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

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9 April 2013

EXECUTIVE SUMMARY

KK179 Product Description

Lucerne is grown to produce forage for direct use on farm or for sale of hay as animal feed. Growers consider both forage yield and forage quality as critical factors in determining the value of the crop. As the crop grows and forage biomass increases, the quality of conventional lucerne begins to decrease rapidly due to increased lignin levels in the stems of maturing plants. Growers, therefore, must decide whether to harvest forage to obtain higher quality forage or higher yield.

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 lucerne at the same growth stage.

KK179 lucerne reduces lignin in forage through the suppression of caffeoyl CoA 3-*O*-methyltransferase (CCOMT), a key enzyme in the lignin biosynthetic pathway. KK179 was produced by insertion of *CCOMT* gene segments, derived from lucerne, assembled to form an inverted repeat DNA sequence. The inverted repeat sequence produces double-stranded RNA (dsRNA) that 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 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.

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

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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.

History of Use of the Host and Donor Organisms

The insert present in KK179 contains a partial gene segment of *CCOMT* from *Medicago sativa* configured into an inverted repeat sequence.

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.

Nature of the Genetic Modification

KK179 was developed through *Agrobacterium tumefaciens*-mediated transformation of conventional lucerne, R2336, with the plasmid vector PV-MSPQ12633. The transformed plant was crossed with Ms208, an elite, male sterile, conventional lucerne plant, to produce KK179. PV-MSPQ12633 contains two separate T-DNAs, each delineated by Left and Right Border sequences to facilitate transformation. The first T-DNA, designated T-DNA I, contains the *CCOMT* suppression cassette, the *Pal2* promoter and the *nos* 3′ UTR regulatory elements. The second T-DNA, designated T-DNA II, contains the *nptII* expression cassette under the regulation of 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 *in vitro* selection of transformed plantlets. Subsequent traditional lucerne breeding methods and meiotic segregation, along with a combination of analytical techniques, were used to isolate a subset of transformed plants that contained the *CCOMT* suppression cassette (T-DNA I) but did not contain the *nptII* expression cassette (T-DNA II). This resulted in the subsequent identification of a single marker-free plant, KK179.

Molecular characterisation of KK179 by Southern blot analyses confirmed that one copy of the CCOMT suppression cassette (T-DNA I) was integrated into the lucerne genome at a single locus. No T-DNA II or backbone DNA sequences from plasmid vector PV-MSPQ12633 were detected in KK179. The complete DNA sequence of the insert and adjacent genomic DNA sequence in KK179 confirmed the integrity of the inserted CCOMT suppression cassette within the inserted sequences and identified the 5′ and 3′ insert-to-genomic DNA junctions. Additionally, Southern blot analysis of progeny from KK179 demonstrated that the inserted DNA has been maintained through four generations of breeding, thereby confirming the stability of the insert over multiple generations.

Characterisation of Novel Proteins or Other Novel Substances

The safety of KK179 expression products was assessed by taking into account multiple factors. The KK179 insert contains a *CCOMT* suppression cassette. RNA-based suppression of *CCOMT* in KK179 is mediated by dsRNA molecules transcribed from the suppression cassette, which decrease the level of endogenous *CCOMT* transcripts resulting in reduced levels of G lignin. Double-stranded RNAs are commonly found in eukaryotes, including plants, and function to suppress endogenous gene expression. Nucleic acids have a long history of safe consumption. There is no evidence to suggest dietary consumption of RNA is associated with mammalian toxicity, adverse health issues, or allergenicity. Several biotechnology-derived plant products previously approved by several international regulatory authorities were developed using RNA-based suppression mechanisms, including high-oleic soybean, plum pox virus-resistant plum trees, virus-resistant papaya, virus-resistant squash, and delayed-ripening tomatoes. The hairpin secondary structure of the dsRNA produced by the *CCOMT* suppression cassette precludes translation initiation and protein synthesis, thus production of the CCOMT protein is highly unlikely. Even in the unlikely event that a protein were produced from the suppression cassette, bioinformatics analyses demonstrate the lack of similarity with known allergens, toxins, or other biologically active proteins for all putative peptides derived from the suppression cassette, or the entire inserted DNA sequence of KK179. This safety assessment, designed to address the appropriate Codex Alimentarius Commission guidelines including those relevant to products where the function of the expressed sequences is to alter the accumulation of endogenous mRNA or protein, supports the conclusion that there are no meaningful risks to animal (or human) health from dietary exposure to dsRNA produced in KK179, nor to putative proteins.

Analyses of KK179 RNA by northern blot confirm the suppression of endogenous *CCOMT* RNA in lucerne forage. Additionally, analysis of lignin subunits, the building blocks for lignin molecules, confirms that the suppression of *CCOMT* acts to specifically reduce the level of one major lignin subunit, G lignin, while not substantially affecting the levels of the other major lignin subunit, syringyl lignin (S lignin), or a minor lignin subunit, *p*-hydroxyphenyl lignin (H lignin), as predicted by the mode of action. The result is a lower proportion of G lignin and a greater proportion of S lignin, shown by an increase in the S to G lignin ratio. Analysis of total lignin levels, measured as acid detergent lignin (ADL), verifies that the reduction in the G lignin leads to a concurrent reduction in total lignin in KK179 forage compared to the conventional control harvested at the same stage of growth.

Potential Toxicity and Allergenicity of Novel Proteins

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. 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). 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 et al., 2003; Parrott et al., 2010). 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). 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.

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.

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.

Compositional Analyses of the GM Food

OECD has concluded that 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 consensus documents on compositional considerations for new crop varieties emphasize quantitative measurements of essential nutrients and known anti-nutrients. 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. For a biotechnology-derived lucerne product, levels of the components in forage of the biotechnology-derived crop are compared to: 1) corresponding levels in a conventional comparator, a genetically similar conventional line, grown concurrently under field conditions, and 2) ranges of conventional commercial reference varieties grown concurrently and from available data published in the scientific literature. The comparison to data established by concurrently grown commercial reference varieties and to available data in published literature places observed differences between the biotechnology-derived crop and its comparator in the context of the well-established variation in the concentrations of the crop's nutrients and anti-nutrients.

Compositional analyses were conducted based on OECD guidelines for lucerne to compare levels of key nutrients, anti-nutrients and secondary metabolites in KK179 to levels in the conventional lucerne control. Nutrients analysed in forage samples were proximates (ash, fat, moisture, and protein), carbohydrates by calculation, acid detergent fiber (ADF), neutral detergent fiber (NDF), acid detergent lignin (ADL), minerals (Ca, Cu, Fe, Mg, Mn, P, K, Na, and Zn), and amino acids (essential and non-essential). Anti-nutrients included daidzein, glycitein, genistein, coumesterol, formononetin, biochanin A, and saponins (total bayogenin, total hederagenin, total medicagenic acid, total soyasapogenol B, total soyasapogenol E, total zanhic acid and total saponins). Secondary metabolites included *p*-coumaric acid, ferulic acid, sinapic acid, total polyphenols, free phenylalanine, and canavanine.

Compositional analyses on forage samples were conducted to determine statistically significant differences ($\alpha = 0.05$) between KK179 and the conventional control. Statistical results from combined-site data were evaluated using considerations relevant to the safety and nutritional quality of KK179 when compared to the conventional control. Considerations to assess the relevance of each statistically significant difference included: 1) the relative magnitude of the differences in the mean values of nutrient, anti-nutrient, and secondary metabolite components between KK179 and the conventional control; 2) whether the KK179 component mean values were within the range of variability of the components as represented by the 99% tolerance interval of the conventional commercial reference varieties grown concurrently in the same field trial; and 3) an assessment of the differences within the context of natural variability of available commercial lucerne composition published in the scientific literature.

Analysis of the observed significant differences ($p<0.05$) in nutrient, anti-nutrient, and secondary metabolite components with respect to magnitude of differences, comparisons of mean analyte values to the 99% tolerance interval and to published values led to the conclusion that the differences were not biologically meaningful from a feed/food safety or

nutritional perspective. Therefore, the genetic modification in KK179 does not meaningfully impact composition, other than the intended reduction in G lignin and total lignin (ADL), and, therefore, the feed/food safety and nutritional quality of this product are comparable to conventional lucerne, which has a history of safe consumption. Finally, the mode of action for reduction in G lignin and total lignin (ADL) is well characterised and provides no reason to expect interactions with important nutrients, anti-nutrients, or secondary metabolites present in lucerne. When KK179 is used on a commercial scale as a source of feed, lucerne products are not expected to be compositionally different from the equivalent feeds originating from conventional lucerne.

Conclusion

All data and information contained within this document strongly support the conclusion that food and feed derived from KK179 and its progeny will be as safe and nutritious as food and feed derived from conventional lucerne.