine preparation. *Microchemical Journal* 29(1):26-43.

Odell, J.T., Nagy, F. and Chua, N.-H. (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature* 313:810-812.

OECD (2003) *Considerations for the Safety Assessment of Animal Feedstuffs Derived from Genetically Modified Plants*. Series on the Safety of Novel Foods and Feeds, No. 9. Organisation for Economic Cooperation and Development, Paris. http://www.olis.oecd.org/olis/2003doc.nsf/LinkTo/NT0000426A/$FILE/JT00147696.PDF.

OECD (2005) *Consensus document on compositional considerations for new varieties of alfalfa and other temperate forage legumes: key feed nutrients, anti-nutrients and secondary plant metabolites*. The Safety of Novel Foods and Feeds. Report No. ENV/JM/MONO(2005)13, Organisation for Economic Co-operation and Development, Paris.

Ong, S.S. and Wickneswari, R. (2012) Characterization of microRNAs Expressed during Secondary Wall Biosynthesis in *Acacia mangium*. *PLoS ONE (open access)* 7(11):e49662.

Parrott, W., Chassy, B.M., Ligon, J., Meyer, L., Petrick, J.S., Zhou, J., Herman, R.A., Delaney, B. and Levine, M. (2010) Application of food and feed safety assessment principles to evaluate transgenic approaches to gene modulation in crops. *Food and Chemical Toxicology* 48:1773-1790.

Patisaul, H.B. and Jefferson, W. (2010) The pros and cons of phytoestrogens. *Front Neuroendocrinal* 31(4):400-419.

Pearson, W.R. and Lipman, D.J. (1988) Improved tools for biological sequence comparison. *Proceedings of the National Academy of Sciences* 85(8):2444-2448.

Petrick, J.S., Brower-Toland, B., Jackson, A.L. and Kier, L.D. (2013) Safety assessment of food and feed from biotechnology-derived crops employing RNA-mediated gene regulation to achieve desired traits: A scientific review. *Regulatory Toxicology and Pharmacology* 66:167-176.

Rosenthal, G.A. and Nkomo, P. (2000) The natural abundance of L-canavanine, an active anticancer agent, in alfalfa, Medicago sativa (L.). *Pharmaceutical Biology* 38(1):1-6.

Schenk, R.U. and Hildebrandt, A.C. (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Canadian Journal of Botany* 50(1):199-204.

Setchell, K.D.R. (1998) Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. *American Journal of Clinical Nutrition* 68(suppl):1333S-1346S.

Small, E. (2011) *Alfalfa and relatives: evolution and classification of Medicago*. National Research Council of Canada (NRC Press), Ottawa, Ontario.

Smith, D. (1969) Influence of temperature on the yield and chemical composition of 'vernal' alfalfa at first flower. *Agronomy Journal* 61:470-472.

Sticklen, M.B. (2008) Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol. *Nature Reviews Genetics* 9:433-443.

Tzfira, T., Li, J., Lacroix, B. and Citosky, V. (2004) *Agrobacterium* T-DNA integration: molecules and models. *Trends in Genetics* 20(8):375-383.

Vanholme, R., Demedts, B., Morreel, K., Ralph, J. and Boerjan, W. (2010) Lignin biosynthesis and structure. *Plant Physiology* 153:895-905.

Walker, K.A. and Sato, S.J. (1981) Morphogenesis in callus tissue of *Medicago sativa*: the role of ammonium ion in somatic embryogenesis. *Plant Cell, Tissue and Organ Culture* 1(1):109-121.

Weng, J.-K. and Chapple, C. (2010) The origin and evolution of lignin biosynthesis. *New Phytologist* 187:273-285.

Yueago, H. and Cash, D. (2009) Global status and development trends of alfalfa. In: Cash, D. eds. *Alfalfa management guide for Ningxia (TCP/CPR/3104)*. Chapter 1. United Nations Food and Agriculture Organization, Beijing, pp. 1-14.

Zambryski, P. (1988) Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. *Annual Review of Genetics* 22:1-30.

Zambryski, P., Depicker, A., Kruger, K. and Goodman, H.M. (1982) Tumor induction by *Agrobacterium tumefaciens*: analysis of the boundaries of T-DNA. *Journal of Molecular and Applied Genetics* 1(4):361-370.

Zhou, R., Jackson, L., Shadle, G., Nakashima, J., Temple, S., Chen, F. and Dixon, R.A. (2011) Distinct cinnamoyl CoA reductases involved in parallel routes to lignin in *Medicago truncatula*. *Proceedings of the National Academy of Sciences* 107(41):17803-17808.