

**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.

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# 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 |

# 

# 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.

# History of use

## 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 C0) 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]](#footnote-1) and is often harvested for hay, but can also be 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).

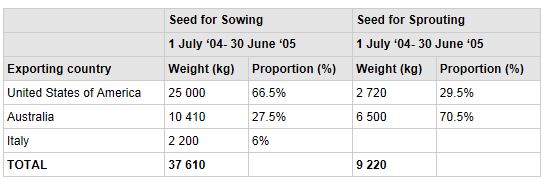
Lucerne was first introduced into Australia in the early 19th century. A second wave of introductions occurred in the 1980s following devastation of the crop by exotic insect pests. Currently Australia has about 3.5 million ha of lucerne under crop in both irrigated and dryland situations in all states,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



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).

## 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).

# 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)

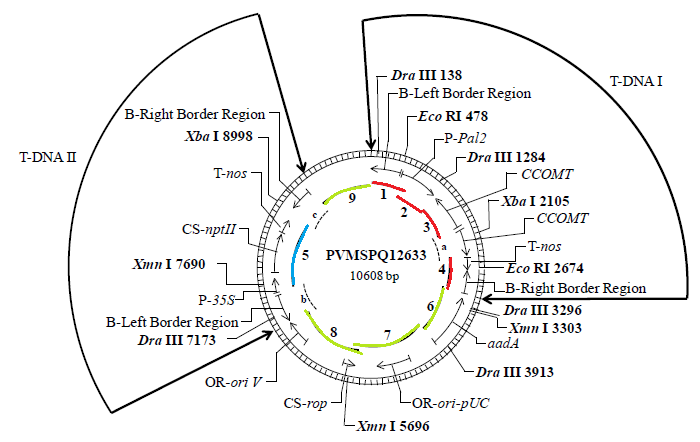
2011. Lignin Analysis of Forage from Multiple Generations of KK179 Alfalfa. **RAR-2011-0129**. Monsanto Company (unpublished).

## 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 (T0) were transferred to the greenhouse for growth and further assessment.

The T0 plants were crossed to a non-GM male sterile line (Ms208) to produce F1 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 F1 plant, was selected as the lead event (P0) based on its superior characteristics and absence of T-DNA II.



*Figure 1: Vector map of plasmid PV-MSPQ12633*

## 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) |
| 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)** | | | | | | |  |  |  |  |

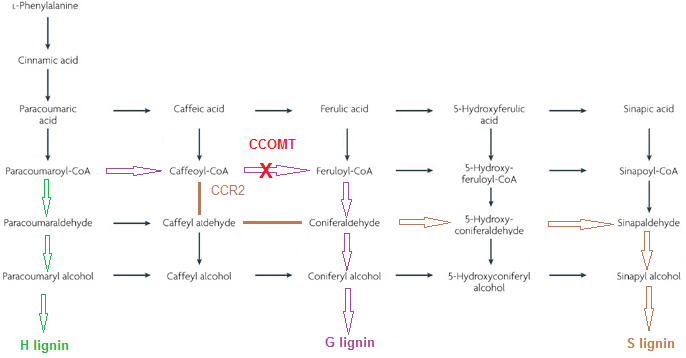
* + 1. ***T-DNA I***

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.

* + 1. ***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.

## Breeding to obtain lucerne line KK179

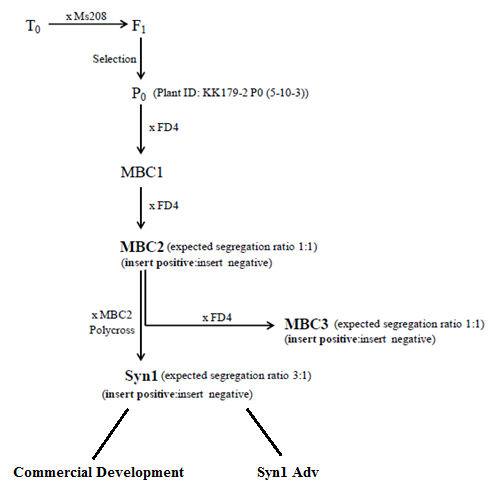
A breeding programme was undertaken for the purposes of:

* 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 (T0 plants) were crossed to a non-GM male sterile line (Ms208) to produce F1 plants. Following selection for plants with desirable characteristics and not containing T-DNA II, a single plant (P0) 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 P0 plant was hand-crossed with each of 10 elite lucerne genotypes with a fall dormancy 4 (FD4)[[2]](#footnote-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 C0 (the cross between ‘R2336’ and ‘Ms208’) plant (see Section 2.1) instead of the T0 plants in order to produce populations that could be used as conventional comparators.



*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 | P0 | R2336; Ms208; C0 |
| Mendelian inheritance | MBC2, MBC3, Syn1 |  |
| Genetic stability | Po; MBC1; MBC2; Syn1 |  |
| Phenotypic stability | MBC1, Syn1, Syn1 Adv | C0-derived equivalents of the 3 generations |
| Northern blot analysis | Syn1 | C0 Syn1 |
| Compositional analyses | Syn1 | C0 Syn1 |

## 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.

* + 1. ***Insert characterisation***

Genomic DNA was obtained from verified leaf tissue of Po 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 P0 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.

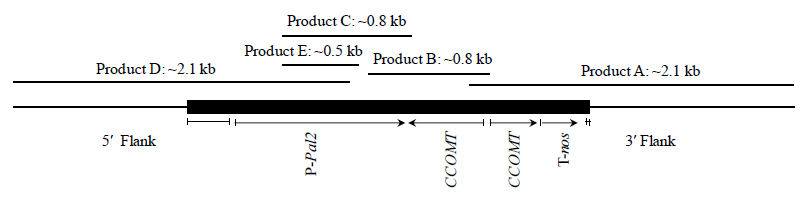
The presence/absence of plasmid backbone was assessed by using four backbone probes (see green lines in the centre of Figure 1). The presence of backbone sequences would be indicated by the appearance of hybridization bands on the Southern blot.

A positive control (DNA from ‘C0’ spiked with either digested PV-MSPQ12633 DNA and/or probe template(s))was also included in the Southern blot analyses. C0 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 C0 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 (P0) 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.

* + 1. ***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.

## 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 C0 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 C0 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.

## 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 P0, MBC1, MBC2 and Syn1 generations (refer to Figure 3) of KK179 and from C0. 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 P0, 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 C0.

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 theKK179 insert. A chi-square (Χ2) 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 Χ2 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 |

## 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.

## 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.

# 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.

## 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/)](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]](#footnote-3) of ≤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 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.

# 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).

## 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)

## Study design, conduct and analysis

The test (PCR-verified KK179, seed of Syn1 generation), and control (PCR-verified C0 Syn1) lines were grown under agronomic conditions pertinent to their geographic regions at six field sites across North America[[4]](#footnote-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]](#footnote-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 C0 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 C0 Syn1 have been compared to a combined literature range for each analyte, compiled from published literature[[6]](#footnote-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 C0 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.

## 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 C0 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.

*Table 5: Mean percentage ± S.E. of proximates and fibre in forage from C0 Syn1 and KK179*

| **Analyte** | **‘C0 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 ± 0.535 | 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 |
| ADF2  (%dw) | 27.02 ± 2.44 | 27.03 ± 2.45 | NS | 7.07 – 39.11 | 21.26 – 42.59 |
| NDF3  (%dw) | 34.46 ± 2.63 | 33.95 ± 2.64 | NS | 18.97 – 49.82 | 26.5 – 53.56 |
| ADL16  (%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 |

1Carbohydrate calculated as 100% - (protein %dw + fat %dw + ash %dw)

2ADF = acid detergent fibre

3NDF = neutral detergent fibre

4ADL = acid detergent lignin

5Mauve shading represents a KK179 mean with a significantly lower value than the C0 Syn1 mean.

6See 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 C0 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 C0 Syn1 and the proportion of G lignin expressed in the total (H + S + G) lignin was significantly lower in KK179 compared to C0 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 C0 Syn1 and KK179*

| **Lignin Component** | **‘C0 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 ± 8.835 | 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 C0 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 C0 Syn1 and KK179*

| **Amino Acid** | **‘C0 Syn’ (A) %dw** | **KK179 (B) %dw1** | **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 |
| 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 C0 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** | **‘C0 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, C0 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 C0 Syn1 but was within the reference range. Both the KK179 and C0 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** | **‘C0 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 |
| 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 ± 13.531 | 0.013 | 11.47 – 151.33 | 600 – 1,200 |
| Ferulic acid  (ppm dw) | 1485.81 ± 58.83 | 1596.41 ± 59.571 | 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 C0 Syn1 mean; orange shading represents a significantly higher mean value for KK179 compared with C0 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 C0 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 C0 Syn1*

| **Analyte** | **‘C0 Syn’** | **KK1791** | **% difference** | **KK179 within Reference range?** | **KK179 within Combined literature range?** |
| --- | --- | --- | --- | --- | --- |
| Ash  (%dw) | 10.79 | 10.381 | 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 | 40.301 | 29% | yes | No – but neither was the control |
| Ferulic acid  (ppm dw) | 1485 | 15961 | 7% | yes | yes |

1 Mauve shading represents a KK179 mean that was significantly lower than the C0 Syn1 mean; orange shading represents a significantly higher mean value for KK179 compared with C0 Syn1.

## 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 (C0-Syn Adv). This was consistent with the findings from the compositional analysis.

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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. [↑](#footnote-ref-1)
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) [↑](#footnote-ref-2)
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. [↑](#footnote-ref-3)
4. The six sites were: Tulare County, CA; Jefferson County, IA; Clinton County, IL; Pawnee County, KS, Armstrong County, TX and Walworth County, WI. [↑](#footnote-ref-4)
5. SAS website - <http://www.sas.com/technologies/analytics/statistics/stat/index.html> [↑](#footnote-ref-5)
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). [↑](#footnote-ref-6)
7. All website references were current as at 1 October 2013 [↑](#footnote-ref-7)