Application to Food Standards Australia New Zealand for the Inclusion of Lucerne KK179 in Standard 1.5.2 - Food Derived from Gene Technology

Submitted by:

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UNPUBLISHED REPORTS BEING SUBMITTED

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[redacted] 2011. Stability of the DNA Insert in KK179 Across Multiple Generations. **MSL0023312.** Monsanto Company.

[redacted] 2012. Bioinformatics Evaluation of the Transfer DNA Insert in KK179 Utilizing the AD_2012, TOX_2012 and PRT_2012 Databases. **MSL0024048**. Monsanto Company.

[redacted] 2012. Bioinformatics Evaluation of DNA Sequences Flanking the 5' and 3' Junctions of Inserted DNA in KK179: Assessment of Putative Polypeptides. **MSL0023975**. Monsanto Company.

[redacted] 2011. Heritability of the KK179 Insert in the MBC2, MBC3, and Syn1 Populations. **RPN-2010-0705**. Monsanto Company.

[redacted] 2011. Lignin Analysis of Forage from Multiple Generations of KK179 Alfalfa. **RAR-2011-0129**. Monsanto Company.

[redacted] 2011. Analysis of the Endogenous CCOMT RNA Level in Alfalfa KK179. **MSL0023329**. Monsanto Company.

[redacted] 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.

[redacted] 2012. Analyses of Lignin in Forage from KK179 Alfalfa Grown in the United States during the 2011 Growing Season. **MSL0024120**. Monsanto Company.

[redacted] 2012. Composition Analyses of Forage from KK179 Alfalfa Grown in the United States during the 2011 Growing Season. **MSL0023847**. Monsanto Company.

[redacted] 2012. Analyses of Saponin Levels of Forage from KK179 Alfalfa Grown in the United States during the 2011 Growing Season. **MSL0023980**. Mo nsanto Company.

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ABBREVIATIONS AND DEFINITIONS[1]

symbol or abbrev.	Definition
~	Approximately
4CL	4-coumarate: CoA ligase
AACC	American Association of Cereal Chemists
aadA	Bacterial promoter, coding sequence, and 3′ UTR for an aminoglycoside-modifying enzyme, 3"(9)-*O*-nucleotidyl-transferase from the transposon Tn*7* that confers spectinomycin and streptomycin resistance
ADF	Acid detergent fiber
ADL	Acid detergent lignin
APHIS	Animal and Plant Health Inspection Service of the United States Department of Agriculture
AOAC	Association of Analytical Chemists
AOCS	American Oil Chemists Society
AOSA	Association of Official Seed Analysts
AOSCA	Association of Official Seed Certification Agencies
AP	Adventitious presence
ASSP	Alfalfa Seed Stewardship Program
B-Left Border Region	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA
B-Right Border Region	DNA region from *Agrobacterium tumefaciens* containing the Right Border sequence used for transfer of the T-DNA
ºC	Degrees Celsius
C_0	The single progeny plant selected from the cross of alfalfa plants R2336 and Ms208 to develop the near-isogenic conventional control
C_0-Syn1	The near-isogenic conventional control for KK179 generation Syn1
C_0-Syn1 Adv	The near-isogenic conventional control for KK179 generation Syn1 Adv
C3H	*p*-coumarate-3-hydroxylase
C4H	Cinnamate-4-hydroxylase
CAD	Cinnamyl alcohol dehydrogenase
CCOMT	Caffeoyl CoA 3-*O*-methyltransferase protein from *Medicago sativa*
CCOMT	Caffeoyl CoA 3-*O*-methyltransferase gene from *Medicago sativa*
CCR	Cinnamoyl-CoA reductase
CFR	Code of Federal Regulations
COMT	Caffeic acid 3-*O*-methyltransferase
CaMV	Cauliflower mosaic virus
cpm	Counts per minute
CRP	Conservation Reserve Program

[1] Alred, G.J., C.T. Brusaw, and W.E. Oliu. 2003. Handbook of Technical Writing, 7th edn., pp. 2-7. Bedford/St. Martin's, Boston, MA.

CS-*rop*	Coding sequence for repressor of primer protein derived from the ColE1 plasmid for maintenance of plasmid copy number in *E. coli*
CS-*nptII*	Coding sequence of the *neo* gene from transposon Tn*5* of *E. coli* encoding neomycin phosphotransferase II (NPT II) that confers neomycin and kanamycin resistance
CTAB	Cetyltrimethylammonium bromide
CWR	Cell wall residue
d	Day
DAP	Days after planting
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dNTP	Deoxynucleotide triphosphate
dw	Dry weight
E. coli	*Escherichia coli*
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
FASTA	Algorithm used to find local high scoring alignments between a pair of protein or nucleotide sequences
FD4	Fall dormancy type 4
fw	Fresh weight
FGI	Forage Genetics International, LLC
F5H	Ferulate 5-hydroxylase
g	Gram(s)
G lignin	Guaiacyl lignin subunits
G:F	Gain:Feed
GRAS	Generally Recognized As Safe
H lignin	*p*-Hydroxyphenyl lignin subunits
HCT	*p*-Hydroxycinnamoyl-CoA:shikimate: hydroxycinnamoyl-transferase
ILSI	International Life Sciences Institute
Kb	Kilobase
kg	Kilogram
LOQ	Limit of quantitation
m	Meter
MBC	Modified backcross
Ms208	FGI proprietary conventional male sterile alfalfa plant
µg	Microgram
µmol	Micromole
mg	Milligram
N	Normal
NDF	Neutral detergent fiber
NFTA	National Forage Testing Association
NPTII	Neomycin phosphotransferase II
NOS	Nopaline synthase
NTO	Non-target organism
OECD	Organisation for Economic Co-operation and Development
ORF	Open Reading Frame
OR-*ori-pUC*	Origin of replication from plasmid pUC for maintenance of plasmid in *E coli*

OR-*ori V*	Origin of replication from the broad host range plasmid *RK2* for maintenance of plasmid in *Agrobacterium*
P_0	The single progeny plant selected from the cross of T_0 and Ms208
P-*35S*	Promoter and leader from the 35S RNA of cauliflower mosaic virus (CaMV)
P-*Pal2*	Promoter of the *Pal2* gene from *Phaseolus vulgaris* encoding the phenylalanine ammonia-lyase
PAL	Phenylalanine ammonia-lyase
PCR	Polymerase chain reaction
PEAQ	Predictive Equations for Alfalfa Quality
polyA$^+$RNA	PolyA enriched RNA
PPA	Plant Protection Act
ppm	Parts per million
PRESS	Predicted residual sums of squares
PRT_2012	GenBank protein database, 187.0 (Released January 30, 2012)
PV-MSPQ12633	Plasmid used to transform the alfalfa genome to produce KK179
R2336	FGI proprietary conventional plant selected for ease of transformation
RNA	Ribonucleic acid
S lignin	Syringyl lignin subunits
SAS	Statistical Analysis System
S.E.	Standard error
S:G lignin ratio	Syringyl lignin subunit divided by guaiacyl lignin subunit
T-DNA	Transfer DNA
Syn1	First generation KK179 synthetic population
Syn1 Adv	Second generation KK179 synthetic population
Tm	Melting temperature
T_0	The transformed R2336 plant selected for KK179 development
T-*nos*	3′ UTR sequence of the *nopaline synthase* (*nos*) gene from *Agrobacterium tumefaciens* pTi encoding NOS that directs polyadenylation
TUG	Technology use guide
USD	U.S. dollars
UTR	Untranslated region
v/v	Volume per volume
VNS	Variety not stated

PART 1 GENERAL INFORMATION

1.1 Applicant Details

(a) Applicant's name/s	[redacted]
(b) Company/organisation name	Monsanto Australia Limited
(c) Address (street and postal)	Level 12 / 600 St Kilda Road, Melbourne, Victoria, 3004 PO Box 6051, St Kilda Road Central, Victoria, 8008
(d) Telephone number	[redacted]
(e) Email address	[redacted]
(f) Nature of applicant's business	Technology Provider to the Agricultural and Food Industries
(g) Details of other individuals, companies or organisations associated with the application	Forage Genetics International LLC N5292 S. Gills Coulee Road West Salem,WI 54669

1.2 Purpose of the Application

This application is submitted to Food Standards Australia New Zealand by Monsanto Australia Limited on behalf of Monsanto Company and Forage Genetics International..

The purpose of this submission is to make an application to vary **Standard 1.5.2** – *Food Produced Using Gene Technology* of the *Australia New Zealand Food Standards Code* to seek the addition of reduced lignin lucerne line KK179[2] and products containing reduced lignin lucerne line KK179 (hereafter referred to as KK179) to the Table to Clause 2 (see below).

Food derived from gene technology	Special requirements
Food derived from reduced lignin lucerne line KK179	None

[2] Lucerne is a forage crop that is referred to as alfalfa in other world areas. The product that is the subject of this application is referred to as Reduced Lignin Alfalfa KK179 in studies attached to this submission. For the purposes of this application, the product will be referred to as Reduced Lignin Lucerne KK179 to distinguish this forage crop from alfalfa which is the term given for food (and not feed) uses in Australia and New Zealand.

1.3 Justification for the Application

1.3(a) The need for the proposed change

Monsanto and Forage Genetics International (FGI) have developed biotechnology-derived lucerne KK179 to provide greater flexibility in harvesting forage without loss of quality. KK179 limits degradation in quality by lowering levels of a major subunit of lignin called guaiacyl lignin (hereafter referred to as G lignin), and thereby reducing accumulation of total lignin. Total lignin levels in KK179 forage are generally similar to those found in conventional forage harvested several days earlier under similar production conditions. Growers, therefore, have the option to harvest KK179 several days later without appreciable loss of forage quality typical in conventional alfalfa at the same growth stage.

KK179 lucerne is intended to be primarily used as an animal feed in northern America. This product is not intended to be introduced into Australia at this time and therefore it is highly unlikely that any foods or feeds derived from KK179 will be introduced into the Australian or New Zealand food supply. However, in the exceptional circumstance that foods derived from KK179 are introduced into the Australian or New Zealand food supply, Monsanto Australia Limited seeks a food safety assessment from FSANZ.

KK179 will be combined, through traditional breeding techniques, with the previously approved Monsanto and FGI approved herbicide-tolerant (*i.e.* glyphosate) Roundup Ready lucerne events, J101 and J163. The combined traits will allow growers planting Roundup Ready × KK179 lucerne in northern America to take advantage of the weed management benefits of the Roundup Ready weed control system: broad spectrum weed control and excellent crop safety with greater application flexibility and simplicity. These growers will also have the flexibility to choose the production strategy that improves forage quality or yield and maximises the profitability of lucerne production for their farming operation. Increased flexibility will allow growers to better manage the yield-quality relationship and harvesting schedules to meet market needs and intended on-farm uses for their lucerne forage production.

1.3(b) The advantages of the proposed change over the status quo, taking into account any disadvantages

The expanded harvest interval that KK179 will allow provides a significant benefit to growers in the form of greater flexibility to dynamically manage harvest strategies to reflect crop production priorities, such as:

- Maximising forage quality: When aiming to maximise forage quality with KK179, the timing of harvest schedules would remain the same as with conventional varieties. KK179 harvested at a typical crop cutting stage will produce lucerne forage with lower levels of lignin compared to conventional lucerne harvested at the same stage. As a result, the quality of the forage is more likely to meet or exceed the quality standard targeted by the grower. The yield will be maintained at the same levels as with conventional lucerne. KK179 does not raise the maximum potential quality attainable for forage, but is more likely to meet or exceed the desired quality compared to conventional lucerne.

- Maximising forage yield: When aiming to maximise the dry matter yield, a grower can delay harvest for several days to accumulate more forage biomass without significantly forfeiting quality. During the reproductive growth stage, lucerne dry matter can increase at the rate of 200 pounds per acre per day (Undersander et al., 2009). Therefore, even a small delay in harvest timing can result in significant gains in forage yield. KK179 can be harvested several days later with quality comparable to that of conventional lucerne harvested several days earlier, but with more forage biomass than conventional systems allow. A similar delay with conventional lucerne would provide a comparable yield, but the forage would have higher lignin content and, therefore, lower quality. From a forage production perspective, the maximum potential attainable yield of lucerne is not raised. Rather, growers can more readily reach the higher end of the potential yield range while maintaining a targeted quality yield range while maintaining a targeted quality standard.

- Tolerating unexpected harvest delays: Unexpected delays in harvesting occur occasionally and can be due to untimely weather events, such as rain; equipment failure; or the pressures of competing farming activities, *e.g.*, labour availability or dairy herd management. During the delay period, forage quality often declines rapidly, leading to potential financial loss. A grower has more flexibility to withstand short delays in forage harvest with KK179, because there is less accumulation of lignin during the delay period and, therefore, less loss of quality by the time harvesting is resumed.

1.4 Regulatory Impact Information

1.4(a) Costs and benefits

A cost/benefit analysis quantified in monetary terms is difficult to determine. In fact, most of the impacts that need to be considered cannot be assigned a dollar value. KK179 is intended to be primarily used as an animal feed and is not intended to be introduced into Australia or New Zealand at this current point in time and therefore it is highly unlikely that any foods or feeds derived from KK179 will be introduced into the Australian or New Zealand food supply.

Given KK179 is an animal feed product and there is no current intention to introduce KK179 into Australia or New Zealand, there are unlikely to be many costs or benefits to Australian consumers of the draft variation to permit the sale and use of food derived from KK179.

Industry sectors affected may be food importers; as mentioned previously KK179 is a feed product but should presence of KK179 find its way into the food chain, approval of KK179 would ensure that importers of foods containing alfalfa derivatives would be compliant with the *Australia New Zealand Food Standards Code.*

Finally, if KK179 was detected in food products, approval would ensure compliance of those products with the *Australia New Zealand Food Standards Code.* This would benefit the Australia and New Zealand Governments by ensuring there is no potential for trade disruption on regulatory grounds and would avoid potential conflicts with WTO responsibilities.

1.4(b) Impact on international trade

Lucerne is one of the most widely cultivated forage crops in the world. Lucerne forage is used almost exclusively for animal feed. However import volumes of lucerne forage into Australia and New Zealand are very low and statistics indicate that lucerne is only imported in years of drought. In addition, there will be stewardship programs in place in countries of KK179 production to prevent any sprouts derived from the genetically modified lucerne from entering the food supply overseas.

However, should there be any unintended presence of KK179 in the Australian or New Zealand food supply in the future, this application – if approved – will ensure any food imports from countries of KK179 production comply with the *Australia New Zealand Food Standards Code.* This will ensure there is no potential for trade disruption on regulatory grounds.

1.5 Assessment Procedure

Monsanto Australia is submitting this application in anticipation that it will fall within the General Procedure category.

1.6 Exclusive Capturable Commercial Benefit

This application is likely to result in an amendment to the Code that provides exclusive benefits and therefore Monsanto intends to pay the full cost of processing the application.

1.7 International and Other National Standards

1.7(a) International standards

Monsanto makes all efforts to ensure that safety assessments are aligned, as closely as possible, with relevant international standards such as the Codex Alimentarius Commission's *Principles for the Risk Analysis of Foods Derived from Modern Biotechnology* and supporting *Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants* (Codex Alimentarius, 2009).

In addition, the composition analysis is conducted in accordance with OECD guidelines and includes the measurement of OECD-defined alfalfa (lucerne) nutrients and anti-nutrients based on conventional commercial alfalfa (lucerne) varieties (OECD, 2005).

1.7(b) Other national standards or regulations

Monsanto has submitted a food and feed safety and nutritional assessment summary for KK179 to the United States Food and Drug Administration (FDA) and has also requested a Determination of Nonregulated Status for KK179, including all progenies derived from crosses between KK179 and other alfalfa, from the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA).

Applications have also been submitted to Canadian Food Inspection Agency (CFIA) and Health Canada (HC), Korea Food and Drug Administration (KFDA) for food, and Rural Development Administration (RDA) for feed use, and Japan's Ministry of Health, Labour, and Welfare (MHLW) for food use.

Regulatory submissions will be made to countries that import significant alfalfa or food and feed products derived from countries where KK179 alfalfa will be grown and have functional regulatory review processes in place. This will result in submissions to a number of additional governmental regulatory agencies including, but not limited to Ministry of Agriculture, People's Republic of China; Japan's Ministry of Agriculture, Forestry, and Fisheries, as well as to regulatory authorities in other alfalfa importing countries with functioning regulatory systems.

PART 2 SPECIFIC DATA REQUIREMENTS FOR SAFETY ASSESSMENT

A. TECHNICAL INFORMATION ON THE GM FOOD

A1 Nature and Identity of the Genetically Modified Food

A1(a) A description of the new GM organism

Monsanto Company and Forage Genetics International have developed biotechnology-derived lucerne KK179 (*Medicago sativa* L.) to provide lucerne growers with greater flexibility in harvesting forage without loss of quality. Forage quality, as defined by market standards, is compromised by the presence of lignin, which is sensitive to timing of harvest.

Plant lignin biosynthesis

An understanding of lignin biosynthesis in lucerne has provided the means to reduce lignin levels and slow the accumulation of lignin during the lucerne growth cycle. Lignin is a high molecular weight, polymeric molecule composed principally of three lignin monomeric subunits: guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) lignin (Figure 1)(Boerjan et al., 2003; Vanholme et al., 2010). The relative proportion of each lignin monomer can vary with plant species and tissue type (Boerjan et al., 2003). In lucerne, G lignin and S lignin subunits comprise up to 95% of all lignin subunits. In the lignin biosynthetic pathway, formation of the G and S subunits requires the activity of two *O*-methyltransferase enzymes for lignin biosynthesis, caffeoyl CoA 3-*O*-methyltransferase (CCOMT) and caffeic acid 3-*O*-methyltransferase (COMT). *O*-methyltransferases are a large family of enzymes that mehtylate the oxygen atom of secondary metabolites such as phenylpropanoids, flavonoids, and alkaloids (Lam et al., 2007). CCOMT methylates caffeoyl CoA in the lignin biosynthetic pathway to produce feruloyl CoA while COMT methylates caffeyl aldehyde to produce coniferyl aldehyde, and methylates 5-hydroxyconiferyl aldehyde to produce sinapyl aldehyde (Figure 1). Current literature on lignin production in alfalfa indicates that the COMT enzyme is specifically involved in the formation of S lignin monomers while the CCOMT enzyme acts in a parallel manner to form G lignin monomers (Figure 1)(Guo et al., 2001; Zhou et al., 2010). Of the two enzymes, CCOMT was identified as the principal enzyme to target suppression in order to lower the production of G lignin subunits.

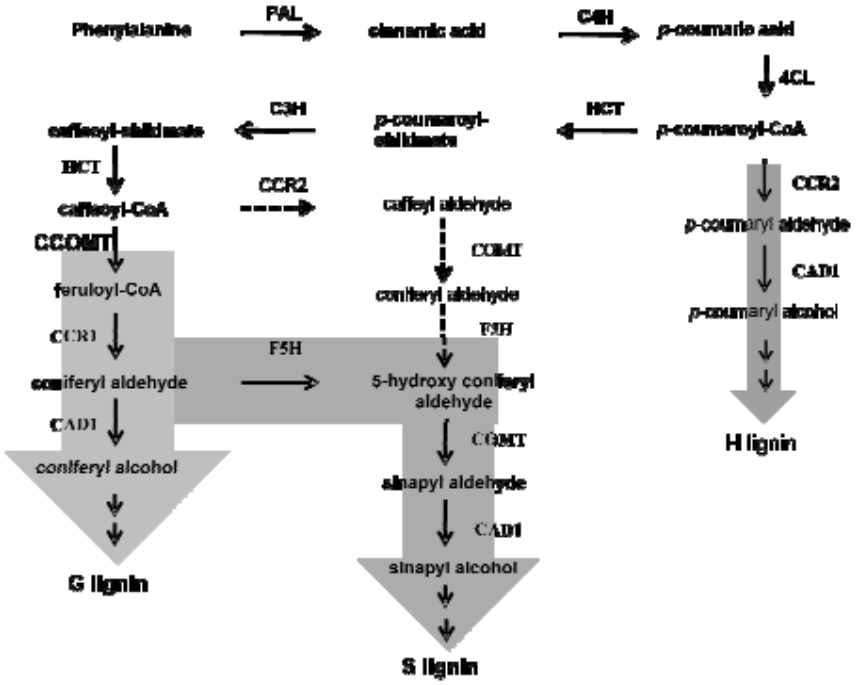


Figure 1. Lignin biosynthetic pathway

PAL: phenylalanine ammonia lyase;
C4H: cinnamate-4-hydroxylase;
4CL: 4-coumarate: CoA ligase;
HCT: hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyl transferase;
C3H: *p*-coumarate-3-hydroxylase;
CCOMT: caffeoyl-CoA *O*-methyltransferase;
COMT: caffeic acid *O*-methyltransferase;
CCR1, CCR2: cinnamoyl-CoA reductase;
F5H: ferulate 5-hydroxylase;
CAD1: cinnamyl alcohol dehydrogenase;

Reactions shown by dotted lines occur at very low rates in the wild type (Zhou et al., 2010);
Shaded arrows indicate committed steps in the production of monolignin subunits.

Mode of Action of KK179

The suppression cassette in KK179 functions by reducing the level of G lignin subunits, which are oxidatively coupled to other lignin subunits to form complex lignin molecules (Boerjan et al., 2003). This specific reduction in G lignin is achieved through the use of endogenous lucerne gene segments configured to suppress the *CCOMT* gene so as to lower CCOMT protein expression, and thereby decrease the synthesis of G lignin (Figure 2). KK179 contains *CCOMT* gene segments under the control of the *Pal2* promoter from the phenylalanine ammonia-lyase (PAL) gene in bean (*Phaseolus vulgaris*). PAL expression responds to endogenous cues for vascularisation and displays a pattern of expression that corresponds with sites of lignin deposition in maturing plants (Guo et al., 2001; Leyva et al., 1992). Therefore, KK179 transgene expression correlates with tissues where higher lignin deposition is observed. The assembled *CCOMT* gene segments produce a transcript with an inverted repeat sequence to form double-stranded RNA (dsRNA), which works via the RNA interference mechanism to suppress the endogenous *CCOMT* gene (Siomi and Siomi, 2009).

The RNAi mechanism is a natural process in eukaryotic organisms for the regulation of gene expression (Dykxhoorn and Novina, 2003; Parrott et al., 2010). The dsRNA molecule that activates the mechanism is first processed by a class of RNAse III enzymes called Dicers into small interfering RNAs (siRNAs, ~21-25 nucleotides)(Hammond, 2005; Zamore et al., 2000; Siomi and Siomi, 2009). The resulting siRNA molecules are then incorporated into multiprotein RNA-induced silencing complexes (RISC), which facilitate target sequence recognition and mRNA cleavage (Hammond, 2005; Tomari and Zamore, 2005); in this case, the degradation of *CCOMT* transcripts. The final outcome of this process is the suppression of the target *CCOMT* mRNA.

When CCOMT enzymatic activity is reduced, an alternative path in the lignin biosynthetic pathway allows S lignin biosynthesis to continue through the conversion of caffeoyl-CoA to caffeyl aldehyde by the CCR2 enzyme (Zhou et al., 2010). As a result, the effect of CCOMT suppression is limited to lowering G lignin production. The decrease in actual amount of G lignin also results in an increase in the proportion of S lignin relative to all subunits, but not in an increase in the actual amount of S lignin. These changes in the subunit proportions result in an increase in the S:G lignin ratio, which is characteristic of CCOMT suppression (Chen et al., 2006).

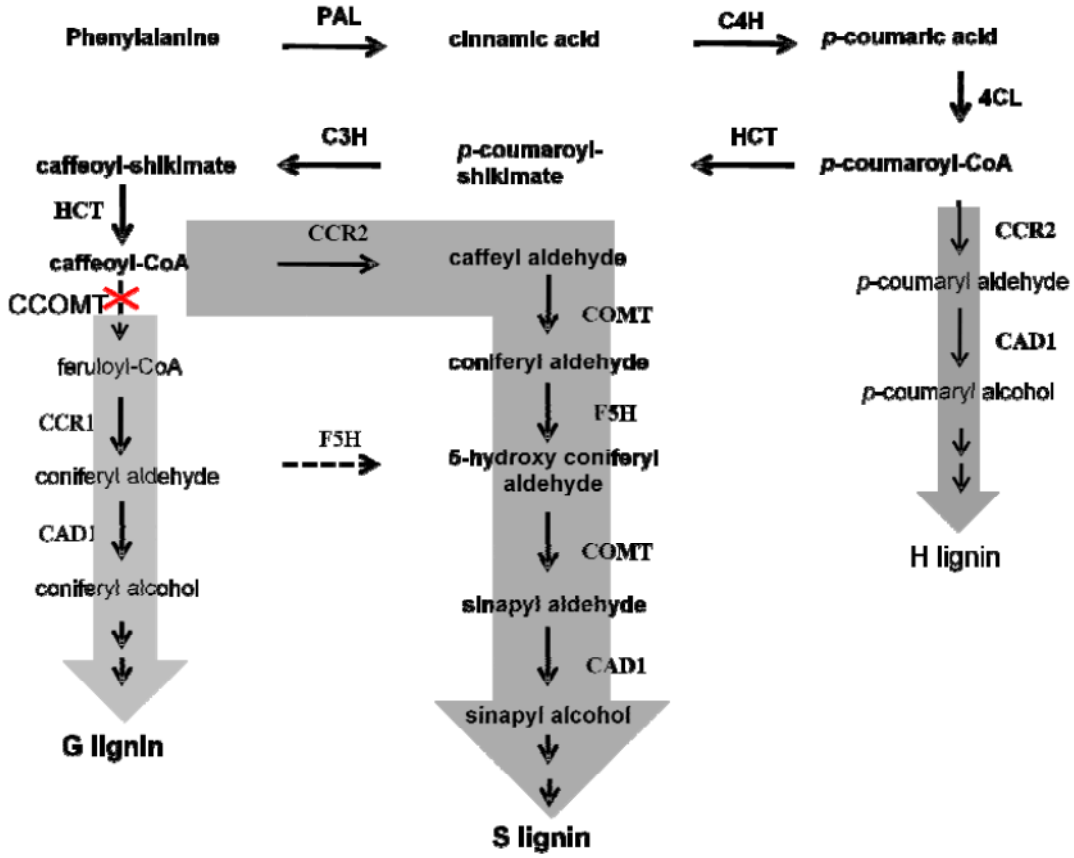


Figure 2. Modified lignin biosynthetic pathway in KK179

Enzymes in pathway are listed in Figure 1 legend:

✕ indicates the suppression of CCOMT enzyme activity.

Reactions shown by dotted lines occur at very low rates when CCOMT activity is reduced (Zhou et al., 2010).

A1(b) Name, number or other identifier of each new line or strain

In accordance with OECD's "Guidance for the Designation of a Unique Identifier for Transgenic Plants" KK179 has been assigned the unique identifier MON-ØØ179-5.

A1(c) The name the food will be marketed under (if known)

Lucerne containing the transformation event KK179 will be produced in North America. There are currently no plans to produce this product in Australia and New Zealand. A commercial trade name for the product has not been determined at the time of this submission and will be available prior to commercial launch of the product in North America.

A1(d) The types of products likely to include the food or food ingredient

The vast majority of lucerne is grown and harvested for animal feed (Higginbotham et al., 2008). Although there are some food uses of the plant (termed alfalfa for food uses in Australia and New Zealand), KK179 is only intended for use as an animal feed and will not be introduced as a forage crop in Australia or New Zealand at this time; therefore there is very little chance that food products containing KK179 will be imported into Australia or New Zealand.

Alfalfa sprouts are used in salads and health food drinks. They are also readily available from the supermarket. Bulk powdered herb or capsules and tablets containing alfalfa leaves or seeds are also available in pharmacies and health food stores for use as dietary supplements and herbal teas. In the unlikely event that food products containing KK179 are imported into Australia or New Zealand, further information on food uses of *Medicago sativa* L. (*i.e.* lucerne or alfalfa) are described in Section A2(a) and Section A2(b).

A2. History of Use of the Host and Donor Organisms

A2(a) Description of all donor organism(s)

A2(a)(i) Common and scientific names and taxonomic classification

The insert present in KK179 contains a partial gene segment of *CCOMT* from *Medicago sativa* configured into an inverted repeat sequence. In Australia and New Zealand, *Medicago sativa* is commonly known as lucerne (animal feed) or alfalfa (human food uses).

The taxonomy of *Medicago sativa* is:

Family: Fabaceae

Tribe: Trifolieae

Genus: *Medicago* L.

Species: *Medicago sativa* L.

Subspecies: *Medicago sativa* L. subsp. *sativa*

A2(a)(ii) Information on pathogenicity, toxicity, allergenicity

There is no evidence of human or animal pathogenicity for any of the donor organisms of the coding and non-coding DNA sequences present in KK179. DNA has always been present in feed and food, and, upon consumption, is quickly degraded to nucleic acids by nucleases present in the gastrointestinal tract of humans and animals. According to the U.S. FDA (U.S. FDA, 1992), nucleic acids, which are present in the cells of every living organism, do not raise concerns as a component of food, and are Generally Recognised As Safe (GRAS). Results from an International Life Sciences Institute (ILSI) workshop on safety considerations of DNA in food were reported (Jonas et al., 2001) and confirmed that: 1) all DNA, including recombinant DNA, is composed of the same four nucleotides; 2) there are no changes to the chemical characteristics or the susceptibility to degradation by chemical or enzymatic hydrolysis of recombinant DNA as compared to non-recombinant DNA; and 3) there is no evidence that DNA from dietary sources has ever been incorporated into the mammalian genome. Additionally, the European Food Safety Authority (EFSA) has reported that a large number of experimental studies have shown that recombinant DNA consumed by livestock has not been subsequently detected in tissues, fluids, or edible products of these farm animals (EFSA, 2007).

A2(a)(iii) History of use of the organism in food supply or human exposure

As mentioned in Section A1(d), the vast majority of lucerne is grown and harvested for animal feed and although there are some food uses of the plant (termed alfalfa for food uses in Australia and New Zealand), KK179 is not intended to be introduced in Australia or New Zealand at this time.

Alfalfa has a history of minor uses by humans as food, dietary supplements, and herbal remedies (OECD, 2005). Greater than 95% of human consumption on a weight basis is in the form of alfalfa seedlings, also referred to as sprouts. In North America, sprouted seedlings of alfalfa and other plant species are available in grocery stores and stores

specialising in natural or health foods. Most people are estimated to consume only small quantities of these foods in the range of 8-20 g per serving (OECD, 2005).

Food uses of alfalfa are also minor in terms of overall alfalfa production (USDA-APHIS, 2010). Less than 2.5% of alfalfa seed is estimated to be used for human consumption, based on testimony provided to the U.S. Food and Drug Administration (FDA) (U.S. FDA, 1998). Seeds used for sprout production are subject to restrictions, because of the potential for residues on food from chemical applications during seed production. At the present time, no agricultural chemicals have been approved for use in food grade alfalfa seed crops. Furthermore, epidemiological investigations have implicated bacterial contamination of alfalfa seeds as the source of sprout-associated illness outbreaks (U.S. FDA, 2011b). Therefore, preventive controls are used to reduce the risk of raw sprouts serving as a vehicle for food borne illness (CFIA, 2007; U.S. FDA, 2004; U.S. FDA, 2011b). Due to these restrictions, seed for sprouts and seed for crop planting purposes are grown and sold separately. In Northern America, Monsanto and FGI restrict use of biotechnology-derived alfalfa seed for sprout production through signed agreements with seed purchasers. Thus, KK179 as a commercial North American product is not intended to be used in the production of sprouts or other alfalfa-derived food products.

In addition to consumption as sprouts, alfalfa also has a history of consumption by humans in the form of dietary supplements and herbal remedies (Bora and Sharma, 2011). Various forms of alfalfa, including leaf concentrates, protein extracts, sprouts, sprout extracts, and seeds have been suggested to be useful for neuroprotective, hypocholesterolemic, antioxidant, antiulcer, antimicrobial, hypolipidemic, and estrogenic effects, as well as in the treatment of atherosclerosis, heart disease, stroke, cancer, diabetes, and menopausal symptoms (Bora and Sharma, 2011). As evidenced in the literature, alfalfa remains an active area of investigation by natural product researchers seeking to support uses in dietary supplements and herbal remedies (Bora and Sharma, 2011).

One notable dietary supplement is alfalfa protein concentrate, which is high in protein and xanthophyll, and is also used as a pigment in animal feed. A review of the food safety of alfalfa protein concentrate by the European Food Safety Authority found that levels of L-canavanine and phytoestrogens in alfalfa protein concentrate that would be consumed at suggested rates are lower than from other common food sources such as lentils and onions and do not raise concerns (EFSA, 2009).

A2(b) Description of the host organism

A2(b)(i) Phenotypic information

Lucerne (*Medicago sativa* L.) is a perennial herbaceous legume (Lesins and Lesins, 1979). Its general morphology was studied by Teuber and Brick (1988) and Barnes and Sheaffer (1995). The mature lucerne plant is characterised by a strong taproot. This taproot can be twenty or more feet in length with several to many lateral roots connected at the crown. The crown, a complex structure near the soil surface, has perennial meristem activity, producing buds that develop into stems. Tri- or multi-foliolate leaves form alternately on the stem, and secondary and tertiary stems can develop from leaf axils. A plant in a typical forage production field has between five and 25 stems and can reach nearly three feet tall. Following harvest, regrowth occurs either directly from crown-produced buds or from auxiliary buds developed in the remaining stubble. Flowers, borne in clusters on a raceme and attached to a central rachis, develop in leaf axils at stem apices. Stems are indeterminate so that vegetative and reproductive growth occurs simultaneously. Flowering will continue for several weeks until either the plant is harvested or the stem becomes senescent.

Cultivated lucerne is widely adapted allowing production across varying climatic regions and geographies under both irrigated and non-irrigated systems. It is typically planted to establish perennial stands that remain in the field from three to seven years depending upon geography and agronomic practice. Forage is harvested from two to eleven times a season depending on the region and the system of management. In certain regions, lucerne is cultivated as a mixture with perennial grasses where it may be harvested as forage or used for grazing livestock. As a legume, it is also desired for rotational use to improve soil characteristics such as nitrogen content (Undersander et al., 2011).

Approximately 30 million hectares (ha) are grown worldwide. The major lucerne producing regions are North America with 11.9 million ha (41%), Europe with 7.1 million ha (25%), South America with 7 million ha (23%), Asia 2.2 million ha (8%), Africa (2%) and Oceania (1%) (FAO, 2009). Approximately 1.2 million metric tonnes (mt) of forage and 1.3 million mt meal and pellets were exported globally in 2010[3]. According to FAOSTAT[3], Australia exported the highest amount of forage while U.S., Spain, France, Italy, Australia and Canada were the principal exporters of meal and pellets. The major importing countries and regions of forage, meal and pellets in 2010 were United Arab Emirates, Occupied Palestinian Territory, Japan, and Belgium. Among Pacific Rim nations, Japan is reported to have imported approximately 109,168 mt of lucerne meal and pellets in 2010, with the largest amounts originating from Canada (33%), Spain (28%) and France (3%). Korea, is reported to have imported 41,869 mt with the largest imports coming from China (27%), U.S. (13%), and Spain (7%). Japan and Korea imported 639,111 mt and 247,013 mt of forage

[3] Source: FAOSTAT database, available online at http://faostat.fao.org/site/535/DesktopDefault.aspx?PageID=535#ancor

respectively from Australia, with no sources of imports reported[4]. For 2010, the U.S. reported lucerne hay exports of 596,620 mt to Japan and 163,427 mt of lucerne to Korea, and meal and pellets of 195,905 to Japan and 39,576 mt to Korea[5].

Genetic improvement has been going on for over a century since the first known trials in the U.S. in 1901 (Volenec et al., 2002). Early lucerne breeding efforts were dedicated to collecting, evaluating and comparing various sources of germplasm around the world. A key milestone in the early 20th century was classification of germplasm into several distinct fall dormancy groups, and selection of more winterhardy types within each group (Melton et al., 1988). A better understanding of autotetraploid genetics and its consequences for breeding and variety synthesis improved genetic gains for forage yield (Rumbaugh et al., 1988). Commercial and public lucerne breeding programs currently focus on developing varieties with improved characteristics in several major areas: 1) greater resistance to insects, nematodes, and diseases; 2) greater yield potential; 3) improved stand persistence, and 4) increased forage quality (Putnam et al., 2008; Undersander et al., 2011).

Selecting for low acid detergent lignin (ADL) has been one breeding strategy to increase forage quality (Hill et al., 1988). This has been met with limited success usually due to negative impacts on yield (Hill et al., 1988; Jung et al., 1997). A broad range of lignin levels can be found in commercial and experimental germplasm (Schwab et al., 2005). Reduced lignin is also known as a trait in certain conventional commercial corn and sorghum silage varieties with the brown mid-rib phenotype, resulting from a natural mutation in a lignin biosynthetic pathway enzyme (Sattler et al., 2010).

[4] Source: FAOSTAT database, available online at http://faostat.fao.org/site/535/DesktopDefault.aspx?PageID=535#ancor

[5] Source: U.S. Dept of Commerce, Bureau of Census, commodity code: 1214900010, available online at http://www.census.gov/foreign-trade/about/index.html#exportstatistics.

A2(b)(ii) How the organism is propagated for food use

Lucerne is grown for forage, grazing and seed production. Lucerne is a deep-rooted and short-lived perennial plant with a long growing season. In Northern America, lucerne grows from early spring until late autumn or early winter. Growth begins when the average temperature reaches 50 degrees fah and continues until a killing freeze occurs. It is adapted to a wide range of climatic and soil conditions. In deep, well-aerated soil, roots may extend 8 to 12 feet deep (Kansas State University, 1998). Because of its deep taproot lucerne can use up to 70% of available soil water without stress or loss of production under arid conditions, thus it is often considered naturally drought-tolerant. It has a high water requirement under normal conditions.

Lucerne is generally regarded as the "queen of forages" because of its high protein content and highly digestible fibre for ruminants and horse (USDA-APHIS, 2010). The highest quality lucerne hay is generally used for dairy cows. For instance, dairy farms consume between 75 to 85% of the lucerne hay production in California (USDA-APHIS, 2010). Another 10 to 15% is consumed by horses. Lucerne hay that is lower in protein and higher in fibre is fed to beef cattle, horses, heifers, and non-lactating dairy cows. Lucerne forage is stored as hay (bales at 18 to 20% moisture), haylage (round bale silage, baled at 50 to 60% moisture and wrapped in plastic), and silage (chopped and stored in silos)(USDA-APHIS, 2010). Grazing lucerne in the vegetative state is practiced sometimes for dormant-season lucerne stubble, a substitute for early or late season cutting, and rotational grazing during the season. However, grazing can cause gastrointestinal bloating in animals and result in stand maintenance problems with over-grazing.

Humans consume a limited amount of alfalfa (term for human food use of Medicago sativa in Australia and New Zealand) in the form of sprouts, dietary supplements, and herbal teas. Over 95% of alfalfa (by weight) used for human consumption is in the form of alfalfa sprouts. An indirect use of alfalfa is its use as a common nectar source for supporting the hives of honey bees.

A2(b)(iii) What part of the organism is used for food

Alfalfa has a history of minor uses by humans as food, dietary supplements, and herbal remedies (OECD, 2005). Greater than 95% of human consumption on a weight basis is in the form of alfalfa seedlings, also referred to as sprouts. Most people are estimated to consume only small quantities of these foods in the range of 8-20 g per serving (OECD, 2005).

Less than 2.5% of alfalfa seed in the U.S. is estimated to be used for human consumption, based on testimony provided to the Food and Drug Administration (FDA) (U.S. FDA, 1998). The production of seeds for sprout production are subject to restrictions. Because of the potential for residues on food from chemical applications during seed production, agricultural chemicals are currently approved in the U.S. or Canada for use only on alfalfa seed crops intended for forage production, not for food sprout production. Furthermore, because epidemiological investigations have implicated bacterial contamination of alfalfa seeds as a major source of sprout-associated illness outbreaks (U.S. FDA, 2011b), preventive controls

are used to reduce the risk of raw sprouts serving as a vehicle for food borne illness (CFIA, 2007; U.S. FDA, 2004; U.S. FDA, 2011b). Due to these restrictions, seed for sprouts and seed for crop planting purposes are grown and sold separately. Monsanto and FGI do not permit use of biotechnology-derived alfalfa seed for sprout production through signed agreements with seed purchasers. Thus, KK179 as a commercial product is not intended to be used in the production of sprouts or other alfalfa-derived food products.

In addition to consumption as sprouts, alfalfa also has a history of consumption by humans in the form of dietary supplements and herbal remedies (Bora and Sharma, 2011). Various forms of alfalfa, including leaf concentrates, protein extracts, sprouts, sprout extracts, and seeds have been suggested to be useful for neuroprotective, hypocholesterolemic, antioxidant, antiulcer, antimicrobial, hypolipidemic, and estrogenic effects, as well as in the treatment of atherosclerosis, heart disease, stroke, cancer, diabetes, and menopausal symptoms (Bora and Sharma, 2011). As evidenced in the literature, alfalfa remains an active area of investigation by natural product researchers seeking to support uses in dietary supplements and herbal remedies (Bora and Sharma, 2011).

One notable dietary supplement is alfalfa protein concentrate, which is high in protein and xanthophyll, and is also used as a pigment in animal feed. A review of the food safety of alfalfa protein concentrate by the European Food Safety Authority found that levels of anti-nutrients, L-canavanine and phytoestrogens, in alfalfa protein concentrate when consumed at suggested rates, are lower than those from other common food sources such as lentils and onions and do not raise concerns (EFSA, 2009).

A2(b)(iv) Whether special processing is required to render food safe to eat

No special processing is required to prepare alfalfa sprouts for human food uses.

A2(b)(v) The significance to the diet in Australia and New Zealand of the host organism

As described in Section A3(iii) and Section A2(b)(ii), alfalfa has a history of minor uses by humans as food, dietary supplements, and herbal remedies (OECD, 2005).

KK179 does not present any concerns with respect to human consumption; however, its intended commercial use will be for forage production, which relies on treated seed and agronomic practices that are incompatible with non-forage purposes. Monsanto and FGI do not permit commercially sold Roundup Ready® alfalfa (J101 and J163) seed to be used for sprout production (Monsanto Company, 2012). This is a restriction enforced through signed agreements between Monsanto/FGI and seed purchasers. This same restriction will apply to KK179 seed as a commercial product.

® Roundup Ready is a registered trademark of Monsanto Technology LLC.

A3 The Nature of the Genetic Modification

A3(a) Method used to transform host organism

KK179 was developed through *Agrobacterium tumefaciens*-mediated transformation of lucerne, based on a published method (Schenk and Hildebrandt, 1972; Walker and Sato, 1981) that allows for the generation of transformed plants without the utilisation of callus. Briefly, lucerne R2336 leaf pieces (explants) were placed in a tissue culture media and co-cultured with *Agrobacterium tumifaciens* carrying the plasmid vector. R2336 is an FGI proprietary single lucerne plant; selected for regenerability from an elite, high yielding, fall dormant lucerne breeding population. After three days, explants were placed on selection medium containing the antibiotics, kanamycin and timentin, to inhibit the growth of untransformed plant cells and excess *Agrobacterium* respectively. The kanamycin-resistant calli are developed with somatic embryos. Somatic embryos were placed in media conducive to shoot and root development. Rooted plants (hereafter called T_0 plants) with normal phenotypic characteristics were selected and transferred to soil for growth and further assessment.

The T_0 plants were crossed to Ms208, a conventional male sterile plant selected from a population with a fall dormancy (FD4) phenotype, to produce F_1 plants, in which the unlinked insertions of T-DNA I and T-DNA II were segregated. Subsequently, plants that were positive for T-DNA I and negative for T-DNA II were identified by a polymerase chain reaction (PCR)-based analysis. KK179 (P_0) was selected as the lead event based on superior phenotypic characteristics and its molecular profile. P_0 is an individual F_1 plant produced from crossing T_0 with Ms208. It has the reduced lignin phenotype without the T-DNA II. The major development steps of KK179 are depicted in Figure 3. The result of this process was the production of marker-free lucerne KK179.

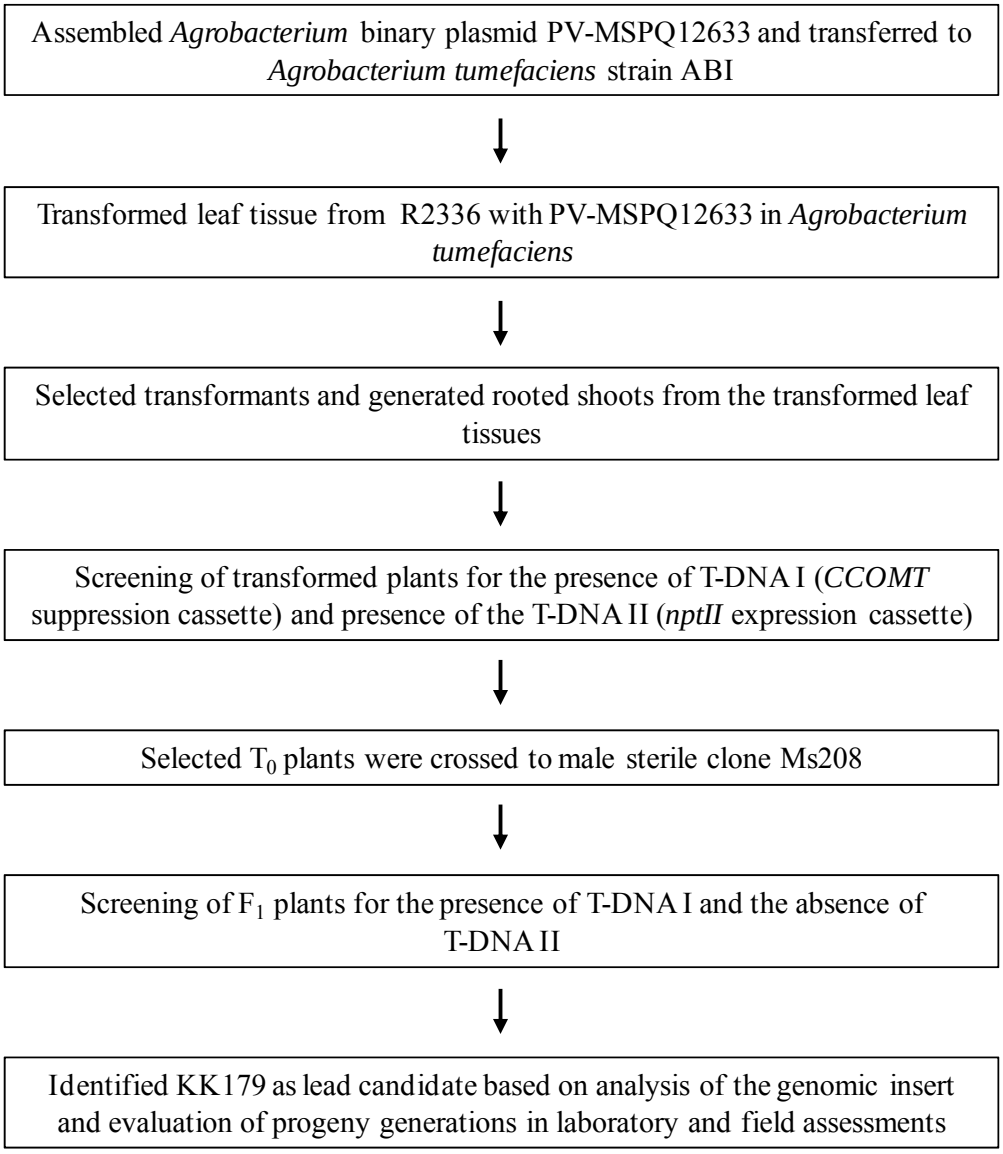


Figure 3. Schematic of the Development of KK179

A3(b) Intermediate hosts (*e.g.* bacteria)

A disarmed strain of *Agrobacterium tumefaciens* was the intermediate host used to transfer the T-DNA containing the *CCOMT surpression cassete* regulated by the Pal2 promoter and nos 3' UTR from plasmid PV-MSPQ12633 into lucerne cells to produce KK179.

A3(c)(i) Gene construct including size, source and function of all elements

PV-MSPQ12633

PV-MSPQ12633 was used for the transformation of conventional lucerne to produce KK179 and is shown in Figure 4. PV-MSPQ12633 is approximately 10.6 kb and contains two T-DNAs, each delineated by Left and Right Border regions to facilitate transformation. The first T-DNA designated as T-DNA I contains the *CCOMT* suppression cassette regulated by the Pal2 promoter and the nos 3′ UTR. The second T-DNA, designated as T-DNA II, contains the *nptII* expression cassette regulated by the 35S promoter and the nos 3′ UTR. During transformation, both T-DNAs were inserted into the lucerne genome where T-DNA II, containing the *nptII* expression cassette, functioned as a marker gene for the selection of transformed plantlets. Subsequently, traditional breeding methods and segregation, along with a combination of analytical techniques, were used to isolate those plants that contained the *CCOMT* suppression cassette (T-DNA I) but did not contain the *nptII* expression cassette (T-DNA II).

The backbone region of PV-MSPQ12633, which is located outside both of the T-DNAs contains two origins of replication (*oriV* and *ori-pUC*) for maintenance of the plasmid vector in bacteria, a bacterial selectable marker gene (*aadA*), and a coding sequence for repressor of primer (ROP) protein for the maintenance of the plasmid vector copy number in *E. coli*. A description of the genetic elements and their prefixes (e.g., P-, OR-, B-, CS-, and T-) in PV-MSPQ12633 is provided in Table 1.

The *CCOMT* Segment Sequence (T-DNA I)

The T-DNA I suppression cassette present in KK179 contains a partial gene segment from *CCOMT* configured into an inverted repeat sequence. The *CCOMT* partial gene segment comprises the *CCOMT* sequence from *Medicago sativa CCOMT* gene that encodes the caffeoyl CoA 3-*O*-methyltransferase protein (Inoue et al., 1998). The suppression cassette is comprised of ~0.8 kb of sequence from the *CCOMT* coding sequence designed to express an RNA that contains an inverted repeat of the *CCOMT* gene segments. The gene transcript with the inverted repeat produces dsRNA that, via an RNA interference (RNAi) pathway (Siomi and Siomi, 2009), suppresses endogenous *CCOMT* RNA levels, which results in reduced biosynthesis of G lignin.

The *nptII* Coding Sequence and NPT II Protein (T-DNA II)

The *nptII* expression cassette (T-DNA II) that is not present in KK179 encodes neomycin phosphotransferase II (NPT II). The *nptII* coding sequence is the *neo* gene from transposon Tn*5* of *E. coli* encoding the NPT II protein (Beck et al., 1982). NPT II protein confers kanamycin resistance (Fraley et al., 1983) and was used as a selectable marker during the

transformation selection process. Plants that did not contain the *nptII* expression cassette were isolated through traditional cross-pollinated breeding methods and segregation, along with a combination of analytical techniques.

Regulatory Sequences

T-DNA I contains an inverted repeat of a *CCOMT* gene segment under the regulation of the *Pal2* promoter and the *nos* 3′ untranslated region. The *Pal2* promoter is the promoter for phenylalanine ammonia-lyase gene from *Phaseolus vulgaris* (Cramer et al., 1989), which functions to direct transcription within vascular tissue and results in a pattern of expression that closely mirrors deposition of lignin as the plant matures (Guo et al., 2001; Leyva et al., 1992). The *nos* 3′ untranslated region is the 3′ untranslated region of the *nopaline synthase* (*nos*) gene from *Agrobacterium tumefaciens* pTi encoding NOS, which functions to direct polyadenylation of the RNA transcripts (Bevan, 1984; Fraley et al., 1983). T-DNA II contains the *nptII* coding sequence under the regulation of the *35S* promoter and the *nos* 3′ untranslated region. The *35S* promoter is the promoter for 35S RNA of cauliflower mosaic virus (CaMV) (Odell et al., 1985), which functions to direct transcription in plant cells.

T-DNA Border Regions

PV-MSPQ12633 contains Left and Right Border regions (Figure 4 and Table 1) that were derived from *Agrobacterium tumefaciens* (Barker et al., 1983; Depicker et al., 1982; Zambryski et al., 1982). The border regions each contain a 24-25 bp nick site that is the site of DNA exchange during transformation. Left and Right Border regions separate the T-DNA from the plasmid backbone region and are involved in the efficient transfer into the lucerne genome. Because PV-MSPQ12633 is a 2T-DNA vector, it contains two Left Border regions and two Right Border regions, where one border region set flanks T-DNA I and the other border region set flanks T-DNA II.

Genetic Elements Outside the T-DNA Border Regions

Genetic elements that exist outside of the T-DNA border regions are those that are essential for the maintenance or selection of PV-MSPQ12633 in bacteria and are referred to as the plasmid backbone. The origin of replication, *oriV*, is required for the maintenance of the plasmid in *Agrobacterium* and is derived from the broad host plasmid RK2 (Stalker et al., 1981). The origin of replication, *ori-pUC*, is required for the maintenance of the plasmid in *E. coli* and is derived from the plasmid vector pUC (Vieira and Messing, 1987). Coding sequence *rop* encodes the repressor of primer (ROP) protein, which is necessary for the maintenance of the plasmid vector copy number in *E. coli* (Giza and Huang, 1989). The selectable marker *aadA* is a bacterial promoter and coding sequence for an aminoglycoside-modifying enzyme, 3"(9)-*O*-nucleotidyltransferase from transposon Tn*7* that confers spectinomycin and streptomycin resistance (Fling et al., 1985) in *E. coli* and *Agrobacterium* during molecular cloning. Because these elements are outside the border regions, they are not expected to be transferred into the lucerne genome (see Section A3(d)(ii)).

Table 1. Summary of Genetic Elements in PV-MSPQ12633

Genetic Element	Location in Plasmid	Function (Reference)
T-DNA I		
B[1]-Left Border Region	1-442	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983)
Intervening Sequence	443-490	Sequence used in DNA cloning
P[2]-*Pal2*	491-1567	Promoter of the *Pal2* gene from *Phaseolus vulgaris* encoding the phenylalanine ammonia-lyase that directs transcription in plant cells (Cramer et al., 1989)
Intervening Sequence	1568-1584	Sequence used in DNA cloning
CCOMT*	1585-2103	Partial coding sequence of the *Medicago sativa CCOMT* gene that encodes the caffeoyl CoA 3-*O*-methyltransferase protein (Inoue et al., 1998) that forms part of the suppression cassette
Intervening Sequence	2104-2110	Sequence used in DNA cloning
CCOMT*	2111-2410	Partial coding sequence of the *Medicago sativa CCOMT* gene that encodes the caffeoyl CoA 3-*O*-methyltransferase protein (Inoue et al., 1998) that forms part of the suppression cassette
Intervening Sequence	2411-2418	Sequence used in DNA cloning
T[3]-*nos*	2419-2671	3′UTR sequence of the *nopaline synthase* (*nos*) gene from *Agrobacterium tumefaciens* pTi encoding NOS that directs polyadenylation (Bevan, 1984; Fraley et al., 1983)
Intervening Sequence	2672-2727	Sequence used in DNA cloning
B-Right Border Region	2728-3084	DNA region from *Agrobacterium tumefaciens* containing the Right Border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)
Vector Backbone		
Intervening Sequence	3085-3199	Sequence used in DNA cloning

Table 1 (continued). Summary of Genetic Elements in PV-MSPQ12633

Genetic Element	Location in Plasmid	Function (Reference)
aadA	3200-4088	Bacterial promoter, coding sequence, and 3′ UTR for an aminoglycoside-modifying enzyme, 3"(9)-*O*-nucleotidyltransferase from the transposon Tn*7* (Fling et al., 1985) that confers spectinomycin and streptomycin resistance
Intervening Sequence	4089-4618	Sequence used in DNA cloning
OR[4]-*ori-pUC*	4619-5196	Origin of replication from plasmid pUC for maintenance of plasmid in *E. coli* (Vieira and Messing, 1987)
Intervening Sequence	5197-5623	Sequence used in DNA cloning
CS[5]-*rop*	5624-5815	Coding sequence for repressor of primer protein from the ColE1 plasmid for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989)
Intervening Sequence	5816-6552	Sequence used in DNA cloning
OR-*oriV*	6553-6949	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium* (Stalker et al., 1981)
Intervening Sequence	6950-7035	Sequence used in DNA cloning
T-DNA II		
B-Left Border Region	7036-7477	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983)
Intervening Sequence	7478-7527	Sequence used in DNA cloning
P-*35S*	7528-7851	Promoter and leader from the 35S RNA of cauliflower mosaic virus (CaMV) (Odell et al., 1985) that directs transcription in plant cells
Intervening Sequence	7852-7884	Sequence used in DNA cloning

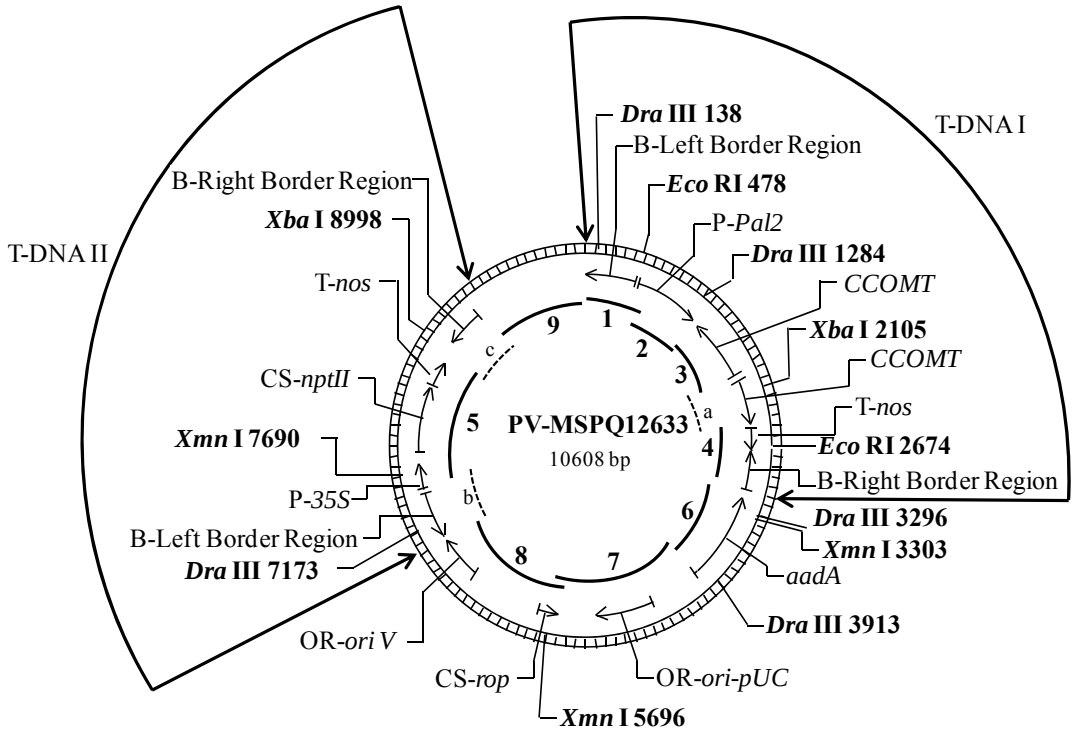
Table 1 (continued). Summary of Genetic Elements in PV-MSPQ12633

Genetic Element	Location in Plasmid	Function (Reference)
CS-*nptII*	7885-8679	Coding sequence of the *neo* gene from transposon Tn*5* of *E. coli* encoding neomycin phosphotransferase II (NPT II) (Beck et al., 1982) that confers neomycin and kanamycin resistance (Fraley et al., 1983)
Intervening Sequence	8680-8710	Sequence used in DNA cloning
T-*nos*	8711-8963	3′UTR sequence of the *nopaline synthase* (*nos*) gene from *Agrobacterium tumefaciens* pTi encoding NOS that directs polyadenylation (Bevan, 1984; Fraley et al., 1983)
Intervening Sequence	8964-9048	Sequence used in DNA cloning
B-Right Border Region	9049-9405	DNA region from *Agrobacterium tumefaciens* containing the Right Border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)
Vector Backbone		
Intervening Sequence	9406-10608	Sequence used in DNA cloning

[1]B, Border
[2]P, Promoter
[3]T, Transcription Termination Sequence
[4]OR, Origin of Replication
[5]CS, Coding Sequence
[*]Within the *CCOMT* suppression cassette, bases 1654-1953 are reverse complement to bases 2111-2410.

A3(c)(ii) Detailed map of the location and orientation of all genetic elements

Plasmid map with locations of genetic elements are shown in Figure 4.



Probe	Probe Type	Start Position (bp)	End Position (bp)	Total Length (~kb)
1	T-DNA I	1	853	0.9
2	T-DNA I	761	1568	0.8
3	T-DNA I	1507	2119	0.6
4	T-DNA I	2411	3084	0.7
5	T-DNA II	7510	9005	1.5
6	Backbone	3085	4219	1.1
7	Backbone	4126	5740	1.6
8	Backbone	5635	7035	1.4
9	Backbone	9406	10608	1.2

Figure 4. Circular Map of PV-MSPQ12633 Showing Probe 1 through Probe 9

A circular map of PV-MSPQ12633 used to develop KK179 is shown. PV-MSPQ12633 contains two T-DNAs, designated as T-DNA I and T-DNA II. Genetic elements and restriction sites (with positions relative to the size of the plasmid) used in Southern blot analyses are shown on the exterior of the map. The probes used in the Southern analyses are shown on the interior of the map. The dashed arcs indicate that probes were not generated for that region.

[a] This portion of the *CCOMT* sequence is contained in Probe 3 and not included in the T-DNA I probes.

[b] The Left Border sequences as well as some intervening sequences of T-DNA II share 100% identity to those of T-DNA I, which are covered by Probe 1 and thus not included in the T-DNA II probe.

[c] The Right Border sequences as well as some intervening sequences of T-DNA II share 100% identity to those of T-DNA I, which are covered by Probe 4 and thus not included in the T-DNA II probe.

A3(d)(i) Molecular characterisation including identification of GM elements

A multi-faceted approach was taken to characterise the genetic modification that produced KK179. The results confirmed that KK179 contains a single copy of *CCOMT* suppression cassette (T-DNA I) that is stably integrated at a single locus and is inherited according to Mendelian principles over multiple generations. Additionally, the results confirmed that T-DNA II and plasmid vector backbone sequences are not detected in KK179. These conclusions were based on several lines of evidence: 1) Southern blot analyses assayed the entire lucerne genome and demonstrated the presence of T-DNA I sequences and the absence of T-DNA II and plasmid vector backbone sequences derived from PV-MSPQ12633, and confirmed that a single copy of T-DNA I was inserted at a single locus; 2) DNA sequence analyses to determine the exact sequence of the inserted DNA and the DNA sequence flanking the 5′ and 3′ end of the insert; 3) DNA sequence comparison of the inserted DNA sequence to the T-DNA I sequence in PV-MSPQ12633 confirmed that only the expected sequences were integrated; 4) sequence comparison of the DNA sequences flanking the 5′ and 3′ ends of the T-DNA I insert to the insertion site sequence in conventional lucerne demonstrated the lack of any rearrangements that occurred at the insertion site during transformation; 5) Southern blot analysis demonstrated insert stability across multiple generations, and 6) segregation analysis further confirmed T-DNA I resides at a single locus and is inherited according to Mendelian principles of inheritance. Taken together, the characterisation of the genetic modification demonstrates that a single copy of T-DNA I was inserted at a single locus of the lucerne genome and that no plasmid vector backbone sequences are present in KK179.

Southern blot analyses were used to determine the number of copies, to characterise the insertion site of T-DNA I, as well as to assess the presence or absence of T-DNA II and plasmid vector backbone sequences (Section A(d)(ii)). The Southern blot strategy was designed to ensure that all potential inserted segments would be identified. The entire alfalfa genome was assayed with probes that spanned the complete plasmid vector PV-MSPQ12633 to detect the presence of T-DNA I, as well as the absence of T-DNA II and plasmid vector backbone sequences. This was accomplished by using probes that were less than 2 kb in length, ensuring a high level of sensitivity. This high level of sensitivity was demonstrated for each blot by detection of a positive control added at 0.1 copies per genome equivalent. Two sets of restriction enzymes were specifically chosen to fully characterise T-DNA I and detect any potential segments from the plasmid vector PV-MSPQ12633. The restriction enzyme sets were chosen such that each enzyme set cleaves once within the inserted T-DNA and at least once within the known DNA sequence flanking the 5′ or 3′ end of the insert. As a result, the enzyme sets produce overlapping segments that contain the entire insert sequence and adjacent 5′ or 3′ flanking DNA sequence. Therefore, at least one segment containing a portion of the insert with the adjacent 5′ flanking DNA generated by one set of the enzyme(s) is of a predictable size and overlaps with another predictable size segment containing a portion of the insert with the adjacent 3′ flanking DNA generated by another set of the enzyme(s). This two set enzyme design ensures that the entire insert is identified in a predictable hybridisation pattern. Additionally, this two enzyme set design also maximises

the possibility of detecting an insertion elsewhere in the genome that could be overlooked if that band co-migrated with an expected band.

To determine the number of copies and the insertion sites of T-DNA I, and the presence or absence of T-DNA II and the plasmid vector backbone sequences, duplicated samples that consisted of equal amounts of digested DNA were run on the agarose gel (Figures 4 through 10). One set of samples was run for a longer period of time (long run) than the second set (short run). The long run allows for greater resolution of large molecular weight DNA, whereas the short run allows the detection of small molecular weight DNA. The molecular weight markers on the left of the figures were used to estimate the sizes of the bands present in the long run lanes of the Southern blots, and the molecular weight markers on the right of the figures were used to estimate the sizes of bands present in the short run lanes of the Southern blots. Southern blot results demonstrated that KK179 contains a single copy of T-DNA I at a single insertion site in the lucerne genome, and no T-DNA II or backbone sequences from PV-MSPQ12633 were detected in KK179.

PCR and DNA sequence analyses of KK179, which complement the Southern blot analyses, determined the complete DNA sequence of the insert, confirmed the organisation of the elements within the insert, and determined the 5′ and 3′ insert-to-plant junctions (Figures 11 and 12)(Section A3(d)(iii)). In addition, DNA sequencing analyses confirmed each genetic element in the insert and the sequence of the insert matches the corresponding sequence in PV-MSPQ12633. Furthermore, genomic organisation at the KK179 insertion site was determined by comparing the 5′ and 3′ flanking sequences of the insert to the sequence of the insertion site in conventional lucerne.

The stability of T-DNA I present in KK179 across multiple generations was demonstrated by Southern blot fingerprint analysis (Section A3(f)). Genomic DNA from four generations (P0, MBC1, MBC2, and Syn1) of KK179 (Figure 17) was digested with one of the enzyme sets used for the insert and copy number analysis and was hybridised with a probe that detects restriction segments that encompass the entire T-DNA I insert (Figure 5). This fingerprint strategy consists of two border segments that assess not only the stability of T-DNA I, but also the stability of genomic DNA directly adjacent to T-DNA I. Generational stability analysis demonstrated that the expected Southern blot fingerprint of KK179 was maintained through four generations of the breeding history, thereby confirming the stability of T-DNA I in KK179 (Figure 18).

Segregation analysis showed that heritability and stability of the insert occurred as expected across multiple generations (Figure 17 and Table 4) which corroborates the molecular insert stability analysis and establishes that T-DNA I in KK179 is inherited according to Mendelian principles of inheritance.

A circular map of PV-MSPQ12633 annotated with the probes used in the Southern blot analysis is presented in Figure 4. A linear map depicting restriction sites within the insert, as well as the DNA flanking the insert in KK179 is shown in Figure 5. Based on the plasmid map and the linear map of the insert, a table summarising the expected DNA segments for Southern analyses is presented in Table 2. The genetic elements within the KK179 insert are summarised in Table 3. The results from the Southern blot analyses are presented in Figures

4 through 10 and Section A3(d)(ii). PCR amplification of the KK179 insert and the insertion site in conventional control for DNA sequence analysis are presented in Section A3(d)(iii) and Figure 13 and Figure 14, respectively. The generations used in the generational stability analysis are depicted in the breeding history shown in Figure 17, and the results from the generational stability analysis are presented in Section A3(f) and Figure 18. The breeding path for the segregation data is shown in Figure 17, and the results for the segregation analysis are presented in Table 4.

Please also refer to [redacted] 2011 (MSL0023299) and [redacted] 2011 (MSL0023312).

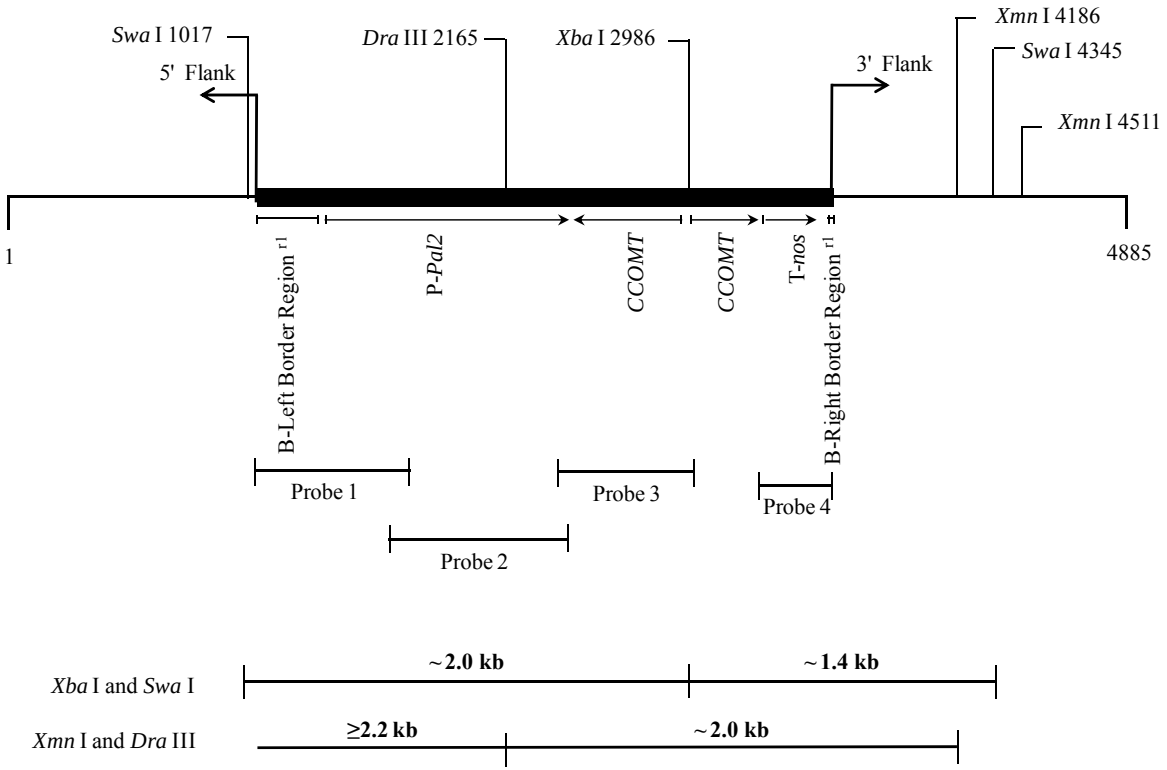


Figure 5. Schematic Representation of the Insert and Flanking DNA in KK179

DNA derived from T-DNA I of PV-MSPQ12633 integrated in KK179. Right-angled arrows indicate the ends of the integrated T-DNA I and the beginning of the flanking sequence. Identified on the map are genetic elements within the insert, as well as restriction sites with positions relative to the size of the DNA sequence (flanks and insert). The relative sizes and locations of the T-DNA I probes and the expected sizes of restriction fragments are indicated. This schematic diagram is not drawn to scale. Locations of genetic elements and T-DNA I probes are approximate. Probes are described in Figure 4

Table 2. Summary Chart of the Expected DNA Segments Based on Hybridising Probes and Restriction Enzymes Used in KK179 Analysis

Southern Blot Analysis		**T-DNA I**				**T-DNA II**	**Backbone**	
Figure		**4**	**5**	**6**	**7**	**8**	**9**	**10**
Probe Used		1	2, 4	3	3	5	6, 8	7, 9
Probing Target	**Digestion enzyme**	**Expected Band Sizes on each Southern Blot**						
PV-MSPQ12633	*Xba* I	~6.9 kb ~3.7 kb	~6.9 kb ~3.7 kb	~~[2]	~~[2]	~6.9 kb	~6.9 kb ~3.7 kb	~6.9 kb ~3.7 kb
	Eco RI	~~[2]	~~[2]	~2.2 kb	~2.2 kb	~~[2]	~~[2]	~~[2]
Probe Templates[1]	N/A	--[3]	~0.8 kb ~0.7 kb	--[3]	--[3]	--[3]	~1.1 kb ~1.4 kb	~1.6 kb ~1.2 kb
KK179	*Xba* I and *Swa* I	~2.0 kb	~2.0 kb ~1.4 kb	~2.0 kb ~1.4 kb	~~[2]	~1.4 kb	NA[4]	NA
	Xmn I and *Dra* III	≥2.2 kb	≥2.2 kb ~2.0 kb	~~[2]	~2.0 kb	~2.0 kb	NA	NA

[1] probe template spikes were used as positive hybridisation controls in Southern blot analyses when multiple probes were hybridised to the blot simultaneously
[2] '~~' indicates that this digest was not performed.
[3] '--' indicates that probe templates were not used.
[4] Not Applicable.

Table 3. Summary of Genetic Elements in KK179

Genetic Element	Location in Sequence	Function (Reference)
5′ flank	1-1047	Sequence flanking the 5′ end of the insert
B[1]-Left Border Region[r1]	1048-1322	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983)
Intervening Sequence	1323-1370	Sequence used in DNA cloning
P[2]-*Pal2*	1371-2447	Promoter of the *Pal2* gene from *Phaseolus vulgaris* encoding the phenylalanine ammonia-lyase that directs transcription in plant cells (Cramer et al., 1989)
Intervening Sequence	2448-2464	Sequence used in DNA cloning
CCOMT*	2465-2983	Partial coding sequence of the *Medicago sativa CCOMT* gene that encodes the caffeoyl CoA 3-*O*-methyltransferase protein (Inoue et al., 1998) that forms part of the suppression cassette
Intervening Sequence	2984-2990	Sequence used in DNA cloning
CCOMT*	2991-3290	Partial coding sequence of the *Medicago sativa CCOMT* gene that encodes the caffeoyl CoA 3-*O*-methyltransferase protein (Inoue et al., 1998) that forms part of the suppression cassette
Intervening Sequence	3291-3298	Sequence used in DNA cloning
T[3]-*nos*	3299-3551	3′ UTR sequence of the *nopaline synthase* (*nos*) gene from *Agrobacterium tumefaciens* pTi encoding NOS that directs polyadenylation (Bevan, 1984; Fraley et al., 1983)
Intervening Sequence	3552-3607	Sequence used in DNA cloning
B-Right Border Region[r1]	3608-3629	DNA region from *Agrobacterium tumefaciens* containing the Right Border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)
3′ flank	3630-4885	Sequence flanks the 3′ end of the insert

[1]B, Border
[2]P, Promoter
[3]T, Transcription Termination Sequence
[r1]Superscript in Left Border and Right Border regions that indicates the sequences in KK179 were truncated compared to the sequences in PV-MSPQ12633.
[*]Within the *CCOMT* suppression cassette, bases 2534-2833 are reverse complement to bases 2991-3290.

A3(d)(ii) Determination of number of insertion sites, and copy number

Insert and Copy Number of T-DNA I in KK179

The copy number and insertion sites of T-DNA I sequences in the KK179 genome were evaluated by digesting the P0 generation of KK179 and the appropriate control genomic DNA samples with two sets of restriction enzymes, a combination of *Xmn* I and *Dra* III and a combination of *Xba* I and *Swa* I, and hybridised Southern blots with probes that span the T-DNA I (Figure 4). Each restriction digest is expected to produce a specific banding pattern on the Southern blots (Table 2). Any additional copies and/or integration sites would be detected as additional bands.

The combination of *Xmn* I and *Dra* III cleaves once within the inserted DNA and at least once within the known 3′ flanking sequence in KK179 (Figure 5). Therefore, if T-DNA I sequences were present as a single copy at a single integration site in KK179, the digestion with *Xmn* I and *Dra* III was expected to generate two border segments with expected sizes of ≥2.2 kb and ~2.0 kb (Figure 5 and Table 2). The combination of *Xba* I and *Swa* I cleaves once within the inserted DNA and once within the known 5′ and 3′ flanking sequences in KK179 (Figure 5). Therefore, if T-DNA I sequences were present as a single copy at a single integration site in KK179, the digestion with *Xba* I and *Swa* I was expected to generate two border segments with expected sizes of ~2.0 kb and ~1.4 kb (Figure 5 and Table 2).

The Southern blots were hybridised with probes spanning the entire T-DNA I sequence (Figure 4, Probes 1, 2, 3, and 4). Each Southern blot contains at least one negative control and one or more positive controls. Conventional control genomic DNA digested with appropriate restriction enzymes was used as a negative control in all Southern blots. The conventional control, C_0, is derived from a cross of the untransformed R2336 with the elite conventional male sterile plant Ms208 resulting in a near isogenic line comparator to KK179. Lucerne is an autotetraploid (Yang et al., 2009) and, therefore, contains multiple copies of each endogenous gene, which are randomly segregating. Southern blots hybridised with sequences specific to the *CCOMT* gene are expected to have different hybridisation banding patterns due to random segregation of the endogenous *CCOMT* gene. Therefore, for blots that were probed with *CCOMT*-containing sequences (probe 3), the conventional parental plants, R2336 and Ms208, were also included as negative controls. Conventional control genomic DNA spiked with either digested PV-MSPQ12633 DNA and/or probe template(s) served as positive hybridisation controls. The results of this analysis are shown in Figure 6 through Figure 12. Please also refer to Paul et al., 2011 (MSL0023299).

Probe 1

Conventional control genomic DNA, digested with a combination of *Xmn* I and *Dra* III (Figure 6, Lane 1 and Lane 8) or with a combination of *Xba* I and *Swa* I (Figure 6, Lane 3 and Lane 10) and hybridised with Probe 1 (Figure 4), showed no detectable hybridisation bands, as expected. Conventional control genomic DNA digested with *Xmn* I and *Dra* III and spiked with PV-MSPQ12633 DNA previously digested with *Xba* I produced two expected size bands at ~6.9 kb and ~3.7 kb (Figure 6, Lane 6 and Lane 7). The ~3.7 kb band

and ~6.9 kb band were both detected because the Left Border region contained in Probe 1 sequence is present in both the ~3.7 kb and the ~6.9 kb *Xba* I segments from PV-MSPQ12633. Detection of the spiked controls indicates that the probe hybridised to its target sequences.

KK179 genomic DNA, digested with a combination of *Xmn* I and *Dra* III and hybridised with Probe 1 (Figure 4), produced a band at ~4.5 kb (Figure 6, Lane 2 and Lane 9). The ~4.5 kb band is the expected band representing the 5′ end of the inserted DNA, and the adjacent DNA flanking the 5′ end of the insert; this correlates with the expected border fragment size of ≥2.2 kb.

KK179 genomic DNA, digested with a combination of *Xba* I and *Swa* I and hybridised with Probe 1, produced the expected band at ~2.0 kb (Figure 6, Lane 4 and Lane 11). The ~2.0 kb band is the expected band representing the 5′ end of the inserted DNA and the adjacent DNA flanking the 5′ end of the insert. The results presented in Figure 6 indicate that the sequence covered by Probe 1 resides as one copy at a single detectable locus of integration in KK179.

Probe 2 and Probe 4

Conventional control genomic DNA, digested with a combination of *Xmn* I and *Dra* III (Figure 7, Lane 1 and Lane 8) or with a combination of *Xba* I and *Swa* I (Figure 7, Lane 3 and Lane 10) and hybridised with Probe 2 and Probe 4 (Figure 4), showed no detectable hybridisation bands, as expected. Conventional control genomic DNA, digested with a combination of *Xmn* I and *Dra* III and spiked with PV-MSPQ12633 DNA previously digested with *Xba* I, produced two expected bands at ~6.9 kb and ~3.7 kb (Figure 7, Lane 5). Conventional control genomic DNA, digested with a combination of *Xmn* I and *Dra* III and spiked with probe templates generated from PV-MSPQ12633 (Figure 4, Probe 2 and Probe 4), produced the expected bands at ~0.8 kb and ~0.7 kb, respectively (Figure 7, Lane 6 and Lane 7). Detection of the spiked controls indicates that the probes hybridised to their target sequences.

KK179 genomic DNA, digested with a combination of *Xmn* I and *Dra* III and hybridised with Probe 2 and Probe 4 (Figure 4), produced two bands at ~4.5 kb and ~2.0 kb (Figure 7, Lane 2 and Lane 9). The ~4.5 kb band is the expected band representing the 5′ end of the inserted DNA and the adjacent DNA flanking the 5′ end of the insert; this correlates with the expected border fragment size of ≥2.2 kb. The ~2.0 kb band is the expected band representing the 3′ end of the inserted DNA and the adjacent DNA flanking the 3′ end of the insert.

KK179 genomic DNA, digested with a combination of *Xba* I and *Swa* I and hybridised with Probe 2 and Probe 4 (Figure 4), produced two expected bands at ~2.0 kb and ~1.4 kb (Figure 7, Lane 4 and Lane 11). The ~2.0 kb band is the expected band representing the 5′ end of the inserted DNA and the adjacent DNA flanking the 5′ end of the insert. The ~1.4 kb band is the expected band representing the 3′ end of the inserted DNA and the adjacent DNA flanking the 3′ end of the insert. The results presented in Figure 7 indicate that the sequence

covered by Probe 2 and Probe 4 resides as one copy at a single detectable locus of integration in KK179.

Probe 3

Lucerne is an autotetraploid (Yang et al., 2009), and thus contains multiple copies of each endogenous gene that are randomly segregating. Probe 3 covers the *CCOMT* region of PV-MSPQ12633 which contains sequences specific to the endogenous *CCOMT* gene in the lucerne genome. Therefore, the random segregation of the endogenous *CCOMT* in the lucerne genome is expected to lead to different hybridisation banding patterns with Probe 3. In order to show all endogenous *CCOMT* alleles, both parental plants R2336 and Ms208 were included as conventional parental controls in addition to conventional control C_0 when probed with Probe 3. A hybridisation band in KK179 that corresponds with a band detected in either one or both of the conventional parental plants, R2336 and Ms208, would indicate that it is an endogenous hybridisation signal and, therefore, not specific to the inserted DNA in KK179.

The conventional control, conventional parental controls R2336 and Ms208, and KK179 genomic DNA were digested with a combination of *Xba* I and *Swa* I (Figure 8), or with a combination of *Xmn* I and *Dra* III (Figure 9) and probed with Probe 3 (Figure 4). As expected, different hybridisation bands were present in the conventional control and conventional parental controls. All observed bands in the conventional and conventional parental controls represent hybridisation with the endogenous *CCOMT* gene in the alfalfa genome.

Conventional control genomic DNA, digested with *Xba* I and *Swa* I (Figure 8, Lane 1 and Lane 8) and hybridised with Probe 3 (Figure 4), displayed hybridisation bands at ~7.9 kb and ~11.0 kb. Conventional parental control R2336 genomic DNA digested with *Xba* I and *Swa* I (Figure 8, Lane 2 and Lane 9) and hybridised with Probe 3 (Figure 4) displayed hybridisation bands at ~7.9 kb and ~10.0 kb. Conventional parental control Ms208 genomic DNA digested with *Xba* I and *Swa* I (Figure 8, Lane 3 and Lane 10) and hybridised with Probe 3 (Figure 4) displayed hybridisation bands at ~7.9 kb, ~11.0 kb, and ~14.0 kb, ~16.0 kb, and ~20.0 kb. Since the conventional control is derived from a cross between R2336 and Ms208, as expected, the hybridisation bands detected in the conventional control are present in either R2336 or Ms208.

The conventional control genomic DNA, digested with *Xba* I and *Swa* I and spiked with PV-MSPQ12633 DNA previously digested with *Eco* RI, produced the expected band at ~2.2 kb (Figure 8, Lane 6 and Lane 7) in addition to the endogenous hybridisation bands at ~7.9 kb and ~11.0 kb. Detection of the positive control indicates that the probe hybridised to its target sequences.

KK179 genomic DNA, digested with *Xba* I and *Swa* I (Figure 8, Lane 4 and Lane 11) and hybridised with Probe 3 (Figure 4), displayed bands at ~1.4 kb, ~2.0 kb, ~7.9 kb, ~10.0 kb, ~11.0 kb, ~14.0 kb and ~16.0 kb. The ~7.9 kb, ~10.0 kb, ~11.0 kb, ~14.0 kb, and ~16.0 kb bands represent endogenous hybridisation, as these bands have also been observed in either

the R2336 or Ms208 conventional parental controls (Figure 8, Lanes 2, 3, 9, and 10). The ~1.4 kb and ~2.0 kb bands are the expected hybridisation bands (Table 2) from the inserted T-DNA. The ~2.0 kb band is the expected band representing the 5′ end of the inserted DNA and the adjacent DNA flanking the 5′ end of the insert. The ~1.4 kb band is the expected band, representing the 3′ end of the inserted DNA and the adjacent DNA flanking the 3′ end of the insert.

The conventional control genomic DNA, digested with *Xmn* I and *Dra* III (Figure 9, Lane 1 and Lane 8) and hybridised with Probe 3 (Figure 4), displayed hybridisation bands at ~6.9 kb, ~7.9 kb, ~11.0 kb, ~14.0 kb, and ~16.0 kb. The conventional parental control R2336 genomic DNA, digested with *Xmn* I and *Dra* III (Figure 9, Lane 2 and Lane 9) and hybridised with Probe 3 (Figure 4), displayed the hybridisation bands at ~4.2 kb, ~5.9 kb ~6.2 kb, ~11.0 kb, ~14.0 kb, and~15.0 kb. The conventional parental control Ms208 genomic DNA, digested with *Xmn* I and *Dra* III (Figure 9, Lane 3 and Lane 10) and hybridised with Probe 3 (Figure 4), displayed the hybridisation bands at ~6.9 kb, ~7.9 kb, ~11.0 kb, ~14.0 kb, ~16.0 kb and ~20.0 kb. Since the conventional control is derived by a cross between R2336 and Ms208, as expected, the hybridisation bands detected in the conventional control are present in either R2336 or Ms208.

The conventional control genomic DNA, digested with *Xmn* I and *Dra* III and spiked with PV-MSPQ12633 DNA previously digested with *Eco* RI, produced an expected band at ~2.2 kb (Figure 9, Lane 6 and Lane 7) in addition to the endogenous hybridisation bands at ~6.9 kb, ~7.9 kb, ~11.0 kb, ~14.0 kb, and ~16.0 kb. Detection of the positive control indicates that the probe hybridised to its target sequences.

KK179 genomic DNA, digested with *Xmn* I and *Dra* III (Figure 9, Lane 4 and Lane 11) and hybridised with Probe 3 (Figure 4), displayed bands at ~2.0 kb, ~4.2 kb, ~6.2 kb, ~6.9 kb, ~11.0 kb, ~14.0 kb, and ~20.0 kb. The ~4.2 kb, ~6.2 kb, ~6.9 kb, ~11.0 kb, ~14.0 kb and ~20.0 kb bands are endogenous, as these bands have also been observed in either the R2336 or Ms208 conventional parental controls (Figure 9, Lanes 2, 3, 9, and 10). The ~2.0 kb band is the expected band from the inserted DNA representing the 3′ end of the inserted DNA and the adjacent DNA flanking the 3′ end of the insert.

The results presented in Figure 8 and Figure 9 indicate that the sequence covered by Probe 3 resides as one copy at a single detectable locus of integration in KK179.

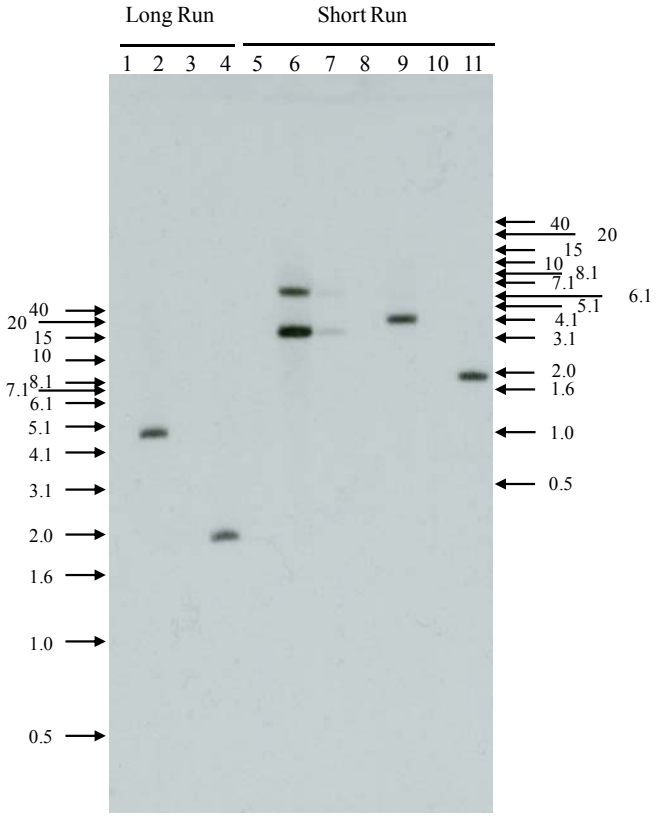


Figure 6. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA I in KK179: Probe 1

The blot was hybridised with one ^{32}P-labeled probe that spanned a portion of the T-DNA I sequence (Figure 4, Probe 1). Each lane contains ~10 µg of digested genomic DNA isolatedfrom leaf tissue. Lane designations are as follows:

Lane

1 Conventional Control (*Xmn* I and *Dra* III)
2 KK179 (*Xmn* I and *Dra* III)
3 Conventional Control (*Xba* I and *Swa* I)
4 KK179 (*Xba* I and *Swa* I)
5 Blank
6 Conventional Control (*Xmn* I and *Dra* III) spiked with PV-MSPQ12633 (*Xba* I) [~1.0 genome equivalent]
7 Conventional Control (*Xmn* I and *Dra* III) spiked with PV-MSPQ12633 (*Xba* I) [~0.1 genome equivalent]
8 Conventional Control (*Xmn* I and *Dra* III)
9 KK179 (*Xmn* I and *Dra* III)
10 Conventional Control (*Xba* I and *Swa* I)
11 KK179 (*Xba* I and *Swa* I)

Arrows denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb DNA Extension Ladder (Invitrogen) on the ethidium bromide stained gel.

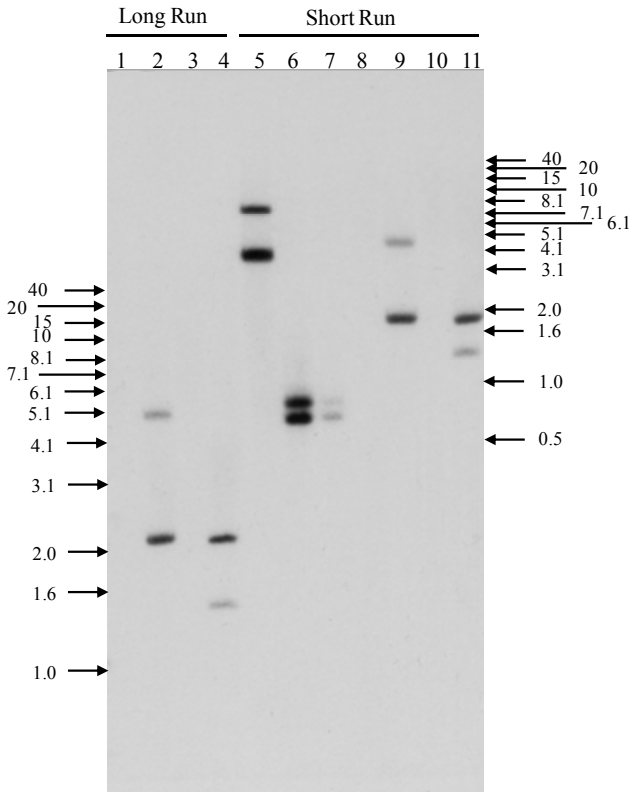


Figure 7. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA I in KK179: Probe 2 and Probe 4

The blot was hybridised with two ^{32}P-labeled probes that spanned a portion of the T-DNA I sequence (Figure 4, Probe 2 and Probe 4). Each lane contains ~10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane

1 Conventional Control (*Xmn* I and *Dra* III)
2 KK179 (*Xmn* I and *Dra* III)
3 Conventional Control (*Xba* I and *Swa* I)
4 KK179 (*Xba* I and *Swa* I)
5 Conventional Control (*Xmn* I and *Dra* III) spiked with PV-MSPQ12633 (*Xba* I) [~1.0 genome equivalent]
6 Conventional Control (*Xmn* I and *Dra* III) spiked with Probe 2 and Probe 4 [~1.0 genome equivalent]
7 Conventional Control (*Xmn* I and *Dra* III) spiked with Probe 2 and Probe 4 [~0.1 genome equivalent]
8 Conventional Control (*Xmn* I and *Dra* III)
9 KK179 (*Xmn* I and *Dra* III)
10 Conventional Control (*Xba* I and *Swa* I)
11 KK179 (*Xba* I and *Swa* I)

Arrows denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb DNA Extension Ladder (Invitrogen) on the ethidium bromide stained gel.

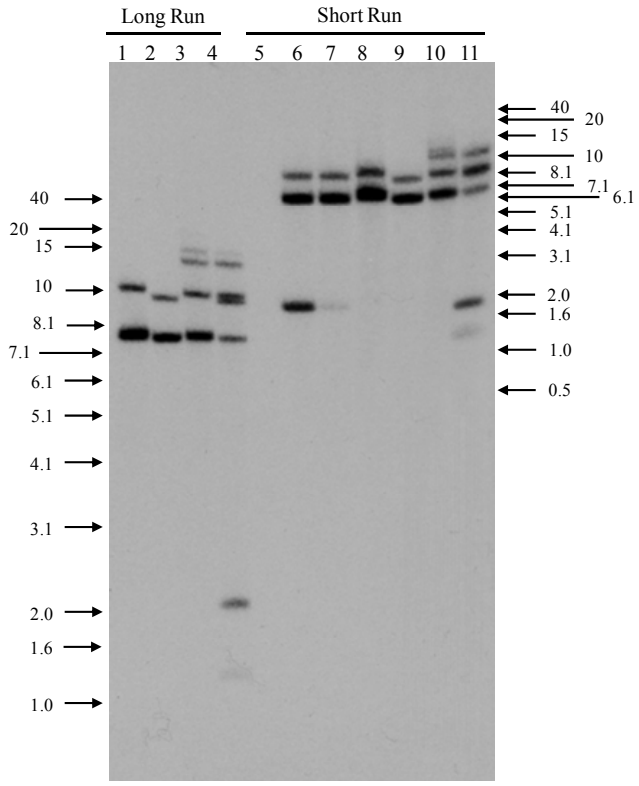


Figure 8. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA I in KK179: Probe 3

The blot was hybridised with one ^{32}P-labeled probe that spanned portions of the T-DNA I sequence (Figure 4, Probe 3). Each lane contains ~10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane	
1	Conventional Control (*Xba* I and *Swa* I)
2	Conventional Parental Control R2336 (*Xba* I and *Swa* I)
3	Conventional Parental Control Ms208 (*Xba* I and *Swa* I)
4	KK179 (*Xba* I and *Swa* I)
5	Blank
6	Conventional Control (*Xba* I and *Swa* I) spiked with PV-MSPQ12633 (*Eco* RI) [~1.0 genome equivalent]
7	Conventional Control (*Xba* I and *Swa* I) spiked with PV-MSPQ12633 (*Eco* RI) [~0.1 genome equivalent]
8	Conventional Control (*Xba* I and *Swa* I)
9	Conventional Parental Control R2336 (*Xba* I and *Swa* I)
10	Conventional Parental Control Ms208 (*Xba* I and *Swa* I)
11	KK179 (*Xba* I and *Swa* I)

Arrows denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb DNA Extension Ladder (Invitrogen) on the ethidium bromide stained gel.

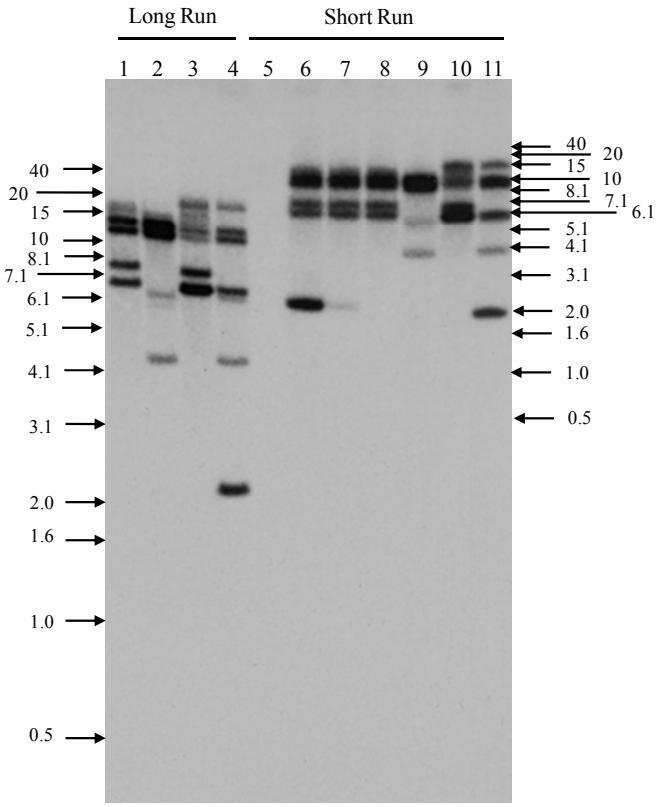


Figure 9. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA I in KK179: Probe 3

The blot was hybridised with one ^{32}P-labeled probe that spanned portions of the T-DNA I sequence (Figure 4, Probe 3). Each lane contains ~10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane

1 Conventional Control (*Xmn* I and *Dra* III)
2 Conventional Parental Control R2336 (*Xmn* I and *Dra* III)
3 Conventional Parental Control Ms208 (*Xmn* I and *Dra* III)
4 KK179 (*Xmn* I and *Dra* III)
5 Blank
6 Conventional Control (*Xmn* I and *Dra* III) spiked with PV-MSPQ12633 (*Eco* RI) [~1.0 genome equivalent]
7 Conventional Control (*Xmn* I and *Dra* III) spiked with PV-MSPQ12633 (*Eco* RI) [~0.1 genome equivalent]
8 Conventional Control (*Xmn* I and *Dra* III)
9 Conventional Parental Control R2336 (*Xmn* I and *Dra* III)
10 Conventional Parental Control Ms208 (*Xmn* I and *Dra* III)
11 KK179 (*Xmn* I and *Dra* III)

Arrows denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb DNA Extension Ladder (Invitrogen) on the ethidium bromide stained gel.

Southern Blot Analysis to Determine the Presence or Absence of T-DNA II Sequences in KK179

The presence or absence of T-DNA II sequences in the KK179 lucerne genome was evaluated by digesting the P_0 generation of KK179 and the conventional control genomic DNA samples with two sets of restriction enzymes: a combination of *Xmn* I and *Dra* III and a combination of *Xba* I and *Swa* I. The Southern blot was hybridised with a probe spanning the T-DNA II sequence, except for the border regions and some of the intervening sequences (Figure 4, Probe 5). Since the border sequences and those intervening sequences of T-DNA II share 100% homology to the border and intervening sequences of T-DNA I, these border regions and intervening sequences were covered by T-DNA I Probes 1 and 4. A portion of Probe 5 contains sequences that are 100% homologous to the *nos* 3′ UTR sequence present in T-DNA I. Therefore, hybridisation with Probe 5 is expected to result in detection of the T-DNA I segment containing the *nos* 3′ UTR in KK179. If T-DNA II sequences are present in KK179, then hybridisation with Probe 5 would result in the detection of unique hybridisation bands in addition to the expected bands from the T-DNA I insert containing the *nos* 3′ UTR. The result of this analysis is shown in Figure 10.

Probe 5

Conventional control genomic DNA, digested with combination of *Xmn* I and *Dra* III (Figure 10, Lane 1 and Lane 8) or a combination of *Xba* I and *Swa* I (Figure 10, Lane 3 and Lane 10) and hybridised with Probe 5 (Figure 4), showed no detectable hybridisation bands, as expected. Conventional control genomic DNA, digested with combination of *Xmn* I and *Dra* III and spiked with PV-MSPQ12633 DNA previously digested with *Xba* I, produced an expected band at ~6.9 kb (Figure 10, Lane 6 and Lane 7). Detection of the spiked controls indicates that the probe hybridised to its target sequences.

KK179 genomic DNA, digested with *Xmn* I and *Dra* III (Figure 10, Lane 2 and Lane 9) and hybridised with Probe 5 (Figure 4), produced the expected band at ~2.0 kb only visible in the longer exposure (data not shown) due to the homology of the *nos* 3′ UTR in Probe 5 with T-DNA I. KK179 DNA, digested with *Xba* I and *Swa* I (Figure 10, Lane 4 and Lane 11) and hybridised with Probe 5 (Figure 4), produced an expected band at ~1.4 kb in a longer exposure (data not shown) due to the homology of the *nos* 3′ UTR in Probe 5 with T-DNA I. This low level of intensity is expected because the *nos* 3′ UTR sequence is AT rich and represents only a small portion of Probe 5. There are no additional hybridisation bands other than the one expected from T-DNA I insert, indicating that KK179 contains no detectable T-DNA II elements from Probe 5 of PV-MSPQ12633.

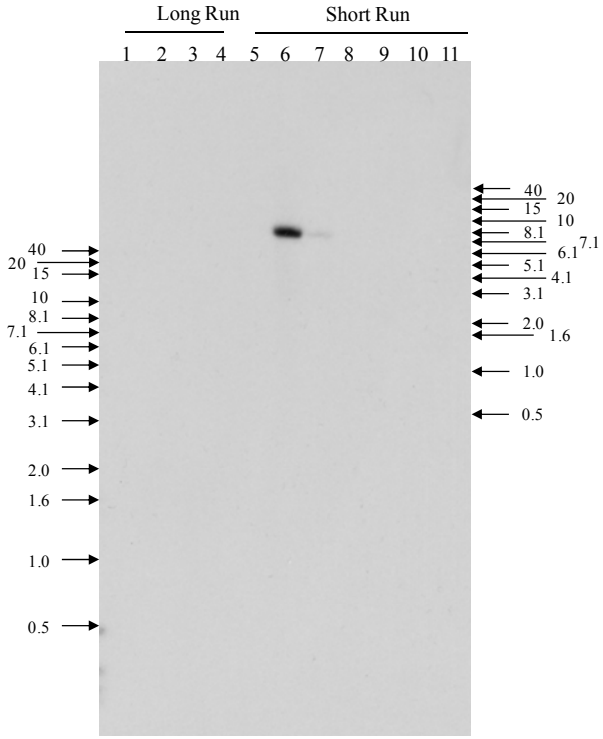


Figure 10. Southern Blot Analysis to Detect the Presence or Absence of T-DNA II Sequences in KK179: Probe 5

The blot was hybridised with one ^{32}P-labeled probe that spanned a portion of the T-DNA II sequence (Figure 4, Probe 5). Each lane contains ~10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane

1 Conventional Control (*Xmn* I and *Dra* III)
2 KK179 (*Xmn* I and *Dra* III)
3 Conventional Control (*Xba* I and *Swa* I)
4 KK179 (*Xba* I and *Swa* I)
5 Blank
6 Conventional Control (*Xmn* I and *Dra* III) spiked with PV-MSPQ12633 (*Xba* I) [~1.0 genome equivalent]
7 Conventional Control (*Xmn* I and *Dra* III) spiked with PV-MSPQ12633 (*Xba* I) [~0.1 genome equivalent]
8 Conventional Control (*Xmn* I and *Dra* III)
9 KK179 (*Xmn* I and *Dra* III)
10 Conventional Control (*Xba* I and *Swa* I)
11 KK179 (*Xba* I and *Swa* I)

Arrows denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb DNA Extension Ladder (Invitrogen) on the ethidium bromide stained gel.

Southern Blot Analysis to Determine the Presence or Absence of PV-MSPQ12633 Backbone Sequences in KK179

The presence or absence of PV-MSPQ12633 backbone sequences in the lucerne genome was evaluated by digesting the P_0 generation of KK179 and the appropriate conventional control genomic DNA samples with two sets of restriction enzymes, a combination of *Xmn* I and *Dra* III and with a combination of *Xba* I and *Swa* I. Digested genomic DNA was hybridised with overlapping probes spanning the backbone sequence of PV-MSPQ12633 (Figure 4, Probes 6, 7, 8, and 9). If backbone DNA sequences were present in KK179, then hybridising with overlapping probes corresponding to the backbone sequence should result in the detection of hybridisation bands on the Southern blot. The results of this analysis are shown in Figure 11 and Figure 12.

Backbone Probe 6 and Probe 8

Conventional control genomic DNA, digested with a combination of *Xmn* I and *Dra* III (Figure 11, Lane 1 and Lane 8) or with a combination of *Xba* I and *Swa* I (Figure 11, Lane 3 and Lane 10) and hybridized with Probe 6 and Probe 8 (Figure 4), showed no detectable hybridisation bands, as expected. Conventional control genomic DNA, digested with *Xmn* I and *Dra* III and spiked with PV-MSPQ12633 previously digested with *Xba* I, produced two expected bands at ~6.9 kb and ~3.7 kb (Figure 11, Lane 5). The ~6.9 kb band was detected because Probe 6 and Probe 8 hybridised with the ~6.9 kb *Xba* I segment from PV-MSPQ12633. The ~3.7 kb band was detected because a small region of the intervening sequence contained in Probe 8 is also present in the ~3.7 kb *Xba* I segment from PV-MSPQ12633 in the region corresponding to the intervening sequence contained in Probe 9 (Figure 4).

Conventional control genomic DNA, digested with a combination of *Xmn* I and *Dra* III and spiked with probe templates (Figure 4, Probe 6 and Probe 8) generated from PV-MSPQ12633, produced the expected bands at ~1.1 kb and ~1.4 kb, respectively (Figure 11, Lane 6 and Lane 7). Detection of the spiked controls indicates that the probes hybridised to their target sequences.

KK179 genomic DNA, digested with a combination of *Xmn* I and *Dra* III (Figure 11, Lane 2 and Lane 9) or with a combination of *Xba* I and *Swa* I (Figure 11, Lane 4 and Lane 11) and hybridised with Probe 6 and Probe 8 (Figure 4), produced no detectable bands, as expected. These data indicate that KK179 contains no detectable backbone elements from Probe 6 and Probe 8 of PV-MSPQ12633.

Backbone Probe 7 and Probe 9

Conventional control genomic DNA, digested with a combination of *Xmn* I and *Dra* III (Figure 12, Lane 1 and Lane 8) or with a combination of *Xba* I and *Swa* I (Figure 12, Lane 3 and Lane 10) and hybridised with Probe 7 and Probe 9 (Figure 4), showed no detectable hybridisation bands, as expected. Conventional control genomic DNA, digested with *Xmn* I and *Dra* III and spiked with PV-MSPQ12633 previously digested with *Xba* I, produced two expected bands at ~6.9 kb and ~3.7 kb, (Figure 12, Lane 5).

Conventional control genomic DNA, digested with a combination of *Xmn* I and *Dra* III and spiked with probe templates generated from PV-MSPQ12633 (Figure 4, Probe 7 and Probe 9), produced the expected bands at ~1.6 kb and ~1.2 kb (Figure 12, Lane 6 and Lane 7). Detection of the spiked controls indicates that the probes hybridised to their target sequences.

KK179 genomic DNA, digested with a combination of *Xmn* I and *Dra* III (Figure 12, Lane 2 and Lane 9) or with a combination of *Xba* I and *Swa* I (Figure 12, Lane 4 and Lane 11) and hybridised with Probe 7 and Probe 9, produced no detectable bands, as expected. These data indicate that KK179 contains no detectable backbone elements from Probe 7 and Probe 9 of PV-MSPQ12633.

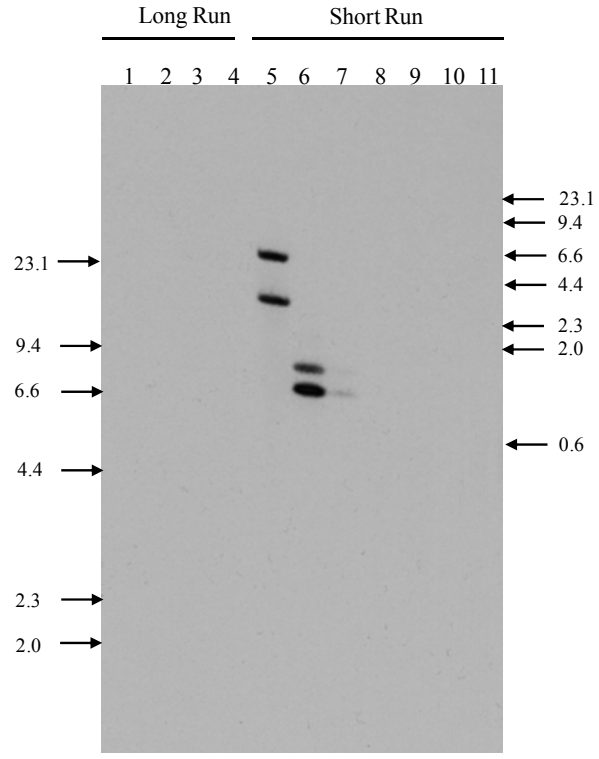


Figure 11. Southern Blot Analysis to Determine the Presence or Absence of PV-MSPQ12633 Backbone Sequences in KK179: Probe 6 and Probe 8

The blot was hybridised with two ^{32}P-labeled probes that spanned a portion of the PV-MSPQ12633 backbone sequence (Figure 4, Probe 6 and Probe 8). Each lane contains ~10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane

1 Conventional Control (*Xmn* I and *Dra* III)
2 KK179 (*Xmn* I and *Dra* III)
3 Conventional Control (*Xba* I *and Swa* I)
4 KK179 (*Xba I and Swa I*)
5 Conventional Control (*Xmn* I and *Dra* III) spiked with PV-MSPQ12633 (*Xba* I) [~1.0 genome equivalent]
6 Conventional Control (*Xmn* I and *Dra* III) spiked with Probe 6 and Probe 8 [~1.0 genome equivalent]
7 Conventional Control (*Xmn* I and *Dra* III) spiked with Probe 6 and Probe 8 [~0.1 genome equivalent]
8 Conventional Control (*Xmn* I and *Dra* III)
9 KK179 (*Xmn* I and *Dra* III)
10 Conventional Control (*Xba* I and *Swa* I)
11 KK179 (*Xba* I and *Swa* I)

Arrows denote the size of the DNA, in kilobase pairs, obtained from the λ DNA/*Hin*d III Fragments (Invitrogen) on the ethidium bromide stained gel.

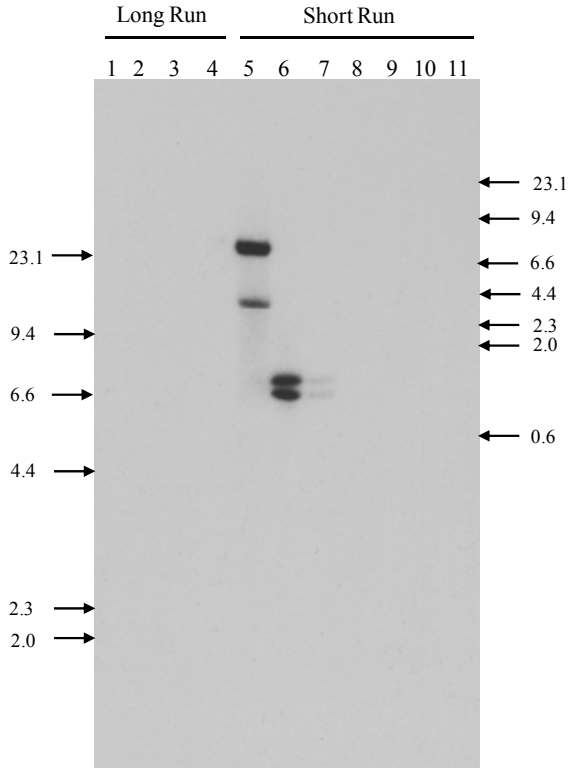


Figure 12. Southern Blot Analysis to Determine the Presence or Absence of PV-MSPQ12633 Backbone Sequences in KK179: Probe 7 and Probe 9

The blot was hybridised with two ^{32}P-labeled probes that spanned portions of PV-MSPQ12633 backbone sequence (Figure 4, Probe 7 and Probe 9). Each lane contains ~10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane

1 Conventional Control (*Xmn* I and *Dra* III)
2 KK179 (*Xmn* I and *Dra* III)
3 Conventional Control (*Xba* I and *Swa* I)
4 KK179 (*Xba* I and *Swa* I)
5 Conventional Control (*Xmn* I and *Dra* III) spiked with PV-MSPQ12633 (*Xba* I) [~1.0 genome equivalent]
6 Conventional Control (*Xmn* I and *Dra* III) spiked with Probe 7 and Probe 9 [~1.0 genome equivalent]
7 Conventional Control (*Xmn* I and *Dra* III) spiked with Probe 7 and Probe 9 [~0.1 genome equivalent]
8 Conventional Control (*Xmn* I and *Dra* III)
9 KK179 (*Xmn* I and *Dra* III)
10 Conventional Control (*Xba* I and *Swa* I)
11 KK179 (*Xba* I and *Swa* I)

Arrows denote the size of the DNA, in kilobase pairs, obtained from the λ DNA/*Hin*d III Fragments (Invitrogen) on the ethidium bromide stained gel.

A3(d)(iii) Full DNA sequence, including junction regions

Organisation and Sequence of the Insert and Adjacent Genomic DNA in KK179

PCR and sequence analyses were performed on genomic DNA that was extracted from KK179 and the conventional parental control R2336 to examine the organisation and sequence of the elements within the KK179 insert. PCR primers were designed with the intent to amplify five overlapping DNA regions that span the entire length of the T-DNA I insert and the associated DNA flanking the 5′ and 3′ ends of the insert (Figure 13). The amplified DNA segments were subjected to DNA sequence analyses. The analyses determined that the DNA sequence of the KK179 insert is 2582 bp long (Table 3) and is identical to the corresponding T-DNA I sequence of PV-MSPQ12633 as described in Table 1. From the sequence analyses, 1047 base pairs flanking the 5′ end of the KK179 insert and 1256 base pairs flanking the 3′ end of the KK179 insert (Table 3) were also determined. Please also refer to [redacted]2011 (MSL0023299).

PCR and DNA Sequence Analyses to Examine the KK179 Insertion Site

PCR and sequence analyses were performed on genomic DNA extracted from KK179 and the conventional parental control R2336 to examine the integrity of the DNA insertion site in KK179. The PCR was performed with a forward primer specific to the genomic DNA sequence flanking the 5′ end of the insert paired with a reverse primer specific to the genomic DNA sequence flanking the 3′ end of the insert (Figure 14). The amplified PCR product from the conventional parental control was subjected to DNA sequence analysis (Figure 14, Lane 2). The reaction containing KK179 genomic DNA also generated a PCR product (Figure 14, Lane 3). This product is not unexpected as lucerne is an autotetraploid and therefore possesses multiple alleles of the insertion site. Only one of the multiple alleles contains the T-DNA I insert while the others do not contain the T-DNA I insert. Sequence alignments were performed between the conventional parental control sequence and the sequences flanking the 5′ and 3′ end of the KK179 T-DNA I insert. The alignment between the sequence flanking the 5′ end of the KK179 insert and the conventional parental control sequence showed that the 5′ flanking sequence of the KK179 insert is identical to the conventional parental control sequence except for one base, which is a G within the 5′ flanking sequence of the KK179 insert and is a G/T heterozygote. The alignment between the 3′ end of the KK179 insert and the conventional parental control sequence showed that the conventional parental control sequence is identical to the sequence flanking the 3′ end of the KK179 insert except for one base, which is a G within the 3′ flanking sequence of the KK179 insert and is a A/G heterozygote. These two heterozygotes were most likely caused by single nucleotide polymorphisms segregating in the autotetraploid lucerne population. The alignment analyses also indicated a 102 base pair deletion from the conventional genomic DNA occurred upon T-DNA I insertion in KK179. This deletion presumably resulted from double stranded break repair mechanisms in the plant during the *Agrobacterium*-mediated transformation process (Salomon and Puchta, 1998). Please also refer to [redacted]2011 (MSL0023299).

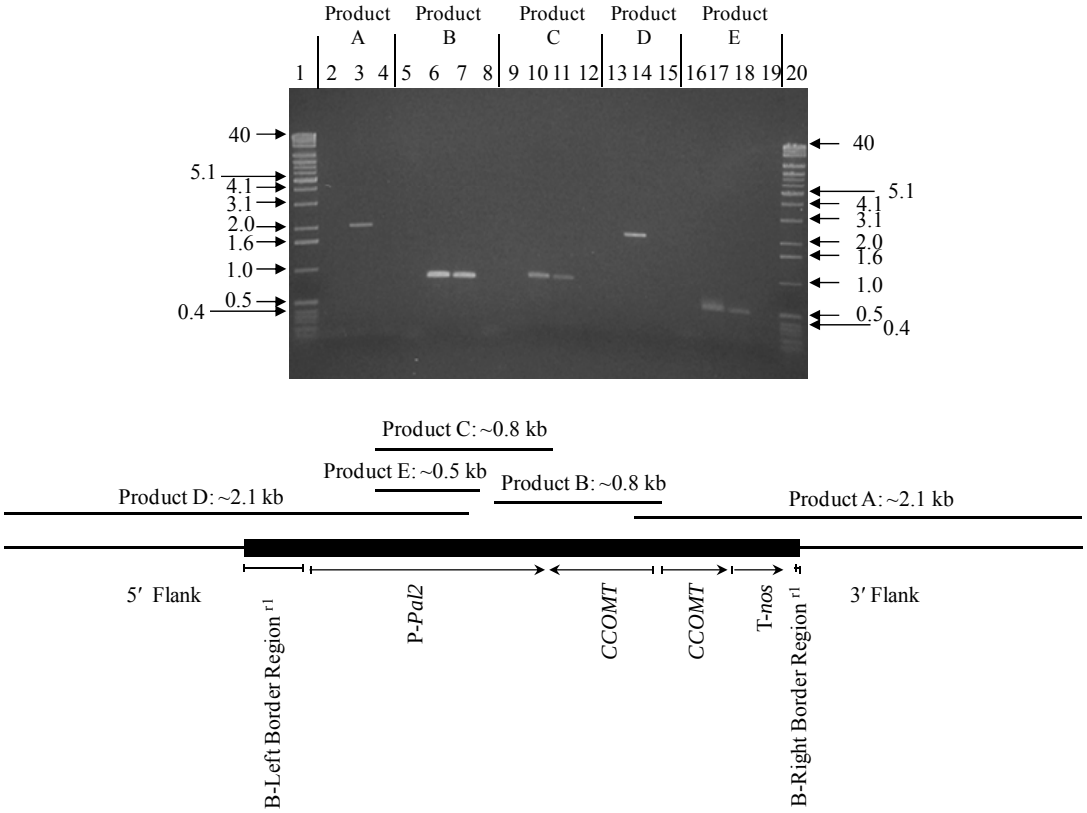


Figure 13. Overlapping PCR Analysis across the Insert in KK179

PCR was performed on both parental control genomic DNA and KK179 genomic DNA using five pairs of primers to generate overlapping PCR fragments from KK179 for sequencing analysis. To verify synthesis of the PCR products, 2-5 µl of each of the PCR reactions was loaded on the gel. The expected product size for each amplicon is provided in the illustration of the insert in KK179 that appears at the bottom of the figure. This figure is a representative of the data generated in the study. Lane designations are as follows:

Lane		Lane	
1	1 Kb DNA Extension Ladder	11	KK179
2	R2336	12	No template DNA control
3	KK179	13	R2336
4	No template DNA control	14	KK179
5	R2336	15	No template DNA control
6	PV-MSPQ12633	16	R2336
7	KK179	17	PV-MSPQ12633
8	No template DNA control	18	KK179
9	R2336	19	No template DNA control
10	PV-MSPQ12633	20	1 Kb DNA Extension Ladder

Arrows on the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb DNA Extension Ladder (Invitrogen) on the ethidium bromide stained gel.

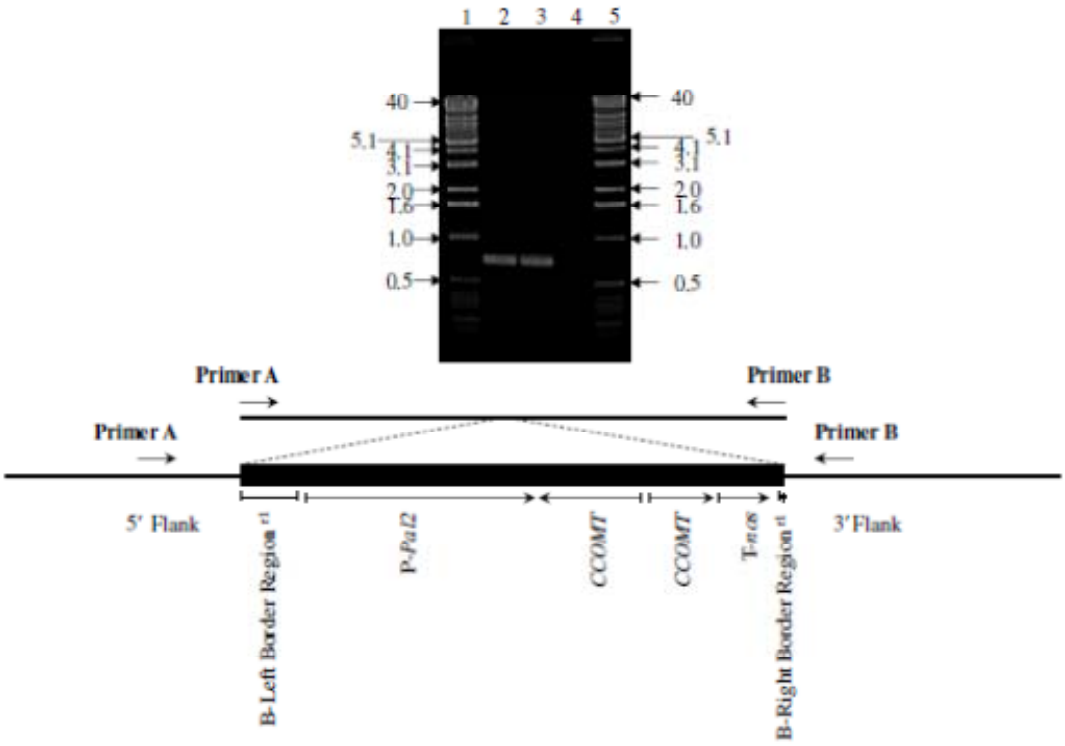


Figure 14. PCR Amplification of the KK179 Insertion Site in Conventional Lucerne

PCR analysis was performed to evaluate the insertion site. PCR was performed on DNA from the conventional parental control R2336 and KK179 using Primer A, specific to the 5′ flanking sequence, and Primer B, specific to the 3′ flanking sequence of the insert in KK179. The DNA generated from the parental control PCR was used for sequencing analysis. This illustration depicts the KK179 insertion site in the conventional parental control (upper panel) and the KK179 insert (lower panel). Approximately 5 µl aliquot of each PCR reaction was loaded on the gel. Lane designations are as follows:

Lane

1 1 Kb DNA Extension Ladder
2 Conventional Parental Control R2336
3 KK179
4 No template DNA control
5 1 Kb DNA Extension Ladder

Arrows on the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb DNA Extension Ladder (Invitrogen) on the ethidium bromide stained gel.

A3(d)(iv) Map of the organisation of the inserted DNA (each site)

PCR and DNA sequence analyses performed on KK179 and the conventional control determined the organisation of the genetic elements within the insert as given in Figure 5 and Table 2.

A3(d)(v) Identification and characterisation of unexpected ORFs

Bioinformatic Assessment of Putative Open Reading Frames (ORFs) of KK179 Insert and Flanking Sequences

The Codex Alimentarius Commission guidelines for the safety assessment of food derived from biotechnology crops (Codex Alimentarius, 2009) includes an assessment element on the identification and evaluation of "open reading frames within the inserted DNA or created by the insertion with contiguous plant genomic DNA." These assessments examine the potential homology of any putative polypeptides or proteins that could be produced from open reading frames (ORFs) in the insert or at the plant-insert junction to known toxins or allergens. These analyses are conducted even if there is no evidence that such ORFs at the plant-insert junction or alternative reading frames in the insert are capable of being transcribed and translated into a protein. Results from these bioinformatics analyses demonstrate that any putative polypeptides in KK179 are unlikely to exhibit allergenic, toxic, or otherwise biologically adverse properties.

Bioinformatic analyses were performed on the KK179 insert and flanking genomic DNA sequences to assess the potential for allergenicity, toxicity, or biological activity of putative polypeptides encoded by all six reading frames present in the KK179 insert DNA, as well as ORFs present in the 5' and 3' flanking sequence junctions (Table 3 and Figure 5). These various bioinformatic evaluations are depicted in Figure 15. The entire KK179 insert DNA sequence was translated in all six reading frames (three forward reading frames and three reading frames in reverse orientation), and the resulting deduced amino acid sequence was subjected to bioinformatic analyses. Similarly, using a conservative approach ORFs spanning the 5' flanking sequence DNA-inserted DNA and 3' flanking sequence DNA inserted DNA junctions were translated from stop codon (TGA, TAG, TAA) to stop codon in all six reading frames (three forward reading frames and three reading frames in reverse orientation). Unique putative peptides/polypeptides of eight amino acids or greater in length from each reading frame junction were then compared to toxin, allergen and all proteins databases using bioinformatic tools. There are no analytical data to indicate that any of the putative polypeptides/proteins subjected to bioinformatic evaluation are produced. Moreover, the data generated from these analyses confirm that even in the highly unlikely occurrence that a translation product was derived from frames 1 to 6 of the insert DNA, or the ORFs spanning the insert junctions; they would not share a sufficient degree of sequence similarity with other proteins to indicate they would be potentially allergenic, toxic, or have other safety implications. Therefore, there is no evidence for concern regarding the relatedness of KK179 putative polypeptides to known toxins, allergens, or biologically active putative peptides.

Bioinformatics Assessment of Insert DNA Reading Frames

Bioinformatic analyses were performed to assess the potential of toxicity, allergenicity or biological activity of any putative peptides encoded by translation of reading frames 1 through 6 of the inserted DNA in KK179 (Figure 15).

The FASTA sequence alignment tool was used to assess structural relatedness between the query sequences and any protein sequences in the AD_2012, TOX_2012, and PRT_2012 databases. Structural similarities between each putative polypeptide and sequences in the database were examined. The extent of structural relatedness was evaluated by detailed visual inspection of the alignment, the calculated percent identity and alignment length (to ascertain if alignments exceeded Codex thresholds of FASTA alignments displaying 35% identity in 80 amino acids or exact 8 amino acid matches with for FASTA searches of the AD_2012 database), and the *E*-score. Alignments having an *E*-score less than 1×10^{-5}* are deemed significant because they may reflect shared structure and function among sequences (Pearson, 2003). In addition to structural similarity, each putative polypeptide was screened for short polypeptide matches using a pair-wise comparison algorithm. In these analyses, eight contiguous and identical amino acids were defined as immunologically relevant, where eight represents the typical minimum sequence length likely to represent an immunological epitope (Silvanovich et al., 2006) and evaluated against the AD_2012 database.

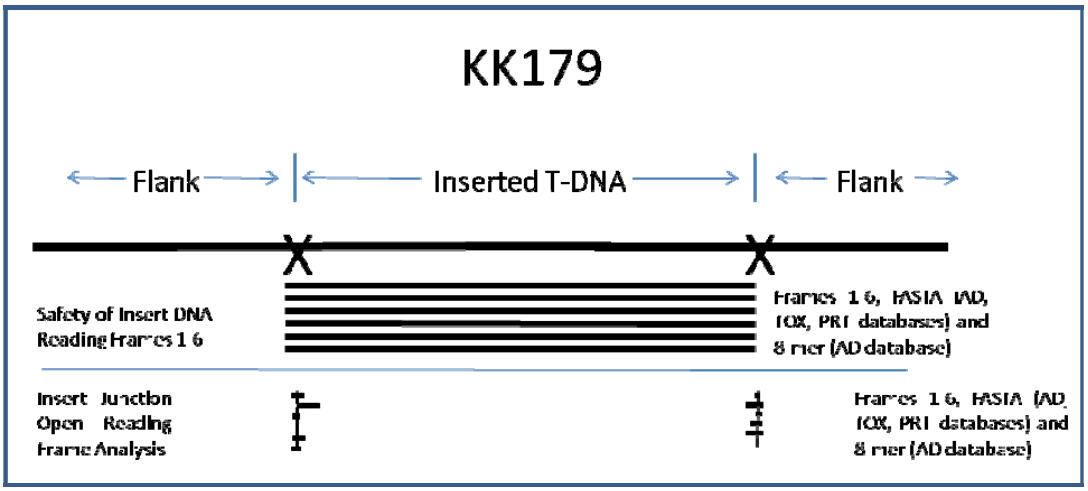


Figure 15. Schematic Summary of KK179 Bioinformatic Analyses

AD= AD_2012, TOX= TOX_2012, and PRT= PRT_2012 (GenBank release 187). 8-mer = the eight amino acid sliding window search.

* The *E*-score is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E*-score indicates a lower degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences require an *E*-score of 1×10^{-5} or smaller to be considered to have sufficient sequence similarity to infer homology.

The results of the search comparisons showed that no relevant structural similarities to known allergens or toxins were observed for any of the putative polypeptides when compared to proteins in the allergen (AD_2012) or toxin (TOX_2012) databases using FASTA. However, two identical short (eight amino acid) polypeptide matches with a protein sequence in the allergen database were identified. These identical matches of the sequence RRSSSSSS were found on frames 2 and 4 and map to the reverse complement and forward strand *CCOMT* gene segment sequences, respectively. Since the mRNA produced by the *CCOMT* gene segment anneals with itself to produce double stranded RNA, that in turn is reduced to smaller fragments by the dicing mechanism, translation of *CCOMT* mRNA resulting in the production of the RRSSSSSS peptide will not occur.

When used to query the PRT_2012 database of protein sequences, all frames yielded alignments that displayed *E*-scores < $1e^{-5}$ (1×10^{-5}). Frame 1 yielded a top alignment that was 33 amino acids in length, displaying an *E*-Score of $2.5e^{-7}$ and 97.0% identity with GI-159141796 a hypothetical protein sequence from *Agrobacterium tumefaciens*. Despite being found in a transcript, the aligning region was derived from the *nos* transcription termination sequence and is therefore unlikely to be translated *in planta*. Frame 2 yielded a top alignment that was 168 amino acids in length, displaying an *E*-Score of $2.4e^{-19}$ and 32.1% identity with GI-223974519 an unknown protein sequence in corn. The frame 2 alignment was punctuated with several stop codons and is therefore unlikely to reflect a conserved structure. Frames 3 and 5 yield alignments displaying 100% identity over 100 or more amino acids with database sequence entries for patent submissions of S-adenosyl-L-methionine trans-caffeoyl coenzyme A 3-*O*-methyltransferase and caffeoyl coenzyme A 3-*O*-methyltransferase. These alignments are not unexpected as the inserted DNA in KK179 contains sense and antisense strands encoding caffeoyl coenzyme A 3-*O*-methyltransferase. Frame 4 yielded a top alignment that was 223 amino acids in length, displaying an *E*-score of $9.3e^{-11}$ and 29.1% identity with GI 260040479, a patent submission sequence. The frame 4 alignment was punctuated with several stop codons and is therefore unlikely to reflect a conserved structure. Frame 6 yielded a top alignment that was 49 amino acids in length, displaying an *E*-score of $1.7e^{-14}$ and 98.0% identity with GI-3327940 a sequence described as belonging to rice ragged stunt polymerase. The frame 6 alignment was bisected with a stop codon and like frame 1 was translated from the *nos* transcription termination sequence, albeit from the reverse complement strand. Since the aligning portion of the query sequence was translated from the reverse complement strand of *nos*, it is unlikely to be transcribed or subsequently translated.

Taken together, these data demonstrate the lack of relevant similarities between known allergens or toxins for putative peptides derived from all six reading frames from the inserted DNA sequence of KK179. As expected, the frame 3 and 5 alignments positively identify the caffeoyl coenzyme A 3-*O*-methyltransferase 1 coding sequence. These alignment data and positive identification of the caffeoyl coenzyme A 3-*O*-methyltransferase coding sequence in the T-DNA provide no indication of adverse biological activity. As a result, in the unlikely event that a translation product was derived from reading frames 1 to 6, these putative polypeptides are not expected to be cross-reactive allergens, toxins, or other proteins that

display adverse biological activity. Please also refer to [redacted] 2012 (MSL0024048).

Insert-Junction Open Reading Frame Bioinformatics Analysis

Analyses of putative polypeptides encoded by DNA spanning the 5' and 3' genomic junctions of the KK179 inserted DNA were performed using a bioinformatic comparison strategy (Figure 15). The purpose of the assessment is to evaluate the potential for novel open reading frames (ORFs) that may have homology to known allergens, toxins, or proteins that display adverse biological activity. Sequences spanning the 5' flanking sequence DNA-inserted DNA and the inserted DNA-3' flanking sequence DNA (Figure 15) were translated from stop codon (TGA, TAG, TAA) to stop codon in all six reading frames. Unique putative peptides/polypeptides from each reading frame that were eight amino acids or greater in length were compared to AD_2012, TOX_2012, and PRT_2012 databases using FASTA, and to the AD_2012 database using an eight amino acid sliding window search.

The FASTA sequence alignment tool was used to assess structural relatedness between the query sequences and protein sequences in the AD_2012, TOX_2012, and PRT_2012 databases. No biologically-relevant structural similarity to known allergens or toxins was observed for any of the putative polypeptides. Furthermore, no short (eight amino acid) polypeptide matches were shared between any of the putative polypeptides and proteins in the allergen database. As a result, in the unlikely event that a translation product was derived from DNA spanning the 5' or 3' genomic DNA-insert junctions of KK179, these putative polypeptides are not expected to be allergens, toxins, or proteins that display adverse biological activity. Please also refer to [redacted] 2012 (MSL0023975).

A3(e) Family tree or breeding process

A single, transformed R2336 lucerne plant (T_0) was crossed with Ms208, a conventional male-sterile lucerne plant (propogated vegetatively via stem cuttings) to produce F_1 progeny plants. A single plant (P_0) was selected from these progeny plants (Figure 16) and used for molecular characterisation of the F_1 generation (Section A3(d)). Separately, a single, untransformed R2336 plant was also crossed with Ms208 to produce conventional F_1 progeny plants. A single plant (C_0) was selected and used as the near-isogenic, conventional comparator to the P_0 plant.

Due to inbreeding depression and self-incompatibility in an outcrossing species like lucerne, it is not possible to breed pure isogenic lines by self-pollination. Therefore, the subsequent generations were developed following traditional population breeding techniques for development of commercial lucerne varieties (Figure 16). The P_0 plant, containing KK179, was hand crossed with each of 10 elite lucerne genotypes with a fall dormancy 4 phenotype (FD4) to produce the next generation; these FD4 plants were used as the female seed parents. This breeding step, known as a modified backcross (MBC), resulted in the first KK179 population with related individuals (MBC1). Repeating this step by hand crossing the MBC1 generation with the same 10 elite lucerne genotypes with the FD4 phenotype resulted in the MBC2 generation, again using FD4 plants as the female seed parents. Finally, a population of MBC2 generation plants (N=80) was hand crossed *inter se* (with itself) in a breeding step known as a polycross. The resulting progeny were the Syn1 generation, as they are the first synthetic population of KK179 and the preferred population for entry into commercial variety development. Analyses of the expressed products, described in Section B.3, and composition of KK179, described in Section B.7, were conducted with the Syn1 generation. Null plants, individuals without KK179, as determined by event-specific PCR analysis, were removed at each generation prior to crossing in the KK179 synthetic populations. All handcrosses were conducted in a greenhouse.

The identical breeding process was followed using the C_0 plant in order to produce a C_0-Syn1 generation and a C_0-Syn1 Adv generation, which are conventional synthetic populations, to serve as the conventional comparators for the Syn1 and Syn1 Adv generations respectively.

The P_0, MBC1, MBC2, and Syn1 generations were used for the insert stability of KK179 reported. The Syn1 generation was used for intended changes to lignin studies and composition studies.

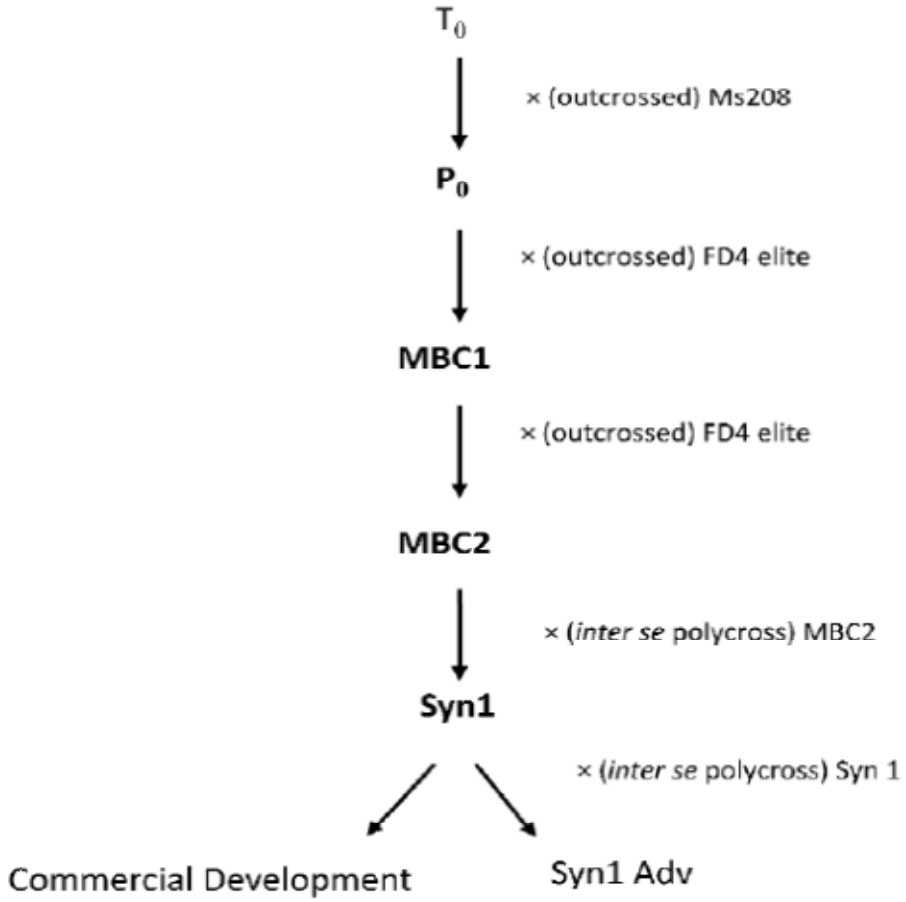


Figure 16. Breeding History of KK179

The **P_0 generation** was used for the **molecular analyses** of KK179 reported in Figures 4 through 10 and is referred to as KK179 in all Southern blot figures.
The **P_0, MBC1, MBC2, and Syn1 generations** were used for the **insert stability** of KK179 reported in Figure 18.
The **Syn1 generation** was used for **intended changes to lignin** studies described in Section B3(c) and **composition studies** described in Section B7.

Lucerne terminology

T_0:	the original transformed R2336 plant;
Ms208:	conventional male sterile lucerne plant;
P_0:	the single KK179 plant selected from the progeny of T_0 × Ms208,
FD4:	10 elite lucerne genotypes with fall dormancy rating 4 phenotype;
MBC1:	KK179 generation produced from crossing P_0 and FD4 elite genotypes through a breeding step called modified backcross;
MBC2:	subsequent KK179 generation produced from crossing MBC1 plants and FD4 elite genotypes through modified backcross;
Syn1:	a synthetic population of KK179 produced by crossing the MBC2 population of plants with each other in a breeding step called polycross; and
Syn1 Adv:	a subsequent synthetic population of KK179 produced by crossing the Syn1 population of plants with each other in a polycross.

A3(f) Evidence of the stability of the genetic changes

A3(f)(i) Pattern of inheritance of insert and number of generations monitored

Inheritance of the Genetic Insert in KK179

During development of KK179, segregation data were generated to assess the heritability and stability of the T-DNA I present in KK179 using Chi square (χ^2) analysis over several generations. The Chi square analysis is based on comparing the observed segregation ratio to the expected segregation ratio according to Mendelian principles.

The KK179 breeding path for generating segregation data is described in Figure 17. The transformed T_0 plant was cross-pollinated to an elite male sterile lucerne plant, Ms208, to produce F_1 seed. From the F_1 segregating population, an individual plant (designated as P_0) that showed negative for T-DNA II and positive for the KK179 insert was identified via Southern blot analysis and construct level gel-based PCR assay.

The selected P_0 plant was crossed with a population of 10 plants with conventional, elite genotypes with a fall dormancy 4 (FD4) rating to give rise to a modified backcrossed (MBC), designated as MBC1 plants. The pollen from 20 MBC1 plants that were positive for the insert by Endpoint TaqMan PCR was used to pollinate the same conventional FD4 population to produce MBC2 seed. The pollen from 24 MBC2 plants positive for the insert by Endpoint TaqMan PCR was used to pollinate the same conventional FD4 population to produce MBC3 seed. Finally, another 80 MBC2 plants shown to be positive for the insert by Endpoint TaqMan PCR were crossed to each other (polycross) to produce Syn1 seed (Figure 17).

The MBC2, MBC3, and Syn1 plants were tested for the expected segregation pattern for the insert using the Endpoint TaqMan PCR assay. Endpoint TaqMan PCR captures sample fluorescence reading, following the completion of the PCR reaction. The Endpoint TaqMan PCR assay was designed to detect specific DNA sequences in flank-insert junction regions and is used to determine the presence or absence of the KK179 insert in the generations evaluated. The MBC2 and MBC3 populations were predicted to segregate at a 1:1 (KK179 positive:KK179 negative) ratio and the Syn1 population was predicted to segregate at a ratio of 3:1 (KK179 positive:KK179 negative) according to Mendelian inheritance principles.

A Chi square (χ^2) analysis was performed using the statistical program R Version 2.12.0 (2010-10-15) to compare the observed segregation ratios to the expected ratios according to Mendelian inheritance principles. The χ^2 was calculated as:

$$\chi^2 = \sum [(\mid o - e \mid)^2 / e]$$

where o = observed frequency of the phenotype and e = expected frequency of the phenotype. The level of statistical significance was predetermined to be 5% ($\alpha = 0.05$).

The results of the χ^2 analysis of the segregating progeny of KK179 are presented in Table 4. The χ^2 value in the MBC2 and MBC3 populations indicated no statistically significant difference between the observed and expected 1:1 segregation ratio (KK179 positive:KK179 negative) of the KK179 insert. Likewise, the χ^2 value in the Syn1 population indicated no statistically significant difference between the observed and expected 3:1 segregation ratio (KK179 positive:KK179 negative) of the KK179 insert. These results support the

conclusion that the KK179 insert sequence in KK179 resides at a single locus within the lucerne genome and is inherited according to Mendelian inheritance principles. These results are also consistent with the molecular characterisation data that indicate KK179 contains a single, intact copy of the *CCOMT* suppression cassette that was inserted into the lucerne genome at a single locus.

Please also refer to [redacted]2011 (RPN-2010-0705).

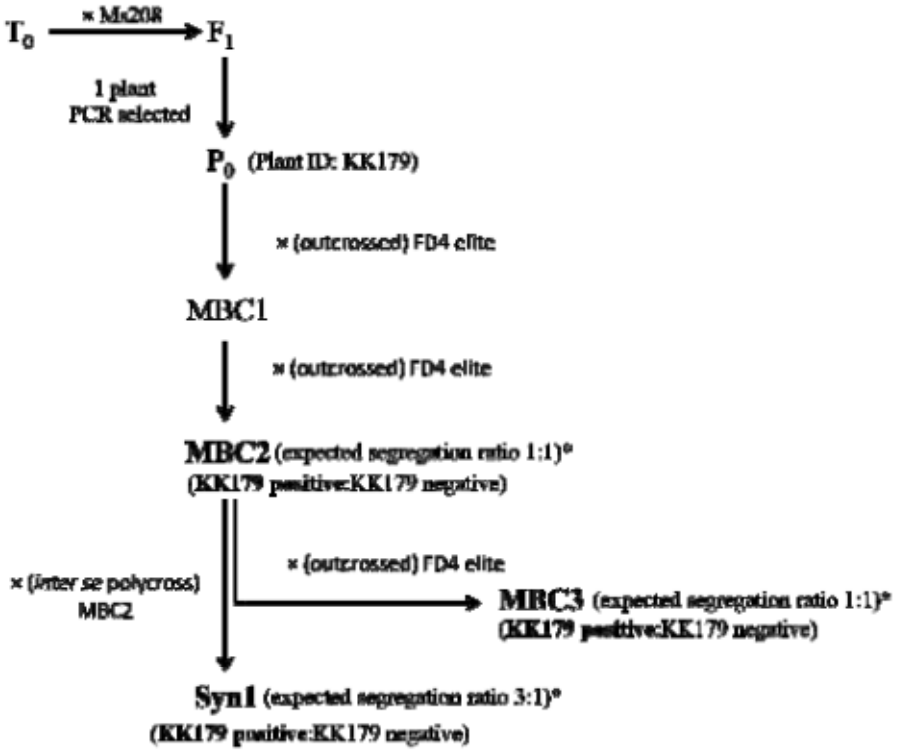


Figure 17. Breeding Path for Generating Segregation Data for KK179

The MBC2, MBC3, and Syn1 generations were used for analysing the inheritance of the insert in multiple generations.

Alfalfa terminology

T_0: the original transformed R2336 plant;

Ms208: conventional male sterile lucerne plant;

F_1: KK179 progeny produced from the cross of T_0 and Ms208.

P_0: the single KK179 plant selected from the progeny of T_0 × Ms208;

FD4: 10 elite lucerne genotypes with fall dormancy rating 4 phenotype;

MBC1: KK179 generation produced from crossing P_0 and FD4 genotypes through a modified backcross;

MBC2: subsequent KK179 generation produced from crossing MBC1 plants and FD4 genotypes through modified backcross;

MBC3: subsequent KK179 generation produced from crossing MBC2 plants and FD4 genotypes through modified backcross; and

Syn1: a synthetic population of KK179 produced by self crossing the MBC2 population of plants in a breeding step called polycross.

*Chi square analysis was conducted on segregation data from the MBC2, MBC3, and Syn1 populations.

Table 4. Segregation of the KK179 Insert During Mendelian Inheritance Testing

				1:1 Segregation			
Generation	**Total Plants[1]**	**Observed # Positives**	**Observed # Negatives**	**Expected # Positives**	**Expected # Negatives**	**χ^2**	**Probability**
MBC2	**261**	**119**	**142**	**130.5**	**130.5**	**2.027**	**0.1545**
MBC3	**263**	**132**	**131**	**131.5**	**131.5**	**0.004**	**0.9508**

				3:1 Segregation			
Generation	**Total Plants[1]**	**Observed # Positives**	**Observed # Negatives**	**Expected # Positives**	**Expected # Negatives**	**χ^2**	**Probability**
Syn1	**504**	**376**	**128**	**378**	**126**	**0.042**	**0.8370**

[1] Plants were tested for the presence of the KK179 insert by Endpoint TaqMan PCR analysis. "Total plants" refers to the total number of plants in which the presence or absence of the insert could be determined using the assay.

Southern Blot Analysis to Examine Insert Stability in Multiple Generations of KK179

In order to demonstrate the stability of the DNA insert in KK179, Southern blot analysis was performed using genomic DNA obtained from four generations of KK179 (Figure 17). Genomic DNA that was isolated from each of the selected generations of KK179 was digested with the restriction enzymes *Xmn* I and *Dra* III (Figure 18) and hybridised with Probe 2 and Probe 4 (Figure 4). Probe 2 and Probe 4 are designed to detect both fragments generated by the *Xmn* I and *Dra* III digest at ≥2.2 kb and ~2.0 kb. Any instability associated with the insert would be detected as novel bands on the Southern blot. The molecular weight markers were used to estimate the band sizes present. The results are shown in Figure 18.

Probe 2 and Probe 4

Conventional control C_0 genomic DNA, digested with *Xmn* I and *Dra* III (Figure 18, Lane 1) and hybridised with Probe 2 and Probe 4 (Figure 4), showed no detectable hybridisation bands, as expected. Conventional control genomic DNA, digested with *Xmn* I and *Dra* III and spiked with PV-MSPQ12633 previously digested with *Xba* I, produced two bands at ~6.9 kb and ~3.7 kb (Figure 18, Lane 2), as expected. Conventional control genomic DNA, digested with a combination of *Xmn* I and *Dra* III and spiked with probe templates generated from PV-MSPQ12633 (Figure 4, Probe 2 and Probe 4), produced the expected bands at ~0.8 kb and ~0.7 kb, respectively (Figure 18, Lane 3 and Lane 4). An additional, faint ~1.4 kb band in the probe templates control lane (Figure 18, Lane 3) was observed and is most likely single stranded DNA formed during purification (Qiagen, 2008) that has partially reannealed in various conformations (Kasuga et al., 2001). Since this ~1.4 kb band was detected in only one conventional control (Lane 3) and not in any other lanes, the detection of this ~1.4 kb band does not affect the conclusion of the analysis on KK179. Detection of the spiked controls indicates that the probes hybridised to their target sequences.

KK179 DNA that was extracted from generations (P_0, MBC1, MBC2, and Syn1), digested with *Xmn* I and *Dra* III (Figure 18, Lanes 5, 6, 7, and 8), and hybridised with Probe 2 and Probe 4 (Figure 4) produced two bands at ~4.5 kb and ~2.0 kb, as expected. The ~4.5 kb band is the expected band representing the 5′ end of the inserted DNA and the adjacent DNA flanking the 5′ end of the insert, which correlates with the expected border fragment size of ≥2.2 kb. The ~2.0 kb band is the expected band representing the 3′ end of the inserted DNA and the adjacent genomic DNA flanking the 3′ end of the insert. The presence of ~4.5 kb and ~2.0 kb bands in the P_0, MBC1, MBC2, and Syn1 generations demonstrates the stability of the T-DNA I insert across multiple generations of KK179.

Please also refer to [redacted]2011 (MSL 0023312).

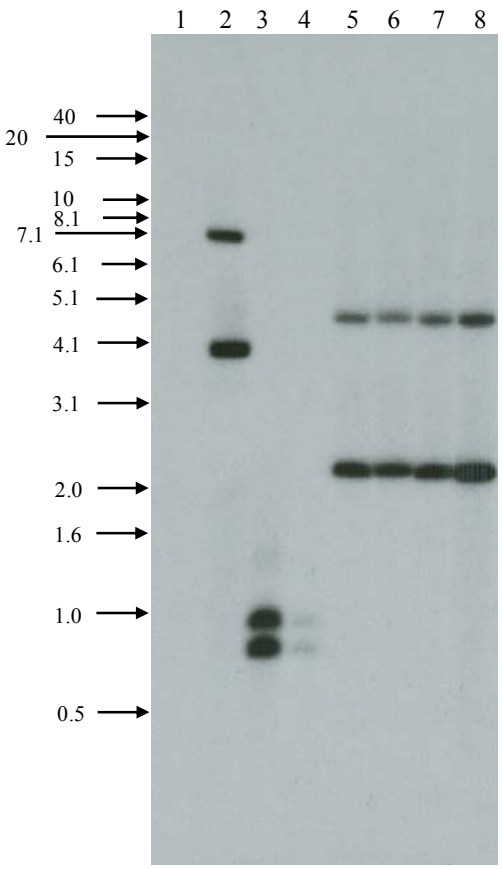


Figure 18. Southern Blot Analysis to Examine Insert Stability in Multiple Generations of KK179: Probe 2 and Probe 4

The blot was hybridised with two ^{32}P-labeled probes that spanned portions of the T-DNA sequence (Figure 4, Probe 2 and Probe 4). Each lane contains ~10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane

1 Conventional Control (*Xmn* I and *Dra* III)

2 Conventional Control (*Xmn* I and *Dra* III) spiked with PV-MSPQ12633 (*Xba* I) [~1.0 genome equivalent]

3 Conventional Control (*Xmn* I and *Dra* III) spiked with Probe 2 and Probe 4 [~1.0 genome equivalent]

4 Conventional Control (*Xmn* I and *Dra* III) spiked with Probe 2 and Probe 4 [~0.1 genome equivalent]

5 KK179 (P_0) (*Xmn* I and *Dra* III)

6 KK179 (MBC1) (*Xmn* I and *Dra* III)

7 KK179 (MBC2) (*Xmn* I and *Dra* III)

8 KK179 (Syn1) (*Xmn* I and *Dra* III)

Arrows denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb DNA Extension Ladder (Invitrogen) on the ethidium bromide stained gel.

A3(f)(ii) Pattern of expression of phenotype over several generations

KK179 does not contain any recombinant genes which encode proteins. The CCOMT suppression cassette in KK179 encodes for an RNA transcript designed to form dsRNA. Analysis of dsRNA-encoding KK179 DNA segments presented indicates that protein production by the dsRNA encoded by the insert in KK179 is highly unlikely. The traditional assessment for the presence of any proteins across multiple generations was not conducted.

dsRNA in KK179 suppresses endogenous *CCOMT* gene expression via the RNA interference (RNAi) pathway. Suppression of the *CCOMT* gene expression leads to lower CCOMT protein expression resulting in reduced production of guaiacyl lignin subunits (hereafter referred to as G lignin) compared to conventional lucerne at the same stage of growth. The reduction in G lignin subunit production leads to reduced accumulation of total lignin, as confirmed through measurement of acid detergent lignin (ADL) by commercial forage testing methods.

As an indirect assessement for the stability of dsRNA in KK179, total lignin content of lucerne forage in three breeding generations of KK179 was compared to conventional controls. Forage of KK179 and control was grown from replicated plots at two U.S. sites in a 2010 field production. Plants were grown in a randomised complete block design with three replicate blocks for each substance. The three KK179 generations were MBC1, Syn1 and Syn1-Adv1 (Figure 16). Forage samples were analysed for total lignin (acid detergent lignin). Forage lignin composition was statistically analysed. The mean, range, and standard error of lignin values of KK179 and the control were calculated across both sites and the difference between the mean values was determined for each generation. Significant differences were predetermined at the 5% level of significance (α=0.05).

Lucerne event KK179 significantly reduced total lignin content in forage from three breeding generations (Table 5). In the combined-site analyses, lignin content was decreased in KK179 by 17.71, 13.27 and 15.34% when compared to the controls in the MBC1, Syn1, and Syn1-Adv1 generations, respectively. These results support the intended reduction in total lignin in all breeding generations confirming the stability of the KK179 event.

Please also refer to [redacted] 2011 (RAR-2011-0129).

Table 5. Statistical Summary of Combined-Site Alfalfa Forage Lignin for KK179 vs. Control

			Difference (Test minus Control)	
Analytical Component (Units)[1]	**KK179 Mean (S.E.)[2] (Range)**	**Control[3] Mean (S.E.) (Range)**	**Mean (S.E.) (Range)**	**Significance (p-Value)**
Lignin (% dw)				
MBC1	4.37 (0.36) (3.55 - 5.63)	5.31 (0.36) (4.42 - 7.11)	-0.94 (0.27) (-2.09 - 1.16)	0.002
Syn1	4.02 (0.36) (3.30 - 4.31)	4.64 (0.36) (4.15 - 5.39)	-0.62 (0.27) (-1.21 - 0.060)	0.034
Syn1-Adv1	3.91 (0.36) (3.61 - 4.42)	4.62 (0.36) (3.72 - 4.89)	-0.71 (0.27) (-1.15 - -0.060)	0.016

[1]dw = dry weight.
[2]Mean (S.E.) = least-square mean (standard error).
[3]Control refers to the non-biotechnology derived, conventional control, C0- MBC1, C0-Syn1, and C0-Syn1-Adv1.

A4. Analytical Method for Detection

The T-DNA insert can be detected by isolating genomic DNA from leaf tissue and digesting with appropriate restriction endonucleases. Southern Blot analysis following digestion of genomic DNA with the appropriate restriction endonucleases will produce banding patterns consistent with the presence of the insert in KK179.

B. Information Related to the Safety of the GM Food

B1 Equivalence Studies

As described in Section A, the KK179 insert contains a *CCOMT* suppression cassette. The *CCOMT* suppression cassette, when transcribed, forms double stranded RNA (dsRNA), which is extremely unlikely to encode for a protein. Therefore, as KK179 does not express a KK179 insert-derived protein, traditional assessments of the safety of an insert-derived protein have not been conducted, including equivalence studies.

B2 Antibiotic Resistance Marker Genes

KK179 does not contain genes that encode resistance to antibiotic markers. Molecular characterisation data presented in Section A demonstrate the absence of the *nptII* antibiotic resistance marker gene in KK179.

B2(a) Clinical importance of antibiotic that GM is resistant to (if any)

Not applicable.

B2(b) Presence in food of antibiotic resistance protein (if any)

Not applicable.

B2(c) Safety of antibiotic protein

Not applicable.

B2(d) If GM organism is micro-organism, is it viable in final food?

Not applicable.

B3 Characterisation of Novel Proteins or Other Novel Substances

B3(a) Biochemical function and phenotypic effects of novel substances

As described in Section A, the KK179 insert contains a *CCOMT* suppression cassette. RNA-based suppression of the *CCOMT* gene leading to the intended reduction of G lignin and total lignin in KK179 is mediated by dsRNA molecules. These dsRNA molecules, which are produced from assembled gene transcripts in KK179 that are composed of an inverted repeat sequence, suppress endogenous *CCOMT* gene via the naturally operating endogenous RNAi pathway. Double-stranded RNAs, which are commonly found in plants and other eukaryotes for endogenous gene suppression, are composed of nucleic acids (Siomi and Siomi, 2009). Nucleic acids have a long history of safe consumption because there is no evidence of mammalian toxicity or allergenicity to RNA or DNA (Burnside et al., 2008; Heisel et al., 2008; Ivashuta et al., 2009; Jonas et al., 2001; Parrott et al., 2010; Reddy et al., 2009; U.S. FDA, 1992; Zhou et al., 2009). Several biotechnology-derived plant products previously reviewed and approved by several international regulatory authorities were developed using RNA-based suppression mechanisms, including improved fatty acid profile soybean MON 87705; high oleic soybean, virus-resistant squash, virus-resistant papaya, delayed-ripening tomatoes, and plum pox virus-resistant plum trees (ANZFA, 2000; CFIA, 2001; 2009; EFSA, 2012; HC, 1999b; 1999a; 2000; 2002; MOE, 2007; U.S. FDA, 1994; 1995a; 1995b; 1997; 2008; 2009b; 2009a; 2011a; USDA-APHIS, 2012).

Analysis of dsRNA-encoding KK179 DNA segments presented in this section indicate that protein production by the dsRNA encoded by the insert in KK179 is highly unlikely. This is based on evidence that, in eukaryotes, dsRNA is refractory to translation, as the 40s ribosomal subunit is unable to melt dsRNA regions as short as 18 nucleotides (Kozak, 1989). As a consequence, it is highly unlikely that the dsRNA produced by the transgene in KK179 would yield a translation product. Based on this information, the inserted DNA and resulting dsRNA are safe and unlikely to produce a protein or polypeptide. Furthermore, even in the highly unlikely event a protein could be produced from the *CCOMT* suppression cassette, bioinformatics analyses demonstrate the lack of relevant similarities with known allergens, toxins or other biologically-active proteins for all putative peptides derived from all six reading frames from the entire inserted DNA sequence of KK179, including the sequences in the suppression cassette described in Section A.

Based on the ubiquitous nature of the RNA-based suppression mechanism utilising dsRNA, the history of safe consumption of RNA with no documented evidence for toxicity or allergenicity of dietary RNA, and the lack of evidence of any expressed protein from the DNA inserted into KK179, the use of RNA-based suppression of endogenous *CCOMT* gene expression in KK179 poses no risks as a result of exposure to expressed products of the DNA insert.

For further explanation of the biochemical function and phenotypic effects of the dsRNA produced in KK179, please refer to Section B3(e).

B3(b) Identification of novel substances (e.g. metabolites), levels and site

Northern blot analyses were used to compare the RNA levels of the endogenous *CCOMT* gene in forage and root tissues of KK179 and conventional lucerne. Forage tissue was chosen because it is the product consumed as animal feed while root tissue comprises the remaining part of the plant. Polyadenylation enriched RNA (polyA$^+$ RNA), extracted from four replicate forage and root tissue samples of KK179 and the conventional control, was subjected to northern blot analysis. A CCOMT probe generated from a portion of the *CCOMT* gene was hybridised to the northern blots in order to compare the *CCOMT* RNA levels in KK179 and the conventional control at an equivalent growth stage. Equivalent RNA loading and quality between the conventional control and KK179 was evaluated using a lucerne actin probe as an endogenous control. The northern blot data demonstrated a clear reduction in the level of *CCOMT* RNA in KK179 compared to the conventional control in both forage and root tissue.

Northern Blot Analysis of *CCOMT* RNA in Forage

PolyA$^+$ RNA from each of four replicate samples of forage tissue from the conventional control produced a strong hybridisation signal at ~1.1 kb, as expected, based on the predicted transcript size of *CCOMT* (Figure 19, Panel A, Lanes 1, 3, 5, and 7); whereas, no detectable hybridisation signal was produced from the polyA$^+$ RNA isolated from the forage tissue of KK179 (Figure 19, Panel A, Lanes 2, 4, 6, and 8). These data demonstrate a clear reduction in the level of *CCOMT* RNA in KK179 compared to the conventional control.

The CCOMT probe was stripped from the blot and the stripped blot was hybridised with the actin probe. The polyA$^+$ RNA from the forage tissue of the conventional control (Figure 19, Panel B, Lanes 1, 3, 5, and 7) and KK179 (Figure 19, Panel B, Lanes 2, 4, 6, and 8) showed a strong hybridisation signal at ~1.5 kb, as expected for the *actin* transcript. The hybridisation signal from the forage tissue of the conventional control and KK179 of each replicate have similar intensities, indicating that the RNA loading, RNA quality, and hybridisation within each replicate of the conventional control and KK179 were similar. When hybridised with the actin probe, in addition to the expected ~0.5 kb band, a very faint ~1.0 kb band was detected (Figure 19, Panel B, Lane 11). This band most likely resulted from a hybridisation of the actin probe to single stranded DNA formed during probe template purification (Qiagen, 2008) that has partially reannealed in various confirmations (Kasuga et al., 2001). Since the actin probe template loaded in this lane serves as a positive hybridisation control and showed that the probe hybridised to the target sequence, the presence of the faint ~1.0 kb band has no impact on the conclusions drawn from this analysis. Therefore, the difference in the CCOMT hybridisation signals between the conventional control and KK179 reflects the difference in the *CCOMT* RNA levels (Figure 19, Panel A).

Northern Blot Analysis of *CCOMT* RNA in Root

PolyA$^+$ RNA from each of the four replicate samples of root tissue from the conventional control produced a strong hybridisation signal at ~1.1 kb, as expected, based on the predicted transcript size of *CCOMT* (Figure 20, Panel A, Lanes 1, 3, 5, and 7); whereas, a greatly reduced signal was produced from the polyA$^+$ RNA isolated from the root tissue of KK179 (Figure 20, Panel A, Lanes 2, 4, 6, and 8). These data demonstrate a clear reduction in the level of *CCOMT* RNA in KK179 compared to the conventional control.

The CCOMT probe was stripped from the blot and the stripped blot was hybridised with the actin probe. The polyA$^+$ RNA from the root tissue of the conventional control (Figure 20, Panel B, Lanes 1, 3, 5, and 7) and KK179 (Figure 20, Panel B, Lanes 2, 4, 6, and 8) showed a strong hybridisation signal at ~1.5 kb, as expected for the *actin* transcript. The hybridisation signal from the root tissue of the conventional control and KK179 of each replicate sample have similar intensities, indicating that the RNA loading, RNA quality, and hybridisation within each replicate of the conventional control and KK179 are similar. As with the forage tissue analysis, a very faint ~1.0 kb band detected (Figure 20, Panel B, Lane 11) when hybridised with the actin probe was not considered to have an impact on the conclusions drawn from this analysis. Therefore, the difference in the CCOMT hybridisation signal intensities between the conventional control and KK179 reflect the difference in *CCOMT* RNA levels (Figure 20, Panel A). In addition to the ~1.5 kb actin transcript, faint ~1.1 kb bands were observed (Figure 20, Panel B, Lanes 1, 3, 5, and 7). Those faint bands likely resulted from the incomplete removal of the CCOMT probe on the stripped blot prior to probing with the actin probe. The expected ~1.5 kb *actin* transcript is larger than the ~1.1 kb *CCOMT* transcript. Therefore, the incomplete removal of the CCOMT probe had no impact on actin probe hybridisation, and no impact on the conclusions made from this analysis. Please also refer to [redacted]2011(MSL0023329).

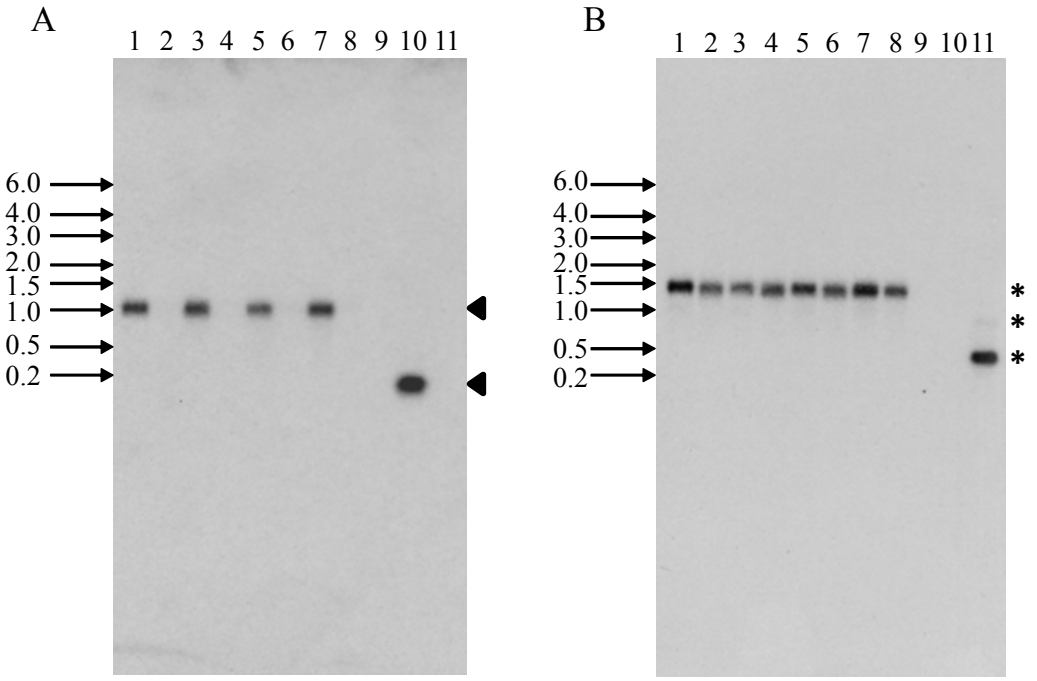


Figure 19. Northern Blot Analysis of *CCOMT* RNA Level in KK179 Forage Tissue

Panel A and Panel B are the same northern blot, which contains polyA$^+$ RNA isolated from forage tissue of the conventional control and KK179. Panel A was hybridised with the CCOMT probe. Panel B was hybridised with the actin probe after stripping the CCOMT probe from the blot. Arrow heads indicate the *CCOMT* hybridisation signal and stars indicate the *actin* hybridisation signal. Lane designations are as follows:

Lane

1 Conventional control (Replicate 1)
2 KK179 (Replicate 1)
3 Conventional control (Replicate 2)
4 KK179 (Replicate 2)
5 Conventional control (Replicate 3)
6 KK179 (Replicate 3)
7 Conventional control (Replicate 4)
8 KK179 (Replicate 4)
9 Empty
10 CCOMT probe template (5 pg)
11 Actin probe template (10 pg)

Arrows denote the size of the RNA, in kilobase pairs, obtained from the RiboRuler™ High Range RNA Ladder on the ethidium stained gel.

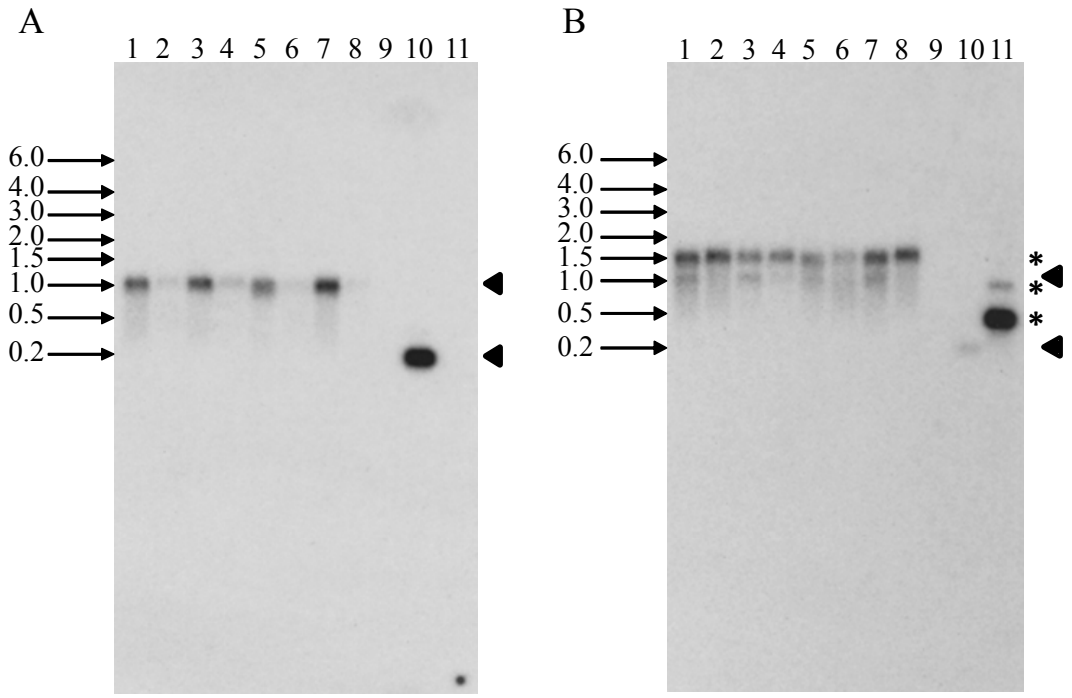


Figure 20. Northern Blot Analysis of *CCOMT* RNA Level in KK179 Root tissue

Panel A and Panel B is the same northern blot containing polyA$^+$ RNA isolated from root tissue of the conventional control and KK179. Panel A was hybridised with the CCOMT probe. Panel B was hybridised with the actin probe after stripping the CCOMT probe from the blot. Arrow heads indicate the *CCOMT* hybridisation signal and stars indicate the *actin* hybridisation signal. Lane designations are as follows:

Lane

1 Conventional Control (Replicate 1)
2 KK179 (Replicate 1)
3 Conventional control (Replicate 2)
4 KK179 (Replicate 2)
5 Conventional control (Replicate 3)
6 KK179 (Replicate 3)
7 Conventional control (Replicate 4)
8 KK179 (Replicate 4)
9 Empty
10 CCOMT probe template (5 pg)
11 Actin probe template (10 pg)

Arrows denote the size of the RNA, in kilobase pairs, obtained from the RiboRuler™ High Range RNA Ladder on the ethidium stained gel.

B3(c) Site of expression of all novel substances and levels

Please refer to Section B3(b).

B3(d) Post-translational modifications to the novel protein(s)

Not applicable.

B3(e) Evidence of silencing, if silencing is the method of modification

Intended Changes to Lignin Levels in KK179 Forage

As described above Section A.1, KK179 reduces G lignin levels through the suppression of CCOMT, a key enzyme in the lignin biosynthetic pathway. Suppression of CCOMT acts to decrease the amount of G lignin, resulting in a lower proportion of G lignin and a greater proportion of the other major lignin subunit, S lignin. The change in lignin subunit proportions can be identified as a change in the ratio of S lignin levels to G lignin levels, or S:G ratio, which is characteristic of CCOMT suppression in alfalfa (Chen et al., 2006). The reduction in G lignin in turn leads to reduced total lignin levels in forage compared to conventional lucerne at the same stage of growth.

To demonstrate that the suppression of CCOMT in KK179 results in the intended reduction of the G lignin subunit, lignin subunit compositional analyses were conducted. Forage samples were collected from KK179, a conventional lucerne control and conventional commercial lucerne varieties grown in the United States from the first cutting of a 2011 field production. The conventional control (C0-Syn1), used as a comparator, was a near-isogenic conventional lucerne population with a genetic background similar to that of KK179. Fourteen different conventional commercial lucerne varieties were included across the field production to provide data on the natural variability of each compositional component analysed. Field production was conducted in typical lucerne-growing regions at six sites located in California (CAPR); Iowa (IARL); Illinois (ILCY); Kansas (KSLA); Texas (TXCL); and Wisconsin (WIDL). The field plots were established from plants started in a greenhouse. Prior to transplanting to the field, the presence or absence of KK179 was verified using PCR. KK179, conventional control, and conventional commercial varieties were planted in a randomised complete block design with four replicated plots per site and grown under normal agronomic field conditions for their respective geographic regions. At the plant growth stage between 1 and 10% bloom, which is a normal stage at which forage is harvested, samples of the whole lucerne plant, 2-3 inches above the soil surface, were harvested at each site from the plants in the center of each individual plot.

The compositional analysis compared levels of the lignin subunits *p*-hydroxyphenyl lignin (H lignin), guaiacyl lignin (G lignin), syringyl lignin (S lignin), caffeyl lignin (derived from caffeyl aldehyde,Figure 2) and 5-hydroxyguaiacyl lignin (derived from 5-hydroxy coniferyl aldehyde, Figure 2). This analysis was performed by researchers at the Samuel Roberts Noble Foundation (Ardmore, OK). The method used to measure the lignin subunitsgenerated values expressed as µmol/g cell wall residue (CWR). Two lignin subunits, caffeyl lignin and 5-hydroxyguaiacyl lignin, had more than 50% of the observations below the assay limit of quantitation (LOQ) and, as a result, were excluded from the

statistical analyses. The S:G lignin ratio was calculated from the values of the individual components expressed as µmol/g CWR. The H, G, and S lignin values were expressed as proportions of each individual lignin subunit calculated as a percentage of the total H, G, and S lignin (total HGS lignin).

To confirm that the reduction in G lignin leads to reduced total lignin in forage, levels of total lignin (as measured by ADL) were determined. From the first cutting in 2011 of stands at six sites, forage samples of KK179 and the conventional control were analysed by the Forage Lab at Dairy One Cooperative, Inc., (hereafter referred to as Dairy One Forage Lab), a facility certified for analytical assessments of forage quality by the National Forage Testing Association (NFTA) , as described in MSL0024403. Dairy One ForNFTAage Lab used a semi-automated ANKOM[6]-based methodology (Weston et al., 2006), as adopted by most commercial forage testing laboratories.

Intended Changes to Lignin Subunits H, G, and S in KK179 Forage

Assessment of KK179 lignin subunit composition compared to the conventional control showed a statistically significant ($p<0.05$) decrease in G lignin when expressed as µmol/g CWR (Table 6). The mean value of G lignin for KK179 was 68.10 µmol/g CWR, a decrease of 15.62 µmol/g CWR or 18.66% compared to the conventional control. The proportion of G lignin for KK179, expressed as a percentage of total HGS lignin in KK179, was 53.69%, a relative decrease of 12.96% compared to the conventional control (Table 7). As a result of this decrease, the S:G ratio increased, as predicted, from 0.58 in the conventional control to 0.80 in KK179 (Table 6) (Chen et al., 2006). These results support the conclusion that suppression of CCOMT in KK179 decreases the production of G lignin resulting in a lower proportion of G lignin in total HGS and an increase in the S:G ratio compared to the conventional control.

Intended Changes to Total Lignin Levels in KK179 Forage

The NFTA-certified Dairy One Forage Lab utilises standards and methods of analysis representative of those commonly adopted by the forage industry to measure forage quality related parameters including total lignin (ADL). It is on the basis of these methods that quality of forage produced by growers and purchased by users is routinely determined; thus the commercial value of the forage as feed is determined. The ANKOM method, which has been adopted by most commercial forage testing laboratories, measures acid detergent lignin based on procedures developed by Goering and Van Soest (Goering and Van Soest, 1970). These procedures involve a series of washes that expose the sample first to an acid detergent solution, then to acetone, followed by sulfuric acid, in order to determine gravimetrically the amount of insoluble residue remaining, which is a measure of total lignin (ADL) in the sample. Compositional analysis of KK179 forage samples at the Dairy One Forage Lab

[6] Refers to ANKOM Technology Corporation, Fairport, NY, a manufacturer of analytical instrumentation and support products for use in laboratory analysis of food and feed products.

confirmed the reduction in total lignin (ADL) levels. The mean value of total lignin (ADL) for KK179 was 5.39% dw, a decrease of 22.15% ($p<0.05$) from the mean value of 6.93% dw for the conventional control (Table 8). This result confirms a measurable reduction in total lignin (ADL) in KK179 relative to conventional alfalfa harvested at the same stage of growth can be observed.

Please also refer to [redacted] 2012 (MSL0024403) and [redacted] 2012 (MSL0024120).

Table 6. Lucerne Forage Lignin Subunit Levels and S:G Ratio for KK179 vs. Conventional Control

				Difference (Test minus Control)			
Analytical (Units)[1]	**Component**	**Test[2] Mean (S.E.)[3] (Range)**	**Control[4] Mean (S.E.) (Range)**	**Mean (S.E.) (Range)**	**95% Confidence Interval**	**Significance (p-Value)**	**Commercial Tolerance Interval[5] (Range)**
Lignin Subunits (µmol/g CWR)							
Guaiacyl (G) lignin		68.10 (9.48) (21.17 - 134.96)	83.72 (9.40) (33.11 - 131.40)	-15.62 (6.12) (-39.11 - 27.03)	-29.16, -2.07	0.027	8.83, 176.39 (25.34 - 153.11)
Syringyl (S) lignin		55.96 (8.83) (9.82 - 87.67)	50.41 (8.78) (12.20 - 91.89)	5.55 (5.11) (-18.80 - 43.57)	-5.82, 16.92	0.302	0, 120.96 (5.64 - 110.93)
p-Hydroxyphenyl (H) lignin		5.05 (0.45) (2.20 - 10.84)	3.88 (0.43) (0.58 - 5.49)	1.17 (0.60) (-1.76 - 7.24)	-0.16, 2.50	0.077	1.59, 6.91 (0.29 - 8.26)
Syringyl to Guaiacyl Subunit Comparison							
S:G Ratio		0.80 (0.061) (0.43 - 1.16)	0.58 (0.060) (0.35 - 0.70)	0.22 (0.027) (-0.16 - 0.50)	0.16, 0.29	<0.001	0.21, 0.96 (0.22 - 0.92)

[1] CWR = Cell Wall Residue; S:G Ratio = Syringyl lignin subunit divided by Guaiacyl lignin subunit.
[2] Test refers to KK179.
[3] Mean (S.E.) = least-square mean (standard error).
[4] Control refers to the conventional lucerne control, C_0-Syn 1.
[5] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lucerne varieties. Negative limits set to zero.

Table 7. Lucerne Forage Lignin Subunit Levels as Percent of Total HGS for KK179 vs. Conventional Control

				Difference (Test minus Control)			
Analytical (Units)[1]	**Component**	**Test[2] Mean (S.E.)[3] (Range)**	**Control[4] Mean (S.E.) (Range)**	**Mean (S.E.) (Range)**	**95% Confidence Interval**	**Significance (p-Value)**	**Commercial Tolerance Interval[5] (Range)**
Lignin Subunits (% Total HGS)							
Guaiacyl (G) lignin		53.69 (1.87) (44.92 - 63.78)	61.69 (1.87) (56.88 - 70.56)	-8.00 (0.71) (-14.63 - 4.22)	-9.42, -6.58	<0.001	46.69, 76.44 (50.02 - 76.69)
Syringyl (S) lignin		42.09 (2.35) (26.98 - 52.01)	35.24 (2.35) (24.60 - 40.26)	6.85 (0.75) (-6.77 - 13.61)	5.34, 8.36	<0.001	17.39, 53.32 (17.07 - 46.14)
p-Hydroxyphenyl lignin	(H)	4.22 (0.54) (2.04 - 9.78)	3.07 (0.54) (0.34 - 5.18)	1.15 (0.28) (-0.85 - 4.60)	0.53, 1.76	0.001	0, 6.74 (0.18 - 6.23)

[1]Total HGS is the sum of *p*-Hydroxyphenyl (H), Guaiacyl (G) and Syringyl (S) lignin subunits (µmol/g CWR); CWR = Cell Wall Residue
[2]Test refers to KK179.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the conventional lucerne control, C_0-Syn 1.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lucerne varieties. Negative limits set to zero.

Table 8. Lucerne Forage Total Lignin (ADL) Levels for KK179 vs. Conventional Control

				Difference (Test minus Control)			
Analytical (Units)[1]	**Component**	**Test[2] Mean (S.E.)[3] (Range)**	**Control[4] Mean (S.E.) (Range)**	**Mean (S.E.) (Range)**	**Relative Difference**	**% Significance (p-Value)**	**Commercial Tolerance Interval[5] (Range)**
Lignin[6] (% dw)		5.39 (0.64) (2.73 - 7.60)	6.93 (0.64) (2.23 - 10.10)	-1.53 (0.42) (-4.33 - 1.30)	-22.15	0.004	1.39, 12.54 (1.70 - 10.03)

[1]dw = dry weight
[2]Test refers to KK179.
[3]Mean (S.E.) = least-square mean (standard error)
[4]Control refers to the conventional lucerne control (C_0-Syn 1).

[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lucerne varieties. Negative limits set to zero.

[6]Determined using the semi-automated ANKOM method (Weston et al., 2006).

B3(f) History of human consumption of novel substances or similarity to substances previously consumed in food

RNA-based suppression of the *CCOMT* gene leading to the intended reduction of G lignin and total lignin in KK179 is mediated by dsRNA molecules. These dsRNA molecules, which are produced from assembled gene transcripts in KK179 that are composed of an inverted repeat sequence, suppress endogenous *CCOMT* gene via the naturally operating endogenous RNAi pathway. Double-stranded RNAs, which are commonly found in plants and other eukaryotes for endogenous gene suppression, are composed of nucleic acids (Siomi and Siomi, 2009). Nucleic acids have a long history of safe consumption because there is no evidence of mammalian toxicity or allergenicity to RNA or DNA (Burnside et al., 2008; Heisel et al., 2008; Ivashuta et al., 2009; Jonas et al., 2001; Parrott et al., 2010; Reddy et al., 2009; U.S. FDA, 1992; Zhou et al., 2009). Several biotechnology-derived plant products previously reviewed and approved by several international regulatory authorities, were developed using RNA-based suppression mechanisms, including virus-resistant papaya, high oleic soybean, virus resistant squash, delayed ripening tomatoes, and plum pox virus-resistant plum trees . (ANZFA, 2000; CFIA, 2001; 2009; EFSA, 2012; HC, 1999b; 1999a; 2000; 2002; MOE, 2007; U.S. FDA, 1994; 1995a; 1995b; 1997; 2008; 2009b; 2009a; 2011a; USDA-APHIS, 2012). Based on the ubiquitous nature of the RNA-based suppression mechanism utilising dsRNA, the history of safe consumption of RNA with no documented evidence for toxicity or allergenicity of dietary RNA, and the lack of evidence of any expressed protein from the DNA inserted into KK179, the use of RNA-based suppression of endogenous *CCOMT* gene expression in KK179 poses no risks as a result of exposure to expressed products of the DNA insert.

B4 Assessment of Potential Toxicity of Novel Proteins

As described in Section A, the KK179 insert contains a *CCOMT* suppression cassette. The *CCOMT* suppression cassette, when transcribed, forms double stranded RNA (dsRNA), which is extremely unlikely to encode for a protein; traditional toxicological assessments of the safety of an insert-derived protein have not been conducted, as KK179 does not express a KK179 insert-derived protein. Information pertaining to the mode-of-action for RNA-based suppression of the expression of an endogenous plant gene can be found in Secton A1. Section B3(f) describes the history of safe use of products developed using RNA-based gene suppression. Section A3.d.(v) summarises a bioinformatics assessment of the potential for allergenicity, toxicity and adverse biological activity of putative polypeptides encoded by the insert and flanking sequences. The results demonstrate the lack of relevant similarities between known allergens, toxins or other biologically-active proteins for all putative peptides derived from all six reading frames from the entire inserted DNA sequence of KK179, including the sequences in the suppression cassette.

B5 Assessment of Potential Allergenicity of Novel Proteins

See Section B4.

B5(a) Source of introduced protein

Not applicable

B5(b) Bioinformatic comparison (aa) of novel protein(s) to allergens

Not applicable

In Section B3(e)., KK179 northern blot data confirmed the expected suppression of endogenous *CCOMT* RNA in alfalfa forage and root tissue. It is extremely unlikely a protein could be produced from the suppression cassette and, even if produced, bioinformatics analyses demonstrate the lack of relevant similarities between known allergens or other biologically-active proteins for all putative peptides derived from all six reading frames from the entire inserted DNA sequence of KK179, including the sequences in the suppression cassette. Therefore, based on the ubiquitous nature of the RNA-based suppression mechanism utilizing dsRNA, demonstration of mode-of-action through *CCOMT* RNA suppression, the history of safe consumption of RNA and the apparent lack of allergenicity of dietary RNA, the RNA-based suppression technology used in KK179 poses no novel risks as a result of expressed products. Please refer to Section A.3.d(v) for further details.

B5(c) Structural properties, including digestion by pepsin, heat treatment

Not applicable

B5(d) Specific serum screening if protein from allergenic source

Not applicable.

B5(e) Protein as a Proportion of Total Protein

Not applicable

B6 Toxicity of Novel Herbicide Metabolites in GM Herbicide-Tolerant Plants

Not applicable

B7 Compositional Assessment

Safety assessments of biotechnology-derived crops follow the comparative safety assessment process (Codex Alimentarius, 2009) in which the composition of forage or other raw agricultural commodities of the biotechnology-derived crop is compared to the appropriate conventional control that has a history of safe use. Compositional assessments of KK179 forage were performed using the principles and analytes outlined in the OECD consensus document for alfalfa (lucerne) composition (OECD, 2005).

A recent review of compositional assessments conducted according to OECD guidelines that encompassed a total of seven biotechnology-derived crop varieties, nine countries and eleven growing seasons concluded that incorporation of biotechnology-derived agronomic traits has had little impact on natural variation in crop composition. Most compositional variation is attributable to growing region, agronomic practices, and genetic background (Harrigan et al., 2010). Compositional quality, therefore, implies a very broad range of endogenous levels of individual constituents. Numerous scientific publications have further documented the extensive variability in the concentrations of crop nutrients, anti-nutrients and secondary metabolites that reflect the influence of environmental and genetic factors as well as extensive conventional breeding efforts to improve nutrition, agronomics, and yield (Harrigan et al., 2010; OECD, 2005).

Compositional equivalence between biotechnology-derived and conventional crops supports an "equal or increased assurance of the safety of foods derived from genetically modified plants" (OECD, 2002). OECD consensus documents on compositional considerations for new crop varieties emphasise quantitative measurements of essential nutrients, known anti-nutrients, and known secondary metabolites. This is based on the premise that such comprehensive and detailed analyses will most effectively discern any compositional changes that imply potential nutritional or safety (*e.g.*, anti-nutritional) concerns. Levels of the components in the biotechnology-derived crop are compared to: 1) corresponding levels in a conventional comparator, which is a genetically similar conventional line, grown concurrently under similar field conditions; and 2) natural ranges generated from an evaluation of commercial reference varieties grown concurrently and/or from data published in the scientific literature. The comparison to data published in the literature places any observed differences between the assessed crop and its comparator in the context of the well-documented variation in the concentrations of crop nutrients, anti-nutrients and secondary metabolites.

Section B7(a) provides analyses of key nutrients, anti-nutrients, and secondary metabolites in KK179 compared to the conventional control grown and harvested under the same conditions. In addition, conventional commercial lucerne reference varieties were included in the composition analyses to establish a range of natural variability for each analyte, defined by a 99% tolerance interval. The production of materials for the compositional analyses used field designs to allow accurate assessments of compositional characteristics over a range of environmental conditions under which KK179 is expected to be grown. Design parameters for all productions included multiple replicated field sites to allow

adequate exposure to the variety of conditions met in agriculture. Methods of analysis were sufficiently sensitive and specific to detect variations in the key components. The information provided in this section addresses the relevant factors in Codex Plant Guidelines, Section 4, paragraphs 44 and 45, for compositional analyses (Codex Alimentarius, 2009).

Compositional Equivalence of KK179 Forage to Conventional Lucerne

Forage samples were collected from the first cutting of KK179, a conventional lucerne control, and conventional commercial lucerne varieties grown at six sites in a 2011 field production. The conventional control (C_0-Syn1) used as a comparator was a near-isogenic conventional lucerne population with a genetic background similar to that of KK179. Fourteen different conventional commercial lucerne varieties were included across the field production to provide data on the natural variability of each compositional component analysed. The field production was conducted in typical lucerne-growing regions at six sites: California (CAPR); Iowa (IARL); Illinois (ILCY); Kansas (KSLA); Texas (TXCL); and Wisconsin (WIDL). The field plots were established from plants started in a greenhouse. Prior to transplanting to the field, the presence or absence of KK179 was verified using PCR. KK179, the conventional control and the conventional commercial varieties were planted in a randomised complete block design with four replicated plots per site and grown under normal agronomic field conditions for their respective geographic regions. At the plant growth stage between 1 and 10% bloom, which is a normal stage for harvesting forage, samples of the whole lucerne plant were harvested at each site from the plants in the center of each individual plot by cutting the plant 2-3 inches above the soil surface.

Compositional analyses were based on OECD consensus document for lucerne (OECD, 2005) to compare levels of key nutrients, anti-nutrients, and secondary metabolites in KK179 to levels in the conventional control. Forage samples were analysed for the following 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). Anti-nutrient and secondary metabolites included daidzein, glycitein, genistein, coumesterol, formononetin, biochanin A, saponins (total bayogenin, total hederagenin, total medicagenic acid, total soyasapogenol B, total soyasapogenol E, total zanhic acid, and total saponins), and canavanine. In addition to the OECD-recommended analytes listed above, *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 biosynthetic pathway and cell wall-associated metabolites.

Methods used in the assessments of nutrients, anti-nutrients, and secondary metabolites are described in [redacted] 2012 (MSL0023847). Prior to compositional analysis, levels of total lignin (ADL) in forage samples from the 2011 field production were measured by Dairy One Forage Lab as described in Section B3(c).

In all, 54 different components of nutrients, anti-nutrients, and secondary metabolites were measured. Of those 54 components, six anti-nutrients (daidzein, glycitein, genistein,

coumesterol, formononetin, and biochanin A) and one secondary metabolite (sinapic acid) had more than 50% of the observations below the assay limit of quantitation (LOQ) and, as a result, were excluded from statistical analyses. Therefore, 47 components were statistically assessed using a mixed-model analysis of variance method. Values for all components were expressed on a dry weight (dw) basis with the exception of moisture, expressed as percent fresh weight (fw).

The statistical comparison was based on compositional data combined across all field sites. Statistically significant differences were identified at the 5% level. Compositional data from the conventional commercial varieties were combined to calculate a 99% tolerance interval for each compositional component to estimate the natural variability of each component in lucerne.

Statistical significance does not imply biological relevance from a feed/food safety or nutritional perspective (EFSA, 2011). Considerations used to assess the relevance of each combined-site statistically significant difference included: 1) the relative magnitude of the difference in the mean values of nutrient, anti-nutrient, and secondary metabolite components of KK179 and the conventional control; 2) whether the KK179 component mean value is within the range of natural variability of that component as represented by the 99% tolerance interval of the commercial reference varieties grown concurrently in the same trial; and 3) an assessment of the differences within the context of natural variability of available commercial lucerne composition published in the scientific literature.

The compositional analysis provided a comprehensive comparative assessment of the levels of key nutrients, anti-nutrients and secondary metabolites in forage of KK179 and the conventional control. Assessment of the results demonstrated that, with the exception of three compositional constituents (ash, canavanine, and ferulic acid), there were no statisitically significant differences in 44 of the 47 constituents statistically compared. For the three constituents where significant differences were detected, an analysis, including the magnitudes of the differences and comparisons of mean values to the 99% tolerance interval and literature values, indicated they were not biologically meaningful from a feed/food safety or nutritional perspective. Further assessment of statistically significant differences observed between KK179 and the conventional control is provided in the following section. These results support the overall conclusion that, with the exception of the intended change in reduced G lignin and total lignin levels compared to conventional lucerne at the same growth stage presented in Section B3(c), forage of KK179 is compositionally equivalent to conventional lucerne.

Please also refer to [redacted] 2012 (MSL0023847) and [redacted] 2012 (MSL0023980).

B7(a) Levels of key nutrients, toxicants and anti-nutrients

The means and ranges for nutrients, anti-nutrients and secondary metabolites from KK179 were consistent with values established from the conventional lucerne control (Tables 10, 11, and 12). No significant differences ($p>0.05$) were identified for protein (and total amino acids), minerals, fat, moisture, ADF, NDF, ADL, carbohydrates by calculation, saponins, total polyphenols, free phenylalanine and p-coumaric acid, although statistically significant differences for ash, canavanine, and ferulic acid were observed. A summary of differences observed in the combined-site analysis can be found in Table 9.

The mean level of ash was significantly lower ($p<0.05$) in KK179 forage than the conventional control in the combined-site analysis (Table 9). The absolute difference in magnitude was 0.41% dw, which is a relative difference of -3.8%. Furthermore, the mean level of ash was within the 99% tolerance interval of the conventional commercial references varieties and within the range of values found in the published literature (Table 13). Therefore, the difference in ash in KK179 forage compared to the conventional control is not considered biologically meaningful from a feed/food safety and nutritional perspective.

The mean level of canavanine was significantly lower ($p<0.05$) in KK179 forage than the conventional control in the combined-site analysis (Table 9). The absolute difference in magnitude was 16.94 ppm, which is a relative difference of -29.6%. Lower levels in animal feed are desirable, therefore, a decrease would not be considered adverse. Also, the mean level of canavanine was within the 99% tolerance interval of the conventional commercial references varieties and within the range of values found in the published literature (Table 13). Therefore, the difference in canavanine in KK179 forage compared to the conventional control is not considered biologically meaningful from a feed/food safety and nutritional perspective.

The mean level of ferulic acid was significantly higher ($p<0.05$) in KK179 forage than the conventional control in the combined-site analysis (Table 9). The absolute difference in magnitude was 110.60 ppm, which is a relative difference of 7.4%. Ferulic acid is an important component of the overall cell wall structure, and may serve as an ‘anchor site’ for lignin deposition (Grabber et al., 2000); therefore, it is not unexpected that alterations in lignin content could result in alterations in ferulic acid levels. Furthermore, the mean level of ferulic acid was within the 99% tolerance interval of the conventional commercial references varieties and within the range of values found in the published literature (Table 13). Therefore, the difference in ferulic acid in KK179 forage compared to the conventional control is not considered biologically meaningful from a feed/food safety and nutritional perspective.

Although the mean level of total lignin (ADL) in KK179 was not significantly lower as reported in Section B3(c), it was numerically lower in KK179 forage compared to the conventional control in the combined-site analysis (Table 10). The absolute difference in magnitude was 0.32% dw, which is a relative difference of -4.89%. The use of different methods, a semi-automated ANKOM-based assay by Dairy One Forage Lab and a manual assay by Covance Lab, likely contributed to the variability in total lignin (ADL) values

determined by the two laboratories. Both methods confirmed a decrease in total lignin in KK179, with Dairy One Forage Lab reporting a significant decrease of 22.15% and Covance a numerical decrease of 4.89%. Despite this variability, it is concluded that Covance Lab's total lignin data are consistent with the observation from Dairy One Forage Lab that total lignin levels in KK179 are reduced relative to the conventional control.

Assessment of these compositional results support the overall conclusion that, with the exception of the intended reduction in G lignin and total lignin as presented in Section B3(c), forage from KK179 is compositionally equivalent to conventional lucerne with regard to the levels of nutrients, anti-nutrients, and secondary metabolites. The statistical differences observed were limited in number. In the case of ash and ferulic acid the relative magnitudes were under 10%. The mean levels of all three analytes with observed statistical differences were within the 99% tolerance interval established from the population of conventional commercial reference varieties grown concurrently with KK179 in the field production, and were within the range of values found in the published literature (Table 13). Therefore, these differences are not considered biologically meaningful from a feed/food safety and nutritional perspective.

Table 9. Summary of Differences Observed in the Combined-Site Analysis (p<0.05) of Lucerne Forage Component Levels for KK179 vs. Conventional Control

Analytical Component (Units)[1]	Test[2] Mean[3]	Control[4] Mean	Mean Difference (Test minus Control)		Test Range	Commercial Tolerance Interval[5]
			Mean Difference (% of Control)	Significance (p-Value)		
Statistical Differences Observed in Combined-Site Analysis						
Forage Proximate (% dw)						
Ash	10.38	10.79	-3.77	0.034	8.43 - 13.26	6.70, 13.54
Forage Metabolite						
Canavanine (ppm dw)	40.30	57.24	-29.60	0.013	11.42 - 87.83	0, 137.35
Ferulic Acid (ppm dw)	1596.41	1485.81	7.44	0.008	1389.38 - 1884.17	854.88, 2061.10

[1]dw = dry weight.
[2]Test refers to KK179.
[3]Mean = least-square mean
[4]Control refers to the conventional alfalfa control (C_0-Syn 1).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference varieties. Negative limits set to zero.

Table 10. Statistical Summary of Lucerne Forage Nutrients for KK179 vs. Conventional Control

Analytical (Units)[1]	Component	Test[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dw)							
Ash		10.38 (0.53) (8.43 - 13.26)	10.79 (0.52) (8.79 - 12.95)	-0.41 (0.19) (-1.80 - 1.06)	-0.78, -0.030	0.034	6.70, 13.54 (7.54 - 13.23)
Carbohydrates		66.55 (1.71) (57.73 - 73.90)	65.97 (1.70) (59.94 - 72.91)	0.58 (0.49) (-3.45 - 8.36)	-0.55, 1.71	0.272	50.57, 81.80 (54.35 - 74.91)
Moisture (% fw)		78.26 (1.54) (73.70 - 84.60)	78.15 (1.54) (70.50 - 83.70)	0.11 (0.33) (-2.70 - 3.60)	-0.64, 0.86	0.748	65.06, 90.61 (66.10 - 85.30)
Protein		20.83 (1.36) (15.50 - 29.03)	21.02 (1.35) (15.98 - 27.30)	-0.19 (0.39) (-6.67 - 3.19)	-0.98, 0.60	0.636	9.26, 33.78 (14.52 - 30.07)
Total Fat		2.28 (0.17) (0.84 - 3.98)	2.28 (0.17) (1.08 - 3.38)	0.0039 (0.16) (-1.30 - 1.46)	-0.31, 0.32	0.980	0.73, 3.59 (0.53 - 4.21)
Fibre (% dw)							
Acid Detergent Fibre		27.03 (2.45) (15.71 - 37.26)	27.02 (2.44) (10.96 - 36.11)	0.015 (0.96) (-10.08 - 9.57)	-2.12, 2.15	0.987	6.16, 49.06 (7.07 - 39.11)

Table 10 (continued). Statistical Summary of Lucerne Forage Nutrients for KK179 vs. Conventional Control

Analytical (Units)[1]	Component	Test[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dw)							
Acid Detergent Lignin		6.22 (0.60) (2.72 - 10.31)	6.54 (0.59) (3.58 - 8.26)	-0.32 (0.27) (-2.08 - 3.39)	-0.91, 0.28	0.265	2.13, 11.99 (3.38 - 9.67)
Neutral Detergent Fibre		33.95 (2.64) (18.57 - 48.67)	34.46 (2.63) (18.94 - 43.32)	-0.52 (0.97) (-9.74 - 11.84)	-2.67, 1.63	0.605	12.04, 58.18 (18.97 - 49.82)
Amino Acid (% dw)							
Alanine		1.11 (0.074) (0.84 - 1.52)	1.13 (0.074) (0.87 - 1.39)	-0.017 (0.020) (-0.19 - 0.13)	-0.062, 0.028	0.417	0.49, 1.79 (0.80 - 1.66)
Arginine		0.99 (0.065) (0.73 - 1.35)	1.01 (0.065) (0.75 - 1.28)	-0.020 (0.020) (-0.17 - 0.11)	-0.065, 0.024	0.326	0.44, 1.59 (0.70 - 1.44)
Aspartic acid		2.77 (0.28) (1.97 - 4.65)	2.74 (0.28) (2.04 - 4.08)	0.027 (0.071) (-0.64 - 0.71)	-0.13, 0.19	0.711	0.44, 5.63 (1.96 - 5.15)
Cystine		0.21 (0.012) (0.15 - 0.30)	0.21 (0.011) (0.15 - 0.29)	0.00079 (0.0074) (-0.041 - 0.062)	-0.016, 0.017	0.916	0.12, 0.32 (0.16 - 0.31)

Table 10 (continued). Statistical Summary of Lucerne Forage Nutrients for KK179 vs. Conventional Control

Analytical (Units)[1]	Component	Test[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)							
Glutamic acid		1.85 (0.12) (1.40 - 2.55)	1.91 (0.12) (1.39 - 2.36)	-0.053 (0.035) (-0.31 - 0.22)	-0.13, 0.024	0.156	0.81, 3.01 (1.31 - 2.80)
Glycine		0.95 (0.055) (0.75 - 1.21)	0.97 (0.055) (0.73 - 1.14)	-0.018 (0.013) (-0.10 - 0.085)	-0.047, 0.011	0.190	0.49, 1.44 (0.70 - 1.33)
Histidine		0.43 (0.020) (0.35 - 0.55)	0.44 (0.020) (0.36 - 0.51)	-0.0064 (0.0058) (-0.053 - 0.059)	-0.018, 0.0053	0.276	0.26, 0.63 (0.34 - 0.61)
Isoleucine		0.86 (0.053) (0.67 - 1.15)	0.88 (0.053) (0.66 - 1.07)	-0.016 (0.014) (-0.12 - 0.12)	-0.048, 0.016	0.284	0.43, 1.36 (0.63 - 1.27)
Leucine		1.43 (0.089) (1.09 - 1.90)	1.47 (0.089) (1.09 - 1.78)	-0.033 (0.023) (-0.22 - 0.16)	-0.086, 0.019	0.187	0.70, 2.25 (1.03 - 2.05)
Lysine		1.14 (0.067) (0.93 - 1.55)	1.17 (0.067) (0.92 - 1.44)	-0.024 (0.017) (-0.15 - 0.12)	-0.058, 0.0094	0.153	0.55, 1.82 (0.82 - 1.73)

Table 10 (continued). Statistical Summary of Lucerne Forage Nutrients for KK179 vs. Conventional Control

Analytical Component (Units)[1]	Test[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Methionine	0.25 (0.024) (0.15 - 0.39)	0.24 (0.024) (0.15 - 0.37)	0.0083 (0.012) (-0.12 - 0.14)	-0.017, 0.033	0.508	0.068, 0.42 (0.14 - 0.45)
Phenylalanine	0.98 (0.061) (0.75 - 1.27)	1.00 (0.061) (0.74 - 1.21)	-0.025 (0.016) (-0.15 - 0.083)	-0.060, 0.0097	0.138	0.48, 1.53 (0.71 - 1.39)
Proline	0.89 (0.054) (0.71 - 1.18)	0.92 (0.053) (0.71 - 1.21)	-0.028 (0.021) (-0.28 - 0.11)	-0.075, 0.018	0.199	0.43, 1.41 (0.65 - 1.24)
Serine	0.87 (0.044) (0.68 - 1.16)	0.88 (0.044) (0.68 - 1.05)	-0.0061 (0.017) (-0.16 - 0.13)	-0.045, 0.033	0.733	0.45, 1.35 (0.66 - 1.25)
Threonine	0.86 (0.050) (0.66 - 1.12)	0.88 (0.050) (0.67 - 1.05)	-0.018 (0.016) (-0.13 - 0.10)	-0.053, 0.018	0.288	0.45, 1.33 (0.63 - 1.23)
Tryptophan	0.37 (0.020) (0.30 - 0.48)	0.37 (0.020) (0.27 - 0.45)	0.0036 (0.0082) (-0.056 - 0.065)	-0.013, 0.020	0.663	0.20, 0.56 (0.25 - 0.50)

Table 10 (continued). Statistical Summary of Lucerne Forage Nutrients for KK179 vs. Conventional Control

Analytical Component (Units)[1]	Test[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Tyrosine	0.71 (0.042) (0.55 - 0.94)	0.71 (0.042) (0.53 - 0.89)	0.0012 (0.015) (-0.098 - 0.10)	-0.033, 0.035	0.939	0.35, 1.09 (0.52 - 1.01)
Valine	1.05 (0.061) (0.79 - 1.38)	1.07 (0.061) (0.81 - 1.32)	-0.017 (0.015) (-0.16 - 0.13)	-0.048, 0.014	0.280	0.52, 1.64 (0.79 - 1.55)
Mineral						
Calcium (% dw)	1.68 (0.16) (1.12 - 2.62)	1.72 (0.16) (1.09 - 2.53)	-0.037 (0.037) (-0.41 - 0.28)	-0.12, 0.045	0.336	0.55, 2.56 (0.95 - 2.07)
Copper (mg/kg dw)	8.86 (0.85) (5.14 - 13.16)	8.34 (0.85) (5.18 - 11.93)	0.52 (0.34) (-1.62 - 4.04)	-0.16, 1.20	0.131	1.87, 14.98 (4.54 - 19.67)
Iron (mg/kg dw)	272.00 (31.45) (123.38 - 473.91)	315.74 (30.93) (163.92 - 547.83)	-43.74 (24.69) (-279.88 - 115.21)	-98.55, 11.07	0.106	41.59, 446.31 (105.45 - 691.43)
Magnesium (% dw)	0.22 (0.023) (0.12 - 0.31)	0.23 (0.023) (0.15 - 0.32)	-0.0082 (0.0050) (-0.048 - 0.037)	-0.018, 0.0019	0.108	0.027, 0.41 (0.11 - 0.34)

Table 10 (continued). Statistical Summary of Lucerne Forage Nutrients for KK179 vs. Conventional Control

Analytical Component (Units)[1]	Test[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Mineral						
Manganese (mg/kg dw)	52.56 (6.30) (30.52 - 106.47)	52.45 (6.27) (30.92 - 77.32)	0.11 (2.65) (-15.31 - 37.75)	-5.86, 6.09	0.966	17.53, 69.85 (23.24 - 98.04)
Phosphorus (% dw)	0.29 (0.019) (0.22 - 0.40)	0.28 (0.019) (0.20 - 0.38)	0.0037 (0.0057) (-0.040 - 0.071)	-0.0079, 0.015	0.523	0.14, 0.46 (0.18 - 0.43)
Potassium (% dw)	2.35 (0.052) (2.16 - 2.65)	2.41 (0.051) (2.18 - 2.71)	-0.055 (0.051) (-0.45 - 0.21)	-0.17, 0.059	0.307	1.82, 3.04 (1.85 - 3.35)
Sodium (% dw)	0.089 (0.024) (0.020 - 0.22)	0.077 (0.024) (0.018 - 0.15)	0.013 (0.0076) (-0.056 - 0.083)	-0.0026, 0.028	0.102	0, 0.24 (0.016 - 0.20)
Zinc (mg/kg dw)	27.83 (2.11) (18.40 - 39.22)	26.81 (2.09) (17.38 - 40.42)	1.02 (1.42) (-5.64 - 11.08)	-2.15, 4.19	0.489	8.89, 47.44 (17.08 - 47.48)

[1] dw = dry weight; fw = fresh weight
[2] Test refers to KK179.
[3] Mean (S.E.) = least-square mean (standard error)
[4] Control refers to the conventional lucerne control (C_0-Syn 1).
[5] With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference varieties. Negative limits set to zero.

Table 11. Statistical Summary of Lucerne Forage Secondary Metabolites for KK179 vs. Conventional Control

			Difference (Test minus Control)			
Analytical Component (Units)[1] Metabolite	**Test[2] Mean (S.E.)[3] (Range)**	**Control[4] Mean (S.E.) (Range)**	**Mean (S.E.) (Range)**	**95% Confidence Interval**	**Significance (p-Value)**	**Commercial Tolerance Interval[5] (Range)**
Canavanine (ppm dw)	40.30 (13.53) (11.42 - 87.83)	57.24 (13.51) (12.69 - 134.50)	-16.94 (5.69) (-79.53 - 5.32)	-29.62, -4.27	0.013	0, 137.35 (11.47 - 151.33)
Ferulic Acid (ppm dw)	1596.41 (59.57) (1389.38 - 1884.17)	1485.81 (58.83) (1103.96 - 2007.38)	110.59 (40.34) (-301.26 - 503.18)	29.36, 191.83	0.008	854.88, 2061.10 (1103.32 - 1906.86)
Free Phenylalanine (ppm dw)	266.99 (28.84) (111.86 - 409.20)	283.70 (28.69) (154.07 - 457.63)	-16.71 (12.62) (-125.58 - 75.77)	-42.11, 8.69	0.192	0, 627.23 (133.05 - 579.05)
Total Polyphenols (mg/g dw)	8.19 (0.34) (6.35 - 10.19)	7.99 (0.34) (6.57 - 10.21)	0.20 (0.23) (-2.13 - 1.72)	-0.30, 0.71	0.390	4.86, 11.15 (6.17 - 11.17)
p-Coumaric Acid (ppm dw)	639.50 (37.62) (458.33 - 870.13)	623.54 (37.34) (442.08 - 819.59)	15.97 (19.93) (-112.64 - 226.29)	-28.25, 60.18	0.441	188.81, 949.95 (326.19 - 945.58)

[1] dw = dry weight.
[2] Test refers to KK179.
[3] Mean (S.E.) = least-square mean (standard error)
[4] Control refers to the conventional lucerne control (C_0-Syn 1).
[5] With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference varieties. Negative limits set to zero.

Table 12. Statistical Summary of Lucerne Forage Anti-Nutrients for KK179 vs. Conventional Control

Analytical Component (Units)[1]	Test[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (Test minus Control)			Commercial Tolerance Interval[5] (Range)
			Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	
Saponins (response units/µg)						
Total Bayogenin	5.10 (0.76) (2.54 - 13.97)	5.67 (0.76) (2.20 - 11.28)	-0.57 (0.47) (-2.85 - 5.81)	-1.53, 0.38	0.230	0.92, 8.86 (1.46 - 11.28)
Total Hederagenin	2.94 (0.35) (1.70 - 5.80)	3.47 (0.35) (1.58 - 6.85)	-0.53 (0.32) (-3.51 - 1.21)	-1.24, 0.19	0.131	0.85, 7.20 (0.90 - 10.31)
Total Medicagenic Acid	21.88 (2.44) (9.09 - 45.08)	23.39 (2.44) (9.43 - 51.04)	-1.51 (2.51) (-22.95 - 15.92)	-6.57, 3.55	0.551	0, 44.42 (2.04 - 48.33)
Total Soyasapogenol B	22.17 (3.02) (9.68 - 40.48)	24.53 (3.02) (7.05 - 41.93)	-2.36 (1.44) (-12.47 - 8.72)	-5.56, 0.84	0.131	7.83, 44.92 (9.22 - 43.87)
Total Soyasapogenol E	2.77 (0.54) (1.20 - 5.02)	3.08 (0.54) (0.84 - 8.89)	-0.31 (0.26) (-4.99 - 1.87)	-0.84, 0.22	0.248	0, 6.59 (0.91 - 7.53)

Table 12 (continued). Statistical Summary of Lucerne Forage Anti-Nutrients for KK179 vs. Conventional Control

Analytical Component (Units)[1]	Test[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Saponins (response units/µg)						
Total Zanhic Acid	4.59 (0.58) (2.25 - 12.08)	5.16 (0.58) (2.62 - 8.69)	-0.57 (0.45) (-3.97 - 3.69)	-1.48, 0.33	0.210	0.32, 12.06 (1.75 - 13.20)
Total Saponins	59.30 (4.94) (36.00 - 122.44)	65.58 (4.94) (29.20 - 96.50)	-6.28 (4.35) (-32.96 - 25.94)	-15.05, 2.49	0.156	21.87, 108.47 (17.38 - 103.19)

[1] Response units equals peak area counts.

[2]Test refers to KK179.

[3]Mean (S.E.) = least-square mean (standard error)

[4]Control refers to the conventional lucerne control, C_0-Syn 1.

[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference varieties. Negative limits set to zero.

Table 13. Literature and OECD Ranges for Compostional Components in Lucerne Forage

Components[1]	Literature Range[2]	OECD Range[3]
Forage Nutrients		
Proximates (% dw)		
Ash	8.62 - 14.81 [a]; 6.86 - 15.25 [b]; 5.8 - 7.5 [c]	8.4 - 15.3
Carbohydrates by calculation	56.63 - 74.80 [b]	NA
Fat, total	1.80 - 3.24 [a]; 1.33 - 4.49 [b] 2.8 - 3.1 [c]	1.3 - 3.2
Moisture (% fw)	7.74 - 18.10 [a]; 70.90 - 83.50 [b]	9.0 - 82.1
Protein	14.91 - 25.35 [a]; 15.29 - 28.34 [b]; 17.0 – 21.3 [c]	15.3 - 25.8
Fiber (% dw)		
Acid detergent fibre	23.17 - 42.59 [a]; 21.26 - 39.25 [b]	23.1 - 33.4
Neutral detergent fibre	29.08 - 53.56 [a]; 26.53 - 51.09 [b]	26.5 - 40.0
Acid Detergent Lignin	5.69 - 9.37 [a]; 2.31 - 13.71 [b]	3.9 - 9.7
Amino Acids (% dw)		
Alanine	0.93 - 1.21 [c]	0.70 - 1.59
Arginine	0.86 - 1.08 [c]	0.62 - 1.54
Aspartic acid	1.97 - 2.15 [c]	1.40 - 3.52
Cystine	NA	0.18 - 0.35
Glutamic acid	1.88 - 2.40 [c]	1.20 - 3.03
Glycine	0.82 - 1.1 [c]	0.60 - 1.47
Histidine	0.48 - 0.60 [c]	0.28 - 0.74
Isoleucine	0.77 - 0.95 [c]	0.50 - 1.26
Leucine	1.35 - 1.62 [c]	0.90 - 2.25
Lysine	1.06 - 1.16 [c]	0.59 - 1.81
Methionine	0.28 - 0.37 [c]	0.18 - 0.48
Phenylalanine	0.87 - 1.08 [c]	0.72 - 1.59
Proline	0.65 - 1.26 [c]	0.70 - 1.34
Serine	0.76 - 0.95 [c]	0.60 - 1.36
Threonine	0.78 - 1.11 [c]	0.60 - 1.15
Tryptophan	NA	0.16 - 0.35
Tyrosine	0.66 - 0.83 [c]	0.50 - 1.16
Valine	0.91 - 1.18 [c]	0.60 - 1.55
Minerals		
Calcium (% dw)	1.03 - 1.93 [a]; 0.90 - 1.86 [b]	0.90 - 1.96
Copper (mg/kg dw)	3.43 - 14.72 [b]	5.3 - 13.4
Iron (ppm dw)	1 - 4749 [a]; 63.49 - 1538.46 [b]	0.2 - 15.4
Magnesium (% dw)	0.20 - 0.40 [a]; 0.11 - 0.45 [b]	0.11 - 0.45
Manganese (ppm dw)	16 - 64 [a]; 15.91 - 109.50 [b]	31.5 - 109.5
Phosphorus (% dw)	0.24 - 0.42 [a]; 0.22 - 0.46 [b]	0.22 - 0.45
Potassium (% dw)	1.59 – 3.21 [a] ; 1.39 – 4.31 [b]	1.39 – 4.31
Sodium (ppm dw)	1 - 3826 [a]; 170 - 5100 [b]	0.2 - 2.1
Zinc (mg/kg dw)	15.20 - 43.62 [b]	18.0 - 36.0

Table 13 (continued). Literature and OECD Ranges for Components in Lucerne Forage

Components[1]	Literature Range[2]	OECD Range[3]
Forage Metabolite		
Ferulic acid (ppm, dw)	627 [d] ; 680 [e]; 770 - 2840 [f]	NA
p-Coumaric acid (ppm, dw)	398 [d] ; 254 [e]; 630 - 1860 [f]	NA
Canavanine (%) (seedling)	1.3 - 2.4 [g]	NA
Free Phenylalanine	NA	NA
Total polyphenols	NA	NA
Forage Anti-nutrients		
Saponins (mg/g dw)		
Bayogenin	NA	NA
Hederagenin	0.03[h]	NA
Medicagenic Acid	0.55 – 1.25[h] ; 0.13 – 1.77[i]	0.013 – 0.165
Soyasapogenol B	0.01[h]	NA
Soyasapogenol E	0.03 – 0.13[h]	NA
Zanhic Acid	0.04 – 0.79[i]	NA
Total Saponins	16.4 – 23.7[h]	4.9 – 17.7

[1] fw=fresh weight; dw=dry weight

[2] Literature range references: [a] (Dairyland Laboratories, 2011); [b] (McCann et al., 2006); [c] (Smith, 1969); [d] (Bourquin et al., 1990); [e] (Cherney et al., 1989); [f] (Jung and Fahey, 1983) [g](Rosenthal and Nkomo, 2000) ; [h] (Tava et al., 1993); [i] (Pecetti et al., 2006)

[3] (OECD, 2005)

[4] NA=not available

Analyses of nutrient, anti-nutrient, and secondary metabolite levels in KK179 and the conventional control were conducted to assess compositional equivalence. The analytes evaluated are consistent with those identified by OECD as important to understanding the safety and nutrition of biotechnology-derived lucerne (OECD, 2005). The compositional comparisons were made by analysing forage harvested from the first cutting during the 2011 field season from six field sites in the U.S. that are representative of normal agricultural regions for lucerne production. The compositional analysis, based on OECD consensus document for lucerne, also included measurement of nutrients, anti-nutrients and secondary metabolites in conventional commercial reference varieties to provide data on the natural variability of each compositional component analysed.

Compositional analyses based on the OECD consensus document confirmed that, other than the intended reduction in G lignin and total lignin presented in Section A1 and Section B3(e), there is no meaningful effect on key nutrient, anti-nutrient, or secondary metabolite components in KK179 compared to a conventional lucerne control. Of the 47 components statistically assessed, only three (ash, canavanine, and ferulic acid) showed a significant difference in combined-site analysis between KK179 and the conventional control. Two of the three observed differences (ash and ferulic acid) are less than 10% in relative magnitude. The mean values for all three components with a statistically significant difference between KK179 and the conventional control fall within the 99% tolerance interval determined from the conventional commercial lucerne varieties grown concurrently with KK179 and the control. In addition, the levels of these three components are also within the ranges published in the scientific literature.

These analyses provide a comprehensive comparative assessment of the levels of key nutrients, anti-nutrients and secondary metabolites in forage of KK179 compared to the conventional control. These results support the overall conclusion that, with the exception of the intended reduction in G lignin and total lignin as presented in Section A1 and Section B3(e), forage of KK179 is compositionally equivalent to that of conventional lucerne and, therefore, the feed/food safety and nutritional quality of this product is comparable to conventional lucerne.

As described in this section, detailed compositional analyses of key components of KK179 have demonstrated that KK179 is compositionally equivalent except for the intended reduction in G lignin and total lignin compared to conventional lucerne at the same stage of growth. Therefore, when KK179 and its progeny are used on a commercial scale as a source of feed, these products are not expected to be different from equivalent feeds originating from conventional lucerne.

B7(b) Levels of other GM-influenced constituents

Not applicable.

B7(c) Levels of naturally-occurring allergenic proteins

Not applicable.

C Nutritional Impact

C1 Data on Nutritional Impact of Compositional Changes

Assessment of the compositional analyses in accordance with OECD guidelines confirmed that, with the exception of the intended reduction in G lignin and total lignin as presented in Section B3(c)., the levels of assessed components in KK179 forage were compositionally equivalent to those of conventional lucerne forage with three exceptions. No significant differences ($p>0.05$) were observed between KK179 and the conventional control for protein (and total amino acids), minerals, fat, moisture, ADF, NDF, ADL, carbohydrates by calculation, saponins, total polyphenols, free phenylalanine, and *p*-coumaric acid; whereas statistically significant differences were observed for ash, canavanine, and ferulic acid. For two of the three components, the magnitude of difference of the component levels in KK179 were less than 10% and all three components were within the 99% tolerance interval established from the variability of conventional commercial reference varieties grown concurrently and within the ranges published in the scientific literature. Thus, observed differences in components were considered not meaningful from a feed/food safety or nutritional perspective. Thus the assessments support the overall conclusion that KK179 is as safe and wholesome as conventional lucerne.

Please also refer to B7 for further details.

C2 Data from an Animal Feeding Study, if Available

The data and information presented in this submission demonstrate that the food and feed derived from KK179 are as safe and nutritious as those derived from commercially-available, conventional alfalfa for which there is an established history of safe consumption.

PART 3 STATUTORY DECLARATION – AUSTRALIA

I, [redacted] Monsanto Australia Limited, make the following declaration under the *Statutory Declarations Act 1959:*

1. The information provided in this application fully sets out the matters required
2. The information provided in this application is true to the best of my knowledge and belief
3. No information has been withheld that might prejudice this application, to the best of my knowledge and belief

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under Section 11 of the *Statutory Declarations Act 1959,* and I believe that the statements in this declaration are true in every particular.

Signature: [redacted]

Declared at Monsanto Australia, Level 12 / 600 St Kilda Road, Melbourne VIC 3004 on.....5.....of April 2013.

Declared before me: ... [redacted] .....

.....

.....

.....

[Full name, qualification and address of person before whom the declaration is made]

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