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Petition for the Determination of Nonregulated Status for Reduced Lignin Alfalfa KK179

The undersigned submits this petition under 7 CFR § 340.6 to request that the Administrator make a determination that the article should not be regulated under 7 CFR Part 340

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RELEASE OF INFORMATION

Monsanto Company and Forage Genetics International are submitting the information in this petition for review by the USDA as part of the regulatory process. Monsanto Company and Forage Genetics International understand that the USDA complies with the provisions of the Freedom of Information Act (FOIA). In the event the USDA receives a FOIA request, pursuant to 5 U.S.C., § 552, and 7 CFR Part 1, covering all or some of the information in this petition, Monsanto Company and Forage Genetics International expect that, in advance of the release of the document(s), USDA will provide Monsanto Company and Forage Genetics International with a copy of the material proposed to be released and the opportunity to object to the release of any information based on appropriate legal grounds, *e.g.*, responsiveness, confidentiality, and/or competitive concerns. Monsanto Company and Forage Genetics International understand that a CBI-deleted copy of this information may be made available to the public in a reading room and upon individual request as part of a public comment period. Monsanto Company and Forage Genetics International also understand that when deemed complete, a copy of the petition may be posted to the USDA-APHIS BRS website or other U.S. government websites (*e.g.*, www.regulations.gov). Except in accordance with the foregoing, Monsanto Company and Forage Genetics International do not authorize the release, publication or other distribution of this information without Monsanto Company's and Forage Genetics International's prior notice and consent.

CERTIFICATION

The undersigned certify that, to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes all relevant data and information known to the petitioner that are unfavorable to the petition.

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EXECUTIVE SUMMARY

The Animal and Plant Health Inspection Service (APHIS) of the United States (U.S.) Department of Agriculture (USDA) has responsibility under the Plant Protection Act (Title IV Pub. L. 106-224, 114 Stat. 438, 7 U.S.C. § 7701-7772) to prevent the introduction and dissemination of plant pests into the U.S. USDA-APHIS regulation 7 CFR § 340.6 provides that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and no longer should be regulated. If USDA-APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

Monsanto Company (hereafter referred to as Monsanto) and Forage Genetics International (hereafter referred to as FGI) are submitting this request to USDA-APHIS for a determination of nonregulated status for the new biotechnology-derived alfalfa product, KK179, any progeny derived from crosses between KK179 and conventional alfalfa, and any progeny derived from crosses of KK179 with biotechnology-derived alfalfa that have previously been granted nonregulated status under 7 CFR Part 340.

Product Description

Monsanto and FGI have developed biotechnology-derived alfalfa KK179 (*Medicago sativa* L.) which has reduced levels of guaiacyl lignin (G), a major subunit component of total lignin, compared to conventional alfalfa at the same stage of growth. This reduction in G lignin leads to reduced accumulation of total lignin in alfalfa forage, the principal feed product derived from alfalfa. Forage quality, as defined by market standards, is compromised by the presence of lignin which is sensitive to timing of harvest. KK179 is designed to provide alfalfa growers with greater flexibility in harvest timing in order to better manage forage quality and improve the ability to meet or exceed intended quality standards for alfalfa forage production.

KK179 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 alfalfa, assembled to form an inverted repeat DNA sequence. The inverted repeat sequence produces double-stranded RNA (dsRNA) which 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 synthesis of G lignin subunit compared to conventional alfalfa at the same stage of growth. The reduction in G lignin subunit synthesis leads to reduced accumulation of total lignin, measured as acid detergent lignin (ADL).

Forage of KK179 and the conventional control were analyzed for monomeric lignin subunits, the building blocks for lignin molecules. These analyses confirm that the suppression of *CCOMT* acts to specifically reduce the level of one major lignin subunit, G lignin by approximately 19%, while not substantially affecting the levels of the other major lignin subunit, syringyl lignin (S lignin), or minor lignin subunits, 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 the same forage for total lignin levels, measured as acid detergent lignin (ADL), by commercial forage testing methods shows a reduction of approximately 22% and 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.

Alfalfa 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 alfalfa forage 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 (tonnage). There is a narrow interval of time, a matter of a few days, depending on the growing region, to harvest high quality forage prior to significant lignin accumulation at which point the quality of the forage declines rapidly. KK179 allows growers more flexibility to schedule harvests and thereby better manage the yield-quality relationship in forage production in order to meet market needs or intended on-farm uses as animal feed. This expanded harvest interval provides valuable flexibility for growers to manage real-time production decisions. For example, over the life of an alfalfa field or stand, a grower using KK179 can, at each cutting, interchangeably: 1) maintain harvest schedule routines and obtain forage that is more likely to meet or exceed the intended quality standard targeted by the grower; or 2) delay a harvest several days and obtain higher tonnage without sacrificing forage quality. At the same time, independently of the production decision on when to harvest, a grower will benefit from flexibility to accommodate unexpected delays in harvesting forage caused by adverse weather conditions, equipment failure, or competing farming activities.

Data and Information Presented Confirm the Lack of Plant Pest Potential and the Feed and Food Safety of KK179 Compared to Conventional Alfalfa

The data and information presented in this petition demonstrate that KK179 is agronomically, phenotypically, and compositionally comparable to conventional alfalfa, with the exception of the introduced trait for reduced G lignin and total lignin. Moreover, the data and information presented demonstrate that KK179 is not expected to pose an increased plant pest risk, including weediness, compared to conventional alfalfa. The feed, food and environmental safety of KK179 was confirmed based on multiple, well established lines of evidence:

- Alfalfa is a familiar crop that has a history of safe consumption as animal feed, and serves as an appropriate basis of comparison for KK179.

- A detailed molecular characterization of the inserted DNA, which confirms the presence of a single, intact *CCOMT* suppression cassette stably integrated at a single locus within the alfalfa genome.

- The *CCOMT* suppression cassette in KK179 is extremely unlikely to produce a protein. The RNA-based suppression of the *CCOMT* alfalfa gene in KK179 is mediated by dsRNA molecules. Double-stranded RNAs are commonly used by

eukaryotes, including plants, for endogenous gene suppression and pose no novel risks from a feed/food and environment perspective. Nucleic acids, as the components of RNA, have a long history of safe consumption and are considered GRAS by the U.S. FDA.

- The compositional and nutritional assessment supports the conclusion that KK179 forage is compositionally equivalent, with the exception of the intended reduction in G lignin and total lignin (ADL), to that of conventional alfalfa at the same stage of growth.

- An extensive evaluation of KK179 phenotypic and agronomic characteristics and environmental interactions demonstrate that KK179 shows no increased plant pest risk potential compared to conventional alfalfa.

- An assessment of potential impacts to non-target organisms (NTOs) including organisms beneficial to agriculture indicates that KK179 is not expected to have an effect on other organisms compared to conventional alfalfa under normal agricultural practices.

- Evaluation of KK179 using current cultivation and management practices for alfalfa concluded that deregulation of KK179 is not expected to have an effect on alfalfa agronomic practices or land use.

Alfalfa is a Familiar Crop Lacking Weedy Characteristics

Alfalfa (*Medicago sativa* L.) is grown for forage in all U.S. states and continues to be an important U.S. crop. It ranks fourth on the list of most widely grown crops by acreage and fourth in terms of value among agricultural crops. Approximately 20 to 24 million acres of alfalfa hay have been harvested annually over the past 10 years. Annual production has ranged from 68 to 82 million tons of hay. Average yields have remained fairly constant at 3.19 to 3.47 tons per acre over that same period. The annual value of production has ranged from $6.7 to $10.7 billion.

Because of its adaptability, alfalfa can survive outside of cultivation as feral populations. However, there is little evidence to suggest that alfalfa behaves as a weed, other than as a volunteer in agricultural settings. Weed control experts from states where alfalfa is cultivated extensively and publicly available weed lists confirm that *Medicago sativa* is not considered or designated a weed or species with weediness potential. This aspect of alfalfa has been comprehensively reviewed by USDA-APHIS for Genuity® Roundup Ready® Alfalfa in its December 2010 Plant Pest Risk Asssessment and Determination and associated Environmental Impact Statement.

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Conventional Alfalfa is an Appropriate Comparator to KK179

A near-isogenic conventional alfalfa comparator was developed using an identical breeding path used for the development of KK179. The same breeding process used to develop subsequent generations of KK179 was then followed to develop subsequent generations of near-isogenic conventional alfalfa comparators for each generation of KK179. All generations of KK179 and corresponding generations of conventional alfalfa comparators used in safety assessments have closely related genetic backgrounds with the exception of the introduced trait for reduced G lignin and total lignin absent in the conventional comparator. Conventional commercial alfalfa varieties (referred to as conventional commercial reference varieties), were used to establish ranges of natural variability or responses representative of commercial alfalfa varieties. The conventional commercial reference varieties used were chosen based on their availability and agronomic fit for the geographic region.

Molecular Characterization Verified the Integrity and Stability of the Inserted DNA in KK179

KK179 was developed through *Agrobacterium tumefaciens*-mediated transformation of conventional alfalfa, R2336, with the plasmid vector PV-MSPQ12633. The transformed plant was crossed with Ms208, an elite, male sterile, conventional alfalfa plant, to produce KK179. The PV-MSPQ12633 plasmid 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 alfalfa genome where T-DNA II, containing the *nptII* expression cassette, functioned as a marker gene for *in vitro* selection of transformed plantlets. Subsequent traditional alfalfa 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 characterization of KK179 by Southern blot analyses confirmed that one copy of the *CCOMT* suppression cassette (T-DNA I) was integrated into the alfalfa 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 and Mendelian segregation 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.

RNA-Based Suppression Technology in KK179 Does not Pose Unique Safety Risks

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* RNA 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. RNA is composed of nucleic acids which have a long history of safe consumption and are generally recognized as safe (GRAS) by the U.S. FDA. 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 reviewed by the U.S. FDA, deregulated by USDA-APHIS, 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, 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 synthesis of the CCOMT protein or a putative polypeptide is highly unlikely. Based on this information, it is concluded that the inserted DNA and resulting dsRNA are safe and unlikely to produce a protein. As a result, the RNA-based suppression technology used in KK179 poses no novel risks from a feed, food or environment perspective.

KK179 is Compositionally Equivalent to Conventional Alfalfa

Detailed compositional analyses were conducted based on OECD guidelines for alfalfa to compare levels of key nutrients, anti-nutrients and secondary metabolites in KK179 forage to levels in the conventional alfalfa control. Nutrients analyzed 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 analyzed related to lignin biosynthesis and phenylpropanoid metabolism in alfalfa 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 alfalfa composition published in the scientific literature.

Analysis of the observed significant differences 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 and 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). As a result, the feed and food safety and nutritional quality of this product are comparable to those of conventional alfalfa, which has a history of safe use and consumption. When KK179 is grown on a commercial scale and used as a source of feed, alfalfa products derived from KK179 are not expected to be compositionally different from the equivalent feeds originating from conventional alfalfa.

KK179 Does Not Change Alfalfa Plant Pest Potential or Environmental Interactions

Plant pest potential of a biotechnology-derived crop is assessed from the basis of familiarity that the USDA recognizes as an important underlying concept in risk assessment. The concept of familiarity is based on the fact that the biotechnology-derived plant is developed from a conventional plant whose biological properties and plant pest potential are well known. Familiarity considers the biology of the plant, the introduced trait, the receiving environment, and the interactions among these factors. This provides a basis for comparative risk assessment between a biotechnology-derived plant and the conventional control. Thus, the phenotypic, agronomic, and environmental interaction assessment of KK179 included the near-isogenic conventional control as a comparator. This evaluation used a weight of evidence approach and considered statistical differences between KK179 and the conventional control with respect to reproducibility, magnitude, and directionality. Characteristics assessed included seed dormancy and germination; pollen and flower morphologies; plant symbiont interactions in the laboratory or greenhouse; plant phenotypic observations; and environmental interaction evaluations conducted in the field. Conventional commercial reference varieties grown concurrently were used to establish a range of natural variability for each assessed characteristic in alfalfa. The phenotypic, agronomic, and environmental interaction assessment demonstrated that KK179 is comparable to the conventional control. Thus, KK179 is not expected to have increased weediness or plant pest potential compared to conventional alfalfa.

Seed dormancy and germination characterization indicated that KK179 seed had no changes relative to the conventional control in dormancy or germination characteristics that could be indicative of increased plant weediness or pest potential of KK179 compared to the conventional control. Some statistically significant differences were detected in which KK179 had higher percent germinated seed and lower percent hard seed. These differences, however, were not considered biologically meaningful in terms of altered weediness of KK179 compared to the conventional control. No statistically significant differences were detected ($\alpha = 0.05$) between KK179 and the conventional control for percent viable pollen or pollen grain diameter. Furthermore, no visual

differences in general pollen morphology were observed between KK179 and the conventional control, demonstrating that the introduced trait for reduced G lignin and total lignin did not alter the overall morphology or viability of pollen of KK179 compared to that of the conventional control. Similarly, no statistically significant differences were detected between KK179 and the conventional control for morphology of flowers.

The field evaluation of phenotypic, agronomic, and environmental characteristics of KK179 also supports the conclusion that KK179 is not expected to have increased weediness or plant pest potential compared to conventional alfalfa. The evaluations were conducted at nine field sites in the U.S. and one field site in Canada to provide a diverse range of environmental and agronomic conditions representative of commercial alfalfa production areas in North America. Trials were managed according to standard, local agronomic practices for forage production in order to harvest forage at a growth stage of 1-10% bloom. For each growing season, assessments were made within each crop growth cycle and at each harvest. In addition to phenotypic and agronomic characteristics, observations were also made for plant responses to abiotic stressors, diseases, and arthropod interactions. These studies were conducted over two complete growing seasons from 2010 to 2012.

The phenotypic characteristics observed during forage production from 2010 to 2012 across 10 field sites were comparable between KK179 and the conventional control. In a combined-site analysis of the phenotypic characteristics in the first growing season (2010-2011), data showed no statistically significant differences between KK179 and the conventional control for seedling emergence and early season vigor during stand establishment; no statistically significant differences for lodging, crop growth stage, and regrowth after cutting for each of three harvests, and no statistically significant differences for fall plant height, total forage yield, spring vigor, spring stand recovery, and spring stand count. In a combined-site analysis of the phenotypic characteristics in the second growing season (2011-2012), data showed no statistically significant differences between KK179 and the conventional control for lodging, crop growth stage, forage yield, and regrowth after cutting for each of five harvests and no statistically significant differences for fall plant height, total forage yield, spring vigor, spring stand recovery, and spring stand count. In a combined-site and combined-year analysis, there were also no statistically significant differences for fall plant height, total forage yield, spring vigor, spring stand recovery, and spring stand count.

The phenotypic characteristics observed during seed production at one field site in 2010 were also comparable between KK179 and the conventional control. Data showed no statistically significant differences between KK179 and the conventional control for seedling establishment, seedling vigor, seed weight, seed per pod, or seed yield. Additionally, no statistically significant differences were detected between KK179 and the conventional control for lodging or split pods, both characteristics that could impact potential weediness.

Environmental interactions were assessed qualitatively within each growing season over two years and included plant response to abiotic stressors, disease damage and arthropod damage. In the first year, no differences were observed between KK179 and the

conventional control for any of the 93 comparisons of plant response to abiotic stressors, the 93 comparisons for plant damage caused by diseases, or the 96 comparisons for plant damage caused by arthropods. In the second year, no differences were observed between KK179 and the conventional control for any of the 129 comparisons of plant response to abiotic stressors, the 129 comparisons for plant damage caused by diseases, or the 129 comparisons for plant damage caused by arthropods.

Environmental interactions were assessed quantitatively within each growing season over two years and included assessments of alfalfa weevil damage and potato leafhopper damage and pest- and beneficial-arthropod abundance. For alfalfa weevil or potato leafhopper damage, no statistically significant differences were detected in combined site analyses for either insect in 2010 and in 2011. For arthropod abundance, four differences out of 69 comparisons were detected at individual sites in the first year and one out of 89 comparisons in the second year. At the sites where statistical differences were observed, the mean abundance values for pests and arthropods from KK179 were within the range of the conventional commercial reference varieties and/or the differences were not consistently detected across collection times or sites. Taken together, these data support the conclusion that compared to conventional alfalfa KK179 is no more susceptible to damage by alfalfa weevil or potato leafhopper and no more likely to promote increased abundance of these species.

In summary, phenotypic, agronomic, and environmental interaction data were evaluated to characterize KK179 and to assess whether the introduced trait for reduced G lignin and total lignin in KK179 alters the plant pest potential compared to conventional alfalfa. The evaluation, using a weight of evidence approach, considered the reproducibility, magnitude, and direction of detected differences between KK179 and the conventional control, and comparison to the range of the conventional commercial reference varieties. Results from the phenotypic, agronomic, and environmental interactions assessment indicated that KK179 does not possess more or enhanced weediness characteristics, increased susceptibility or tolerance to specific abiotic stress, diseases, or arthropods, or characteristics that would confer a plant pest risk compared to conventional alfalfa.

KK179 Will Not Have Effects On NTOs Including Those Beneficial To Agriculture

Evaluation of the impacts of KK179 on non-target organisms (NTOs) is a component of the plant pest risk assessment. Since KK179 does not possess pesticidal activity, all organisms that interact with KK179 are considered to be NTOs. The environmental interactions assessment conducted over two growing seasons under field conditions demonstrated that the introduction of the CCOMT suppression cassette for reduced G lignin and total lignin in KK179 does not unexpectedly alter plant-arthropod interactions, including beneficial arthropods, soil symbiont interactions, or alter disease susceptibility compared to the conventional control. These results support the conclusion that cultivation of KK179 is not expected to effect non-target organisms and no altered incidence of disease in KK179. The evaluation also considered product characterization information including molecular characterization and composition, and the history of environmental exposure to lignin. Taken together, these data support the conclusion that KK179 has no reasonable mechanism for harm to NTOs, including organisms beneficial to agriculture, compared to commercial alfalfa.

Potential for KK179 to outcross with sexually compatible species is not expected since no known related species capable of crossing with cultivated alfalfa are known to be present in North America. Assessments of phenotypic, agronomic, and environmental interactions, including reproductive characteristics indicated that KK179 is not expected to outcross with sexually compatible species compared with conventional alfalfa. Additionally, under forage production conditions, the potential for pollen production and outcrossing is very limited. Cross-pollination of KK179 or its progeny with feral alfalfa plants or with related species, would not be expected to have an effect on the environment, because evaluations have shown the introduced trait for reduced G lignin and total lignin in KK179 does not enhance weediness or plant-pest potential relative to conventional alfalfa. Therefore, the environmental consequence of pollen transfer between KK179 and feral alfalfa or a related species is considered negligible. Outcrossing to feral alfalfa populations and other species has been comprehensively reviewed by USDA-APHIS for Roundup Ready Alfalfa in its December 2010 Plant Pest Risk Assessment and Determination and associated Environmental Impact Statement. USDA-APHIS has concluded that outcrossing to feral alfalfa by deregulated biotechnology-derived alfalfa does not pose greater environmental risk compared with conventional alfalfa.

Deregulation of KK179 Is Not Expected To Change Alfalfa Agronomic Practices or Land Use

An assessment of current alfalfa agronomic practices was conducted to determine whether the cultivation of KK179 has the potential to impact current alfalfa management practices. Alfalfa fields are typically highly managed agricultural areas that are dedicated to forage production. KK179 is likely to be used in common rotations on land previously used for agricultural purposes. Cultivation of KK179 is not expected to differ from current alfalfa cultivation, with the exception of allowing growers greater flexibility to manage the yield-quality relationship in forage production and harvesting schedules to meet market needs or for intended on-farm uses as animal feed. Certified seed production will not be impacted by the introduction of KK179 and germplasm developers and seed producers will continue to use well-established industry practices to deliver a diverse range of high quality alfalfa seed to growers.

KK179 is similar to conventional alfalfa in its agronomic, phenotypic, environmental, and compositional characteristics and has levels of resistance to insects and diseases comparable to conventional alfalfa. Based on this assessment, KK179 is not expected to result in changes to current U.S. alfalfa agronomic practices. Therefore, no impacts on current cultivation and management practices for alfalfa are expected following the introduction of KK179.

Conclusion

Based on the data and information presented in this petition, Monsanto and FGI have concluded that KK179 is not expected to be a plant pest. Therefore, Monsanto and FGI request a determination from USDA-APHIS that KK179 and any progeny derived from crosses between KK179 and conventional alfalfa or deregulated biotechnology-derived alfalfa be granted nonregulated status under 7 CFR Part 340.

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ABBREVIATIONS AND DEFINITIONS[1]

Symbol or Abbreviation	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*

[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.

	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
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
HGS	Combined H lignin, G lignin, and S lignin
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

I. RATIONALE FOR THE DEVELOPMENT OF KK179

I.A. Basis for the Request for a Determination of Nonregulated Status under 7 CFR § 340.6

The Animal and Plant Health Inspection Service (APHIS) of the United States (U.S.) Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (Title IV Pub. L. 106-224, 114 Stat. 438, 7 U.S.C. § 7701-7772) to prevent the introduction and dissemination of plant pests into the U.S. USDA-APHIS regulation 7 CFR § 340.6 provides that an applicant may petition USDA-APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and no longer should be regulated. If USDA-APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

Monsanto Company (hereafter referred to as Monsanto) and Forage Genetics International (hereafter referred to as FGI) are submitting this request to USDA-APHIS for a determination of nonregulated status for the new biotechnology-derived alfalfa product, KK179, any progeny derived from crosses between KK179 and conventional alfalfa, and any progeny derived from crosses of KK179 with biotechnology-derived alfalfa that have previously been granted nonregulated status under 7 CFR Part 340.

I.B. Rationale for the Development of Reduced Lignin Alfalfa KK179

Alfalfa (*Medicago sativa* L.) is the principal forage crop cultivated in the U.S. for animal feed. Approximately 20 to 24 million acres of alfalfa hay have been harvested annually in the U.S. in the majority of states over the past ten years to produce between 68 and 82 million tons of hay annually valued between approximately $6.7 and $10.7 billion USD (USDA-NASS, 2011b) Alfalfa is the fourth largest agricultural crop in the U.S. in terms of acres harvested and fourth highest in value (USDA-NASS, 2012a; b). Approximately 40 percent of U.S. alfalfa acreage is planted as pure stands, while 30 percent is planted with cover or nurse crop and approximately 25 percent with grasses or another companion crop (USDA-APHIS, 2010).

Alfalfa forage products are valued for their high protein content and highly digestible fiber for ruminants and horses (USDA-APHIS, 2010). The principal commercial product is hay, which is forage that has been dried and baled, or cubed if mechanically compressed. Haylage, which is ensiled forage, is typically produced and used on-farm. The forage industry defines the quality of alfalfa hay by nutritional components, including crude protein, acid detergent fiber, neutral detergent fiber, total digestible nutrients, and relative feed value (Putnam et al., 2008b; USDA-APHIS, 2010). Other quality parameters evaluated include observations for the presence of weeds or molds, or anti-palatability factors such as poor texture, evidence of heating, or unpleasant odors (Putnam et al., 2008b). USDA's Agricultural Marketing Service uses the grades of supreme, premium, good, fair, and utility to regularly report average prices for alfalfa hay in major producing areas (USDA-AMS, 2010). Supreme quality is defined as very early maturity, pre-bloom forage with no damage and with the highest nutritive value, while utility is forage harvested at very late maturity, with heavy damage, and with minimal

nutritive value. Price differences between supreme and fair quality can exceed 50% (Putnam et al., 2008b; USDA-AMS, 2012; USDA-APHIS, 2010).

Lignin deposition in maturing plants has a significant impact on the overall quality of alfalfa (Coors et al., 1986; Marten et al., 1988; Schwab et al., 2005). Along with cellulose and hemicellulose constituents, lignin is a cell wall component that accumulates in the plant, particularly in the stem. At alfalfa crop maturity, lignin comprises 5-15% of dry matter (Putnam et al., 2008b), with this large range being dependent on many factors such as climate. After forage is harvested, regrowth of stems and leaves initiates within a short time and the process of lignin accumulation is renewed. While a certain amount of lignin is essential for healthy alfalfa plants, lignin is indigestible and slows down the digestion of cellulose in the rumen of livestock. Therefore, forage producers and commodity purchasers desire alfalfa with lower lignin levels but without loss of nutritional components, including protein and fiber.

Alfalfa is grown as a perennial forage crop that is repeatedly harvested throughout the growing season. After each harvest, alfalfa plants go through a period of recovery followed by regrowth. Harvesting of alfalfa forage from an established stand can take place from two to eleven times in a growing season depending on the climate and region. Deciding when to harvest forage is a critical decision made by the grower that determines both forage yield and quality, and the ultimate profitability and utility of the crop. Growers must compromise between obtaining high yield and high quality because the quality of forage declines rapidly as lignin accumulates in maturing plants. This inverse relationship means that crop maturity at the time of forage harvest affects yield and quality differently. Alfalfa forage yields can double from the pre-bud to full-bloom stages, but this rapid biomass increase is accompanied by a steep decline in quality due to lignin accumulation (Orloff and Putnam, 2008). Conversely, alfalfa harvested at an immature or early maturity growth stage produces the highest level of quality in forage due to lower lignin levels but at a lower yield (Orloff and Putnam, 2008). As a result, the interval of time during which forage quality and yield is optimized is relatively narrow and varies depending on which objective, quality or yield, is the priority for the grower. Planning the optimal schedule to harvest forage, therefore, is a difficult management decision involving market considerations, agronomic factors (Orloff and Putnam, 2008), and weather.

Monsanto and FGI have developed biotechnology-derived alfalfa, KK179, which has reduced levels of guaiacyl (G) lignin subunits (hereafter referred to as G lignin), a primary subunit of total lignin, compared to conventional alfalfa at the same stage of growth. This reduction in G lignin subunits leads to reduced accumulation of total lignin in alfalfa forage. The levels of lignin in KK179 forage are generally similar to those found in conventional forage harvested several days earlier under similar production conditions. As a result, growers will have an expanded harvest interval and experience the benefit of greater flexibility to dynamically manage harvest strategies based on crop production priorities, such as:

- Maximizing forage quality: When aiming to maximize 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 alfalfa forage

with lower levels of lignin compared to conventional alfalfa 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 forage yield will be maintained at the same levels as with conventional alfalfa. KK179 does not raise the maximum potential quality attainable for forage; rather, KK179 is more likely to meet or exceed the desired quality compared to conventional alfalfa harvested at the same stage.

- Maximizing forage yield: When aiming to maximize dry matter yield of KK179, a grower can delay harvest for several days to accumulate more forage biomass without significantly forfeiting quality. During the reproductive growth stage, alfalfa 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 alfalfa harvested several days earlier, but with additional forage yield, a benefit not afforded by conventional systems. A similar delay with conventional alfalfa would provide a comparable yield, but the forage would have higher lignin content and, thus, lower quality. From a forage-production perspective, the maximum potential yield of alfalfa attainable is not raised; rather, growers can more readily reach the higher end of the potential yield range while maintaining a targeted quality standard.

- Tolerating unexpected harvest delays: Unexpected delays in harvesting occur occasionally and are usually due to an untimely event such as rain, equipment failure, or the pressures of competing farming activities, *e.g.*, labor availability and 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 lignin accumulation during the delay period and, thus, less loss of quality by the time harvesting is resumed.

I.B.1 Plant lignin biosynthesis

An understanding of lignin biosynthesis in alfalfa has provided the means to reduce lignin levels and slow the accumulation of lignin during the alfalfa 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 I-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 alfalfa, G lignin and S lignin subunits comprise up to 95% of the lignin subunits. In the lignin biosynthetic pathway, the formation of the G and S lignin 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 methylate 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 acid, while COMT methylates caffeyl aldehyde to produce coniferyl aldehyde, and methylates 5-hydroxyconiferyl aldehyde to produce sinapyl aldehyde

(Figure I-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 I-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.

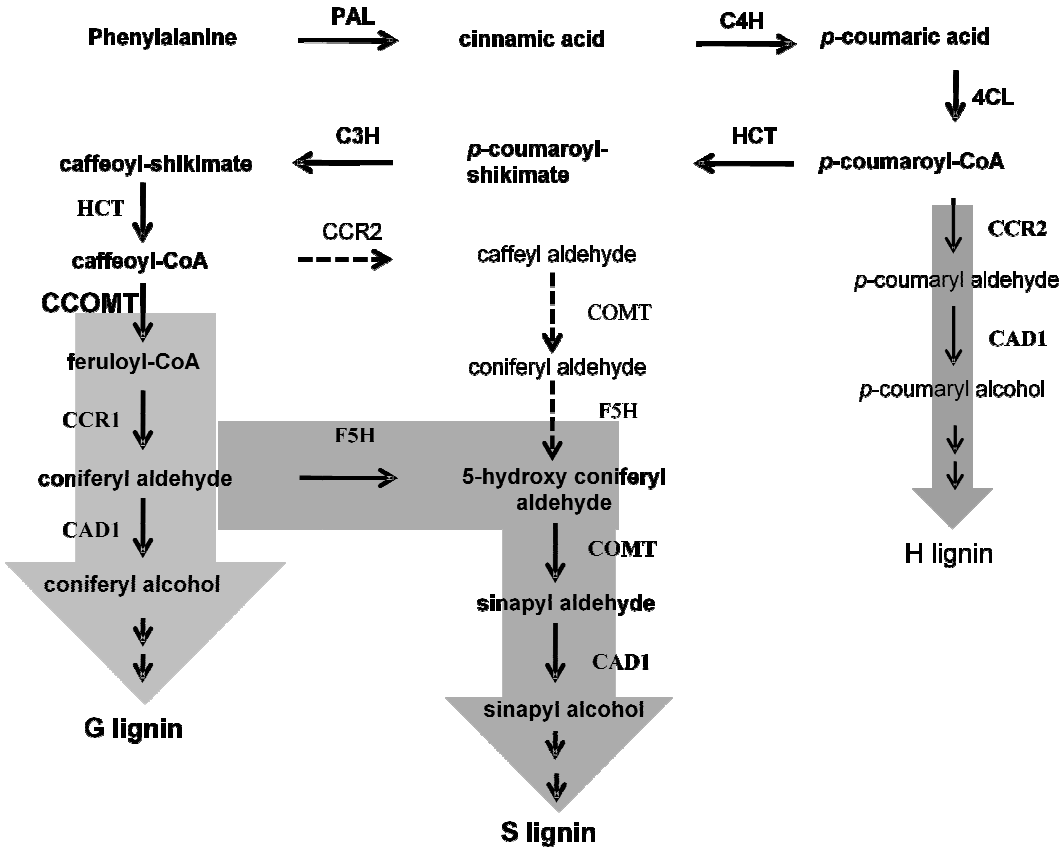


Figure I-1. Lignin biosynthetic pathway

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 3-*O*-methyltransferase;
COMT: caffeic acid 3-*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.

I.B.2 Mode-of-Action and Evidence of Suppression of the *CCOMT* Gene

I.B.2.1 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 use of endogenous alfalfa gene segments configured to suppress the *CCOMT* gene in order to lower CCOMT protein expression and thereby decrease the synthesis of G lignin (Figure I-2). KK179 contains *CCOMT* gene segments under the control of the *Pal2* promoter from the phenylalanine ammonia-lyase 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). Thus, 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 regulation of gene expression (Dykxhoorn et al., 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 (~21-24 nucleotides) small interfering RNAs (siRNAs) (Hammond, 2005; Zamore et al., 2000). 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 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 results in a lower proportion of G lignin relative to all subunits. The decrease in G lignin also results in an increase in the proportion of S lignin relative to all subunits, but not an increase in the actual amount of S lignin. These changes in subunit proportions result in an increase in the S:G lignin ratio, which is characteristic of CCOMT suppression (Chen et al., 2006).

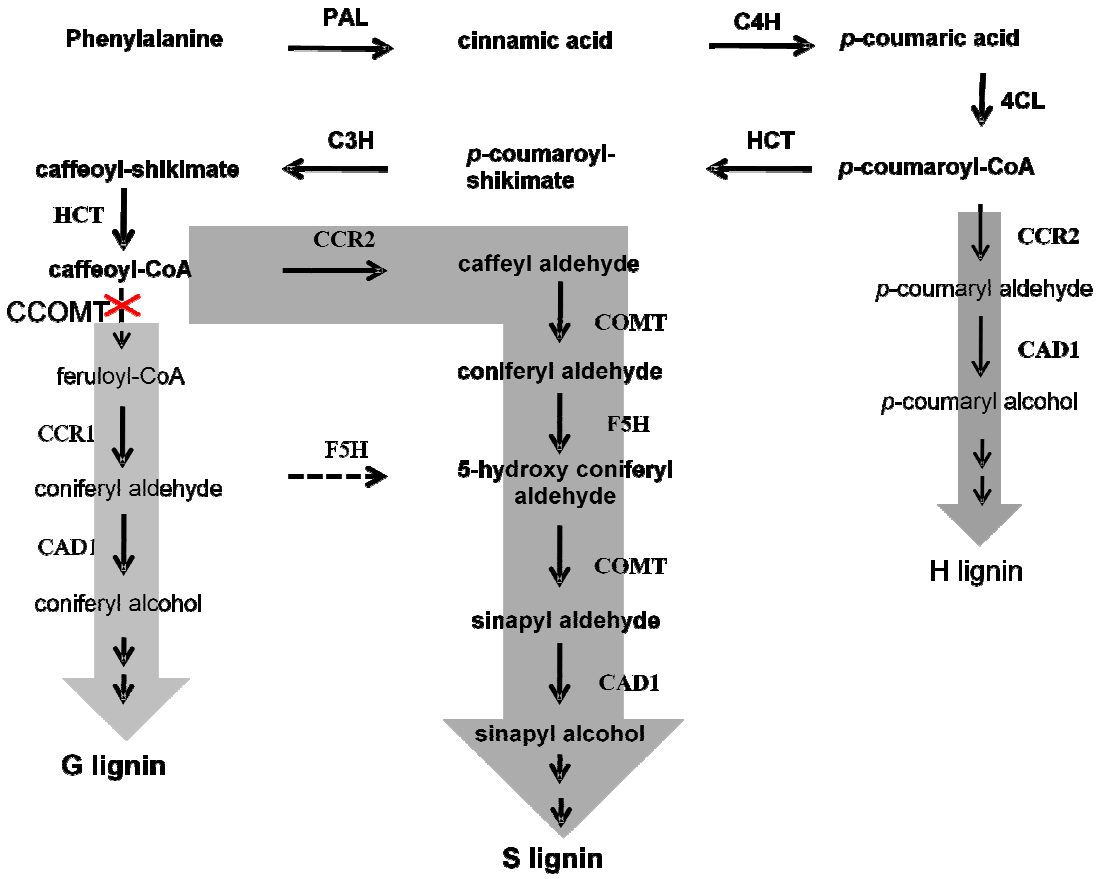


Figure I-2. Modified lignin biosynthetic pathway

Enzymes in pathway are listed in **Figure I-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).

I.B.2.2 Evidence of Suppression of the CCOMT Gene in Forage and Root

Northern blot analyses were used to compare the RNA levels of the endogenous *CCOMT* gene in forage and root tissues of KK179 and conventional alfalfa. 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 analyses. A CCOMT probe generated from a portion of the *CCOMT* gene was hybridized 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 an alfalfa 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. The details of the materials and methods are described in Appendix C.

I.B.2.2.1. 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 hybridization signal at ~1.1 kb, as expected, based on the predicted transcript size of *CCOMT* (Figure I-3, Panel A, Lanes 1, 3, 5, and 7), whereas no detectable hybridization signal was produced from the polyA$^+$ RNA isolated from the forage tissue of KK179 (Figure I-3, 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 hybridized with the actin probe. The polyA$^+$ RNA from the forage tissue of the conventional control (Figure I-3, Panel B, Lanes 1, 3, 5, and 7) and KK179 (Figure I-3, Panel B, Lanes 2, 4, 6, and 8) showed a strong hybridization signal at ~1.5 kb, as expected for the *actin* transcript. The hybridization signals from the forage tissue of the conventional control and KK179 of each replicate have similar intensities, indicating that the RNA loading, RNA quality, and hybridization within each replicate of the conventional control and KK179 were similar. When hybridized with the actin probe template, in addition to the expected ~0.5 kb band, a very faint ~1.0 kb band was detected (Figure I-3, Panel B, Lane 11). This band most likely resulted from a hybridization 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 served as a positive hybridization control and showed that the probe hybridized 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* hybridization signals between the conventional control and KK179 reflects the difference in the *CCOMT* RNA levels (Figure I-3, Panel A).

I.B.2.2.2. Northern Blot Analysis of *CCOMT* RNA in Root

PolyA$^+$ RNA from each of four replicate samples of root tissue from the conventional control produced a strong hybridization signal at ~1.1 kb, as expected, based on the predicted transcript size of *CCOMT* (Figure I-4, 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 I-4, 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 hybridized with the actin probe. The polyA$^+$ RNA from the root tissue of the conventional control (Figure I-4, Panel B, Lanes 1, 3, 5, and 7) and KK179 (Figure I-4, Panel B, Lanes 2, 4, 6, and 8) showed a strong hybridization signal at ~1.5 kb, as expected for the *actin* transcript. The hybridization signals 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 hybridization 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 I-4, Panel B, Lane 11) when hybridized with the actin probe template was not considered to have an impact on the conclusions drawn from this analysis. Therefore, the difference in the *CCOMT* hybridization signal intensities between the conventional control and KK179

reflect the difference in *CCOMT* RNA levels (Figure I-4, Panel A). In addition to the ~1.5 kb actin transcript, faint ~1.1 kb bands were observed (Figure I-4, Panel B, Lanes 1, 3, 5, and 7). These 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 hybridization and no impact on the conclusions made from this analysis.

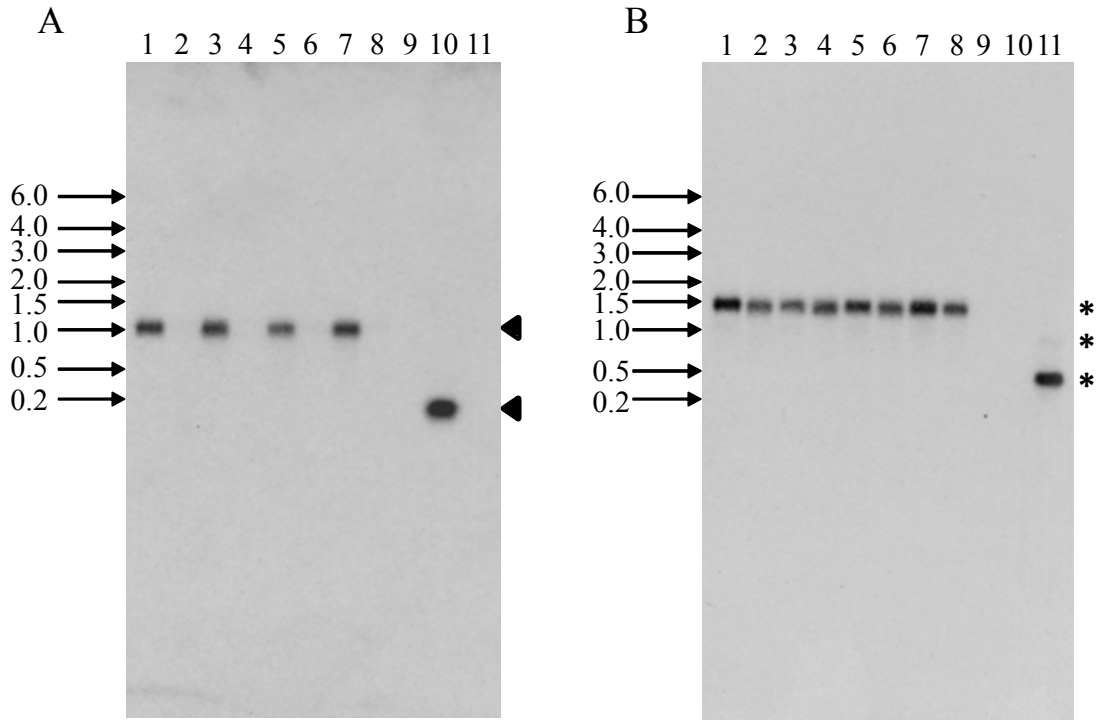


Figure I-3. Northern Blot Analysis of *CCOMT* RNA Level in KK179 Forage Tissue
Panel A and Panel B are the same northern blot containing polyA$^+$ RNA isolated from forage tissue of the conventional control and KK179. Panel A was hybridized with the CCOMT probe. Panel B was hybridized with the actin probe after stripping the CCOMT probe from the blot. Arrow heads indicate the *CCOMT* hybridization signal and stars indicate the *actin* hybridization 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.

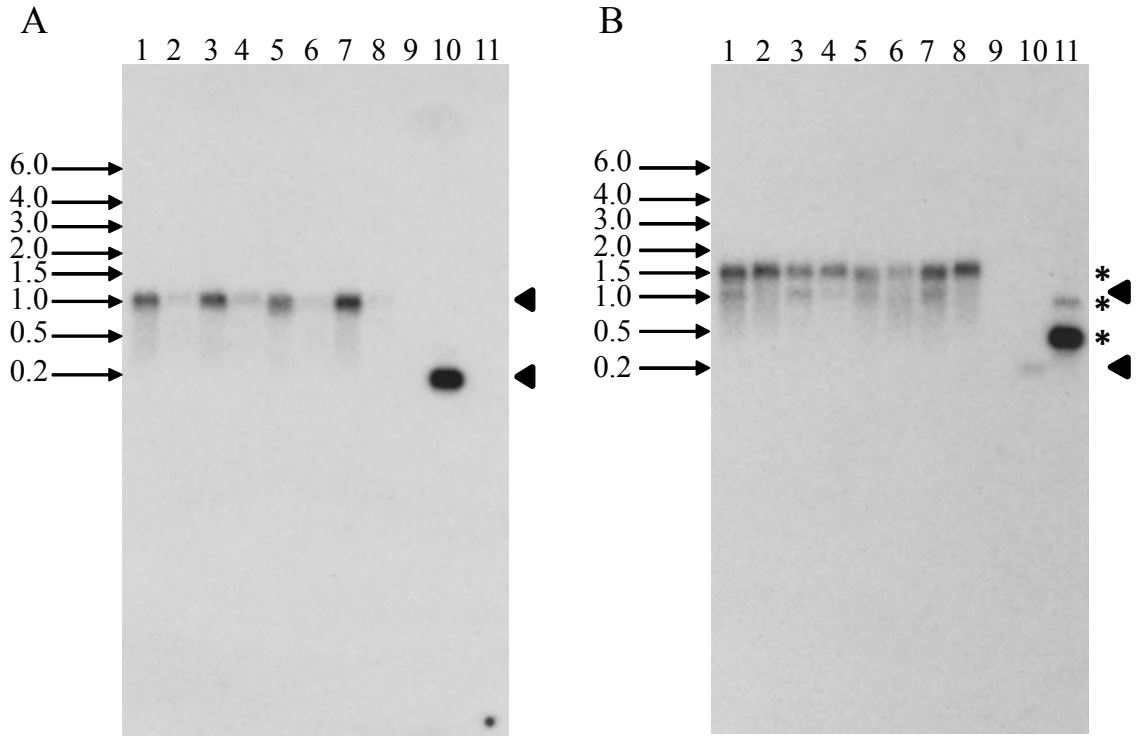


Figure I-4. Northern Blot Analysis of *CCOMT* RNA Level in KK179 Root tissue
Panel A and Panel B is the same northern blot containing polyA$^+$ RNA isolated from root tissue of the conventional control and KK179. Panel A was hybridized with the CCOMT probe. Panel B was hybridized with the actin probe after stripping the CCOMT probe from the blot. Arrow heads indicate the *CCOMT* hybridization signal and stars indicate the *actin* hybridization 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.

I.B.3. Intended Changes to Lignin Levels in KK179 Forage

As described in Section I.B.2.1, KK179 reduces G lignin levels through the suppression of caffeoyl CoA 3-*O*-methyltransferase (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 alfalfa 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 alfalfa control, and conventional commercial alfalfa varieties grown in the United States from the first cutting of a 2011 field production, described in Appendix D. The conventional control (C_0-Syn1) used as a comparator was a near-isogenic conventional alfalfa population with a genetic background similar to that of KK179. Fourteen different conventional commercial alfalfa varieties were included across the field production to provide data on the natural variability of each compositional component analyzed. Field production of forage was conducted in typical alfalfa growing regions at six sites located in California (CAPR); Iowa (IARL); Illinois (ILCY); Kansas (KSLA); Texas (TXCL); and Wisconsin (WIDL). KK179, conventional control and conventional commercial varieties were planted in a randomized 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 alfalfa 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 I-1), and 5-hydroxyguaiacyl lignin (derived from 5-hydroxy coniferyl aldehyde, Figure I-1). This analysis was performed by researchers at the Samuel Roberts Noble Foundation (Ardmore, OK). The method used to measure the lignin subunits, described in Appendix D, generated 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 sum total of 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 on the same samples. Forage samples of KK179 and the conventional control from the first cutting in 2011 at six sites, as described in Appendix D, were analyzed 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). Dairy One Forage Lab used a semi-automated ANKOM-based methodology, described in Appendix D, as adopted by most commercial forage testing laboratories.

I.B.3.1. 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 I-1). 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. As a result, the S:G ratio increased from 0.58 in the conventional control to 0.80 in KK179 (Table I-1), as predicted (Chen et al., 2006). 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 I-2). 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.

I.B.3.2. Intended Changes to Total Lignin Levels in KK179 Forage

The NFTA-certified Dairy One Forage Lab utilizes 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 (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, to gravimetrically determine the amount of insoluble residue remaining. Compositional analysis of KK179 forage samples at the Dairy One Forage Lab 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 I-3). These results confirm that commercial forage testing labs observe a measurable reduction in total lignin (ADL) in KK179, relative to conventional alfalfa harvested at the same stage of growth.

Table I-1. Alfalfa Forage Lignin Subunit Levels and S:G Ratio for KK179 vs. Conventional Control

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (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]Mean (S.E.) = least-square mean (standard error)
[3]Control refers to the conventional alfalfa control, C_0-Syn1.
[4]With 95% confidence, interval contains 99% of the values expressed in the population of commercial alfalfa varieties. Negative limits set to zero.

Table I-2. Alfalfa Forage Lignin Subunit Levels as Percent of Total HGS for KK179 vs. Conventional Control

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (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 (H) lignin	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 Hydroxyphenyl (H), Guaiacyl (G) and Syringyl (S) lignin subunits (µmol/g CWR); CWR = Cell Wall Residue
[2]Mean (S.E.) = least-square mean (standard error)
[3]Control refers to the conventional alfalfa control, C_0-Syn1.
[4]With 95% confidence, interval contains 99% of the values expressed in the population of commercial alfalfa varieties. Negative limits set to zero.

Table I-3. Alfalfa Forage Total Lignin (ADL) Levels for KK179 vs. Conventional Control

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	Difference (Test minus Control) Relative % Difference	Difference (Test minus Control) Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Acid Detergent Lignin[5] (% dw)	5.39 (0.64)	6.93 (0.64)	-1.53 (0.42)	-22.15	0.004	1.39, 12.54
	(2.73 - 7.60)	(2.23 - 10.10)	(-4.33 - 1.30)			(1.70 - 10.03)

[1]dw = dry weight
[2]Mean (S.E.) = least-square mean (standard error)
[3]Control refers to the conventional alfalfa control (C_0-Syn1).
[4]With 95% confidence, interval contains 99% of the values expressed in the population of commercial alfalfa varieties. Negative limits set to zero.
[5]ADL determined using the semi-automated ANKOM method (Weston et al., 2006).

I.C. Submissions to Other Regulatory Agencies

Under the Coordinated Framework for Regulation of Biotechnology, the responsibility for regulatory oversight of biotechnology-derived crops falls primarily on three U.S. agencies: the U.S. Food and Drug Administration (FDA), the United States Department of Agriculture (USDA), and in the case of plant-incorporated protectants, the Environmental Protection Agency (EPA). Deregulation of KK179 by USDA constitutes only one component of the overall regulatory oversight and review of this product. As a practical matter, KK179 cannot be released and marketed until FDA has completed its review and assessment under its respective jurisdiction.

I.C.1. Submission to FDA

KK179 falls within the scope of the 1992 FDA policy statement concerning regulation of products derived from new plant varieties, including those developed through biotechnology (U.S. FDA, 1992). In compliance with this policy, Monsanto and FGI have initiated a consultation with the FDA on KK179, identified under BNF No. 138. A feed/food safety and nutritional assessment summary document was submitted in August 2012.

I.C.2. Submissions to Foreign Government Agencies

Consistent with their commitments to the Biotechnology Industry Organization's Excellence Through Stewardship Program, Monsanto and FGI intend to obtain import approvals from all key alfalfa import markets with functioning regulatory systems prior to commercial planting of KK179. As appropriate, notifications will be made to countries that import significant quantities of alfalfa and alfalfa products and do not have formal regulatory review processes for biotechnology-derived crops.

II. THE BIOLOGY OF ALFALFA

The Canadian Food Inspection Agency document on the biology of alfalfa, Biology Document Bio 2005-02 (CFIA, 2005), provides key information on:

- general description of alfalfa biology
- use of alfalfa as a crop plant
- geographic centers of origin
- taxonomy and genetics
- reproductive biology
- breeding and seed production
- gene flow
- inter-species/genus introgression
- interactions with other organisms
- summary of the ecology of alfalfa

An extensive review of the history and biology of alfalfa can be found in *Alfalfa and Alfalfa Improvement* (Hanson et al., 1988) and in the USDA Final Environmental Impact Statement on Roundup Ready alfalfa (USDA-APHIS, 2010). Taxonomic information for alfalfa is available in the U.S. Department of Agriculture's Natural Resources Conservation Service PLANTS database (USDA-NRCS, 2012).

To support the evaluation of the plant pest potential of KK179 relative to conventional alfalfa, additional information regarding several aspects of alfalfa biology can be found elsewhere in this petition. This includes: agronomic practices for alfalfa in Section VIII; volunteer management of alfalfa in Section VIII; and inter-species/genus introgression potential in Section IX.D.

II.A. Alfalfa as a Crop

Alfalfa (*Medicago sativa* L.), including both cultivated alfalfa and closely related subspecies, originated in Asia Minor, Transcaucasia, Turkmenistan, and Iran. With the spread of agriculture, alfalfa became endemic throughout the Mediterranean region, North Africa, the Middle East, most of Europe, Siberia, northern India, and China (Michaud et al., 1988; Quiros and Bauchan, 1988). Also known as lucerne, it has the longest history of any plant grown solely for forage (Michaud et al., 1988). Due to its importance as an animal feed, it has spread globally and become acclimatized in Australia, New Zealand, North America, South America, and South Africa.

Alfalfa belongs to the order Fabales, family *Fabaceae*, tribe *Trifolium*, genus *Medicago*. *Medicago* includes more than 80 described species, including perennials and annuals (Small and Jomphe, 1989; Steele et al., 2010). Alfalfa is a perennial legume species composed of several subspecies of the same karyotype with the ability to cross with each other, including subsp. *sativa*, subsp. *falcata*, subsp. *coerulea*, and subsp. *glomerata* (Chandra et al., 2011; Quiros and Bauchan, 1988; Small and Jomphe, 1989; USDA-ARS, 2007). In addition, the crossbreeds produced by crossing of the above mentioned subspecies, including subsp. *varia*, subsp. *hemicycla,* and subsp. *tunetana* are also

classified into *Medicago sativa* L. (Quiros and Bauchan, 1988). The subspecies are classified according to ploidy level, flower color, and shape of seedpod. Most alfalfa cultivated worldwide is the tetraploid (2n=4x=32) *M. sativa* L. subsp. *sativa,* which produces predominantly violet flowers and coiled seedpods. The tetraploid *M. sativa* L. subsp. *falcata* L., which produces yellow flowers and straight or sickle-shaped seedpods, has been mostly used for selective breeding with *M. sativa* L. subsp. *sativa* to improve cold-tolerance, drought tolerance, and disease resistance (Quiros and Bauchan, 1988). Modern alfalfa cultivars, especially those adapted to temperate growing zones, contain germplasm originally derived from both subspecies *sativa* and *falcata*.

Early alfalfa breeding efforts were dedicated to collecting, evaluating, and comparing various sources of germplasm around the world. A key milestone in advancing alfalfa breeding and development was reached in the early 20th century with the development of a system for classifying alfalfa germplasm into several distinct fall dormancy groups and selection of more winterhardy types within each group (Melton et al., 1988). This system made it possible to separate fall dormancy from winter hardiness. In recent years, a better understanding of autotetraploid genetics and its effects on breeding and variety synthesis has improved genetic gains for forage yield (Rumbaugh et al., 1988). Commercial alfalfa breeding programs focus on developing varieties with improved characteristics in several major areas, including: 1) greater resistance to insects, nematodes, and diseases; 2) greater yield potential; 3) improved stand persistence, and 4) increased forage quality (Putnam et al., 2008a; Undersander et al., 2011).

Cultivated alfalfa 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 per season, depending on the region and the system of harvest management. In certain regions, alfalfa 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).

Alfalfa is among the most important forage crops in the United States and ranks as the fourth most widely grown crop by acreage, after corn, soybean, and wheat. Approximately 20 million acres of alfalfa and alfalfa-grass mixtures were grown for hay in 2010 across most states, with the highest acreages harvested in California, Colorado, Idaho, Iowa, Michigan, Minnesota, Montana, Nebraska, North Dakota, South Dakota, and Wisconsin (USDA-NASS, 2012a). Alfalfa hay production in 2010 totaled 67,971,000 tons and was valued at approximately $8 billion USD, ranking fourth overall among agriculture crops in terms of total value (USDA-NASS, 2012b).

A small amount of conventional alfalfa is consumed by humans in the United States, predominately in the form of sprouts, but also as dietary supplements and herbal teas. KK179 does not present any concerns with respect to human consumption; however, its intended commercial use will be 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 seed to be used for sprout production (Monsanto Company, 2012), which 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.

II.B. Characteristics of the Recipient Plant

The alfalfa plant used as the recipient of the DNA insertion to create KK179 was R2336, a conventional FGI proprietary plant (propagated vegetatively via stem cuttings), and selected for ease of transformation from an elite, high-yielding breeding population. A single, transformed R2336 alfalfa plant (T_0) was crossed with Ms208, a conventional male-sterile alfalfa plant (propogated vegetatively via stem cuttings) to produce F_1 progeny plants. A single plant (P_0) was selected from these progeny plants (Figure IV-11) and used for molecular characterization of the F_1 generation, as described in Section IV.

Due to inbreeding depression and self-incompatibility in an outcrossing species like alfalfa, 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 alfalfa varieties (Figure IV-11). The P_0 plant, containing KK179, was hand-crossed with each of 10 elite alfalfa 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 alfalfa 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 I.B.2.2., and composition of KK179, described in Section VI.B., 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 hand-crosses were conducted in a greenhouse.

II.C. Alfalfa as a Test System in Product Safety Assessment

The identical breeding process described above was followed using the C_0 plant in order to produce a C_0-Syn1 generation, which is a conventional synthetic population, to serve as the conventional comparator for the Syn1 generation, respectively. A single, non-transformed R2336 plant was crossed with Ms208 to produce conventional F_1 progeny plants, from which a conventional alfalfa comparator C_0 was selected. The same breeding process used to develop subsequent generations of KK179 was then followed to develop subsequent generations of near-isogenic conventional alfalfa comparators for each generation of KK179 as shown in Figure IV-11. As a result, all generations of

KK179 and the conventional controls used in the safety assessment studies have closely related genetic backgrounds, with the exception of the intended trait for reduced G lignin and total lignin. The C_0 and C_0-Syn1 plants are both referred to as the conventional control. R2336 and Ms208 are referred to as conventional parental controls.

In addition, conventional commercial alfalfa varieties (referred to as conventional commercial reference varieties), were used to establish ranges of natural variability representative of commercial alfalfa varieties. The conventional commercial reference varieties used at each location were chosen based on their availability and agronomic adaptation for the respective geographic region.

III. DESCRIPTION OF THE GENETIC MODIFICATION

Molecular analyses are an integral part of the characterization of crop products with new traits introduced by methods of biotechnology. Vectors and methods are selected for transformation to achieve high probability of obtaining the trait of interest and integration of the introduced DNA into a single locus in the plant genome. This helps ensure that only the intended DNA encoding the desired trait is integrated into the plant genome and facilitates the molecular characterization of the product. Information provided here allows for the identification of the genetic material present in the transformation vector delivered to the host plant and for an analysis of the data supporting the characterization of the DNA inserted in the plant found in Section IV.

KK179 was developed through *Agrobacterium tumefaciens*-mediated transformation of conventional alfalfa R2336 leaf tissue utilizing plasmid vector PV-MSPQ12633. This section describes the plasmid vector, the donor genes, and the regulatory elements used in the development of KK179. In this section, transfer DNA (T-DNA) refers to DNA that is transferred to the plant during transformation. The suppression cassette refers to the sequences and regulatory elements necessary for the suppression of the endogenous *CCOMT* RNA transcript. An expression cassette refers to the sequences and regulatory elements necessary for the expression of those sequences.

III.A. The Plasmid Vector PV-MSPQ12633

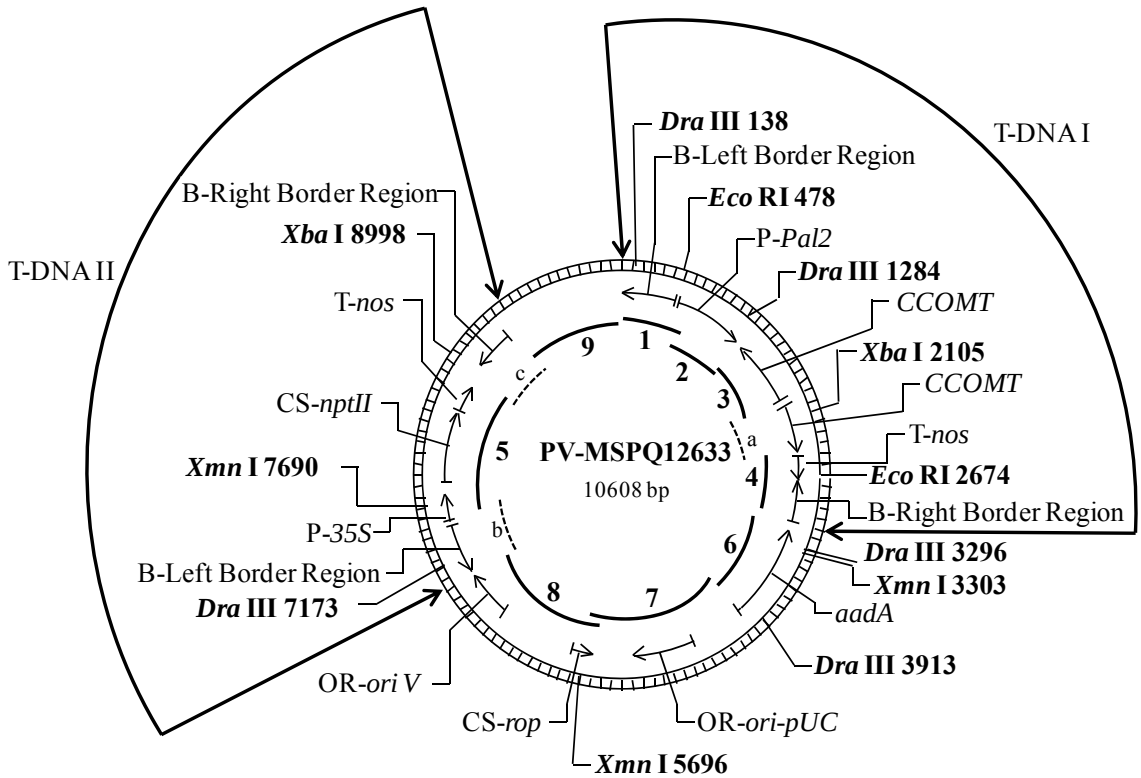
PV-MSPQ12633 was used for the transformation of conventional alfalfa to produce KK179 and is shown in Figure III-1. PV-MSPQ12633 is approximately 10.6 kb in length and contains two T-DNAs, each delineated by Left and Right Border regions to facilitate transformation. The first T-DNA, designated as T-DNA I, contains the *CCOMT* suppression cassette, which is regulated by the *Pal2* promoter and the *nos* 3′ UTR. The second T-DNA, designated as T-DNA II, contains the *nptII* expression cassette, which is regulated by the *35S* promoter and the nos 3′ UTR. During transformation, both T-DNAs were inserted into the alfalfa genome (Section IV.B.) 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 III-1.

III.B. Description of the Transformation System

KK179 was developed through *Agrobacterium*-mediated transformation of alfalfa, based on the published method (Schenk and Hildebrandt, 1972; Walker and Sato, 1981) and allows for the generation of transformed plants. Briefly, alfalfa R2336 leaf cuttings were placed in a tissue culture media and co-cultured with *Agrobacterium* carrying the plasmid vector. R2336 is an FGI proprietary single alfalfa plant, selected for regenerability from an elite, high yielding, fall dormant alfalfa 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 developed with somatic embryos. The 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 with 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 III-2. The result of this process was the production of marker-free alfalfa KK179.



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 III-1. 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.

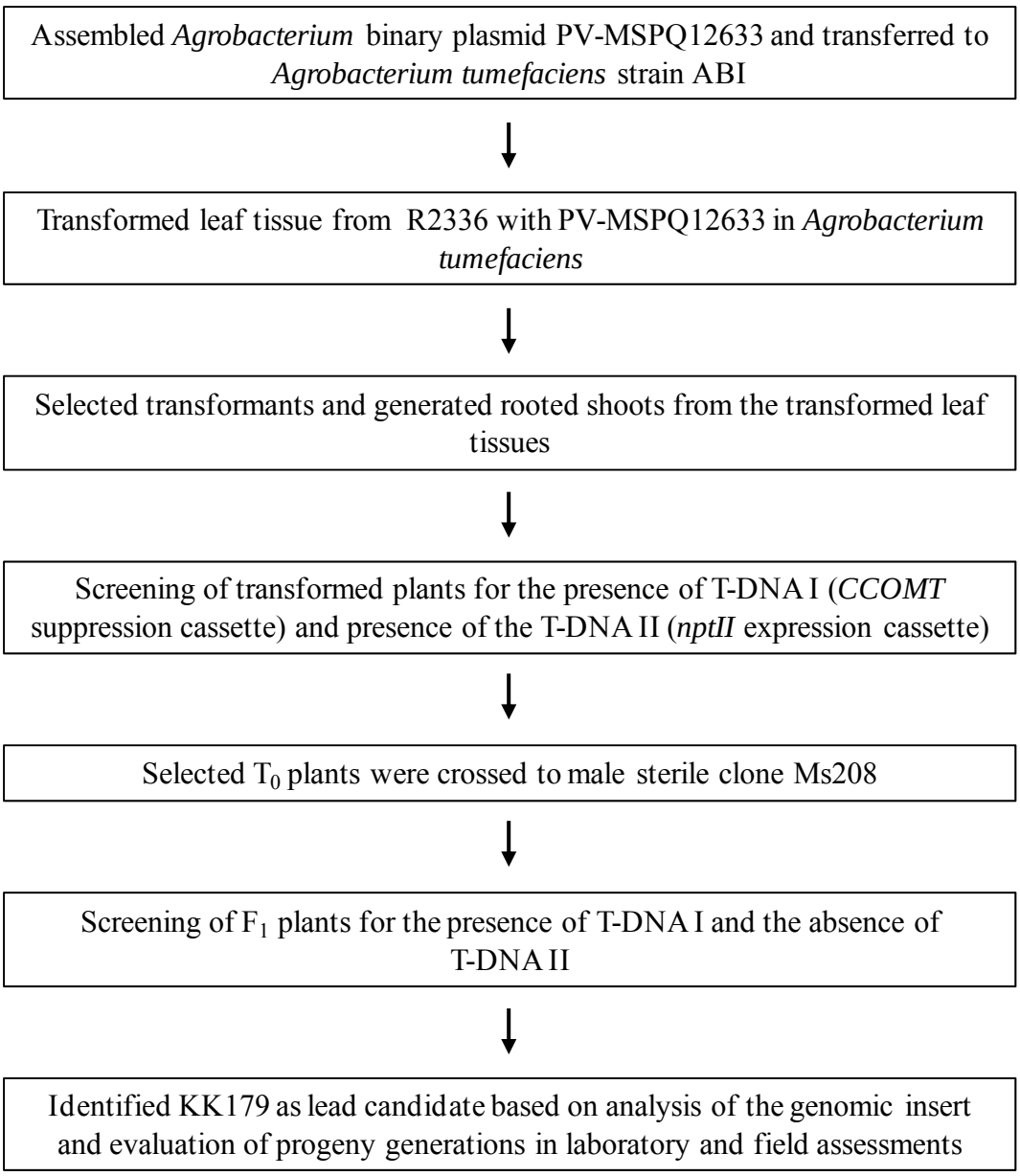


Figure III-2. Schematic of the Development of KK179

III.C. 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 is *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.

III.D. The *nptII* Coding Sequence and nptII 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). The 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.

III.E. 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.

III.F. T-DNA Border Regions

PV-MSPQ12633 contains Left and Right Border regions (Figure III-1 and Table III-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 alfalfa genome. Because PV-MSPQ12633 is a 2-T-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.

III.G. 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 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 plasmid vector copy number in *E. coli* (Giza and Huang, 1989). The selectable marker *aadA* is a bacterial promoter and coding sequence for an enzyme 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 alfalfa genome.

Table III-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 III-1 A (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 III-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 Tn5 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 2410-2111.

IV. CHARACTERIZATION OF THE GENETIC MODIFICATION

This section contains a comprehensive molecular characterization of the genetic modification present in KK179. It provides information on the DNA insertion into the plant genome of KK179, and additional information relative to the arrangement and stability of the introduced genetic material. The information provided in this section addresses the relevant factors in Codex Plant Guidelines, Section 4, paragraphs 30, 31, 32, and 33 (Codex Alimentarius, 2009).

A multi-faceted approach was taken to characterize the genetic modification that produced KK179. The results confirmed that KK179 contains a single copy of the *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 alfalfa 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 alfalfa 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 that T-DNA I resides at a single locus and is inherited according to Mendelian principles of inheritance. Taken together, the characterization of the genetic modification demonstrates that a single copy of T-DNA I was inserted at a single locus of the alfalfa genome and that no plasmid vector backbone sequences are present in KK179.

Southern blot analyses were used to determine the number of copies, to characterize 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. 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 characterize 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 hybridization pattern. Additionally, this two-enzyme set design also maximizes 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 IV-2 through IV-8). 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 alfalfa 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 organization of the elements within the insert, and determined the 5′ and 3′ insert-to-plant junctions (Figure IV-9 and Figure IV-10). 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 organization 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 alfalfa.

The stability of the T-DNA I present in KK179 across multiple generations was demonstrated by Southern blot fingerprint analysis. Genomic DNA from four generations (P_0, MBC1, MBC2, and Syn1) of KK179 (Figure IV-11) was digested with one of the enzyme sets used for the insert and copy number analysis and was hybridized with a probe that detects restriction segments that encompass the entire T-DNA I insert (Figure IV-1). 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 IV-12).

Segregation analysis showed that heritability and stability of the insert occurred as expected across multiple generations (Figure IV-13, Table IV-3), 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 III-1. A linear map depicting restriction sites within the insert, as well as the DNA flanking the insert in KK179 is shown in Figure IV-1. Based on the plasmid map and the linear map of the insert, a table summarizing the expected DNA segments for Southern analyses is presented in Table IV-1. The genetic elements within the KK179 insert are summarized in Table IV-2. The results from the Southern blot analyses are presented in Figure IV-2 through Figure IV-8. PCR amplification of the KK179 insert and the insertion site in conventional control for DNA sequence analysis is shown in Figure IV-9 and Figure IV-10, respectively. The generations used in the generational stability analysis are depicted in the breeding history shown in Figure IV-11 and the results from the generational stability analysis are presented in Figure IV-12. The breeding path for the segregation data is shown in Figure IV-13 and the results for the segregation analysis are presented in Table IV-3. Materials and methods used for the characterization of the insert in KK179 are found in Appendix B.

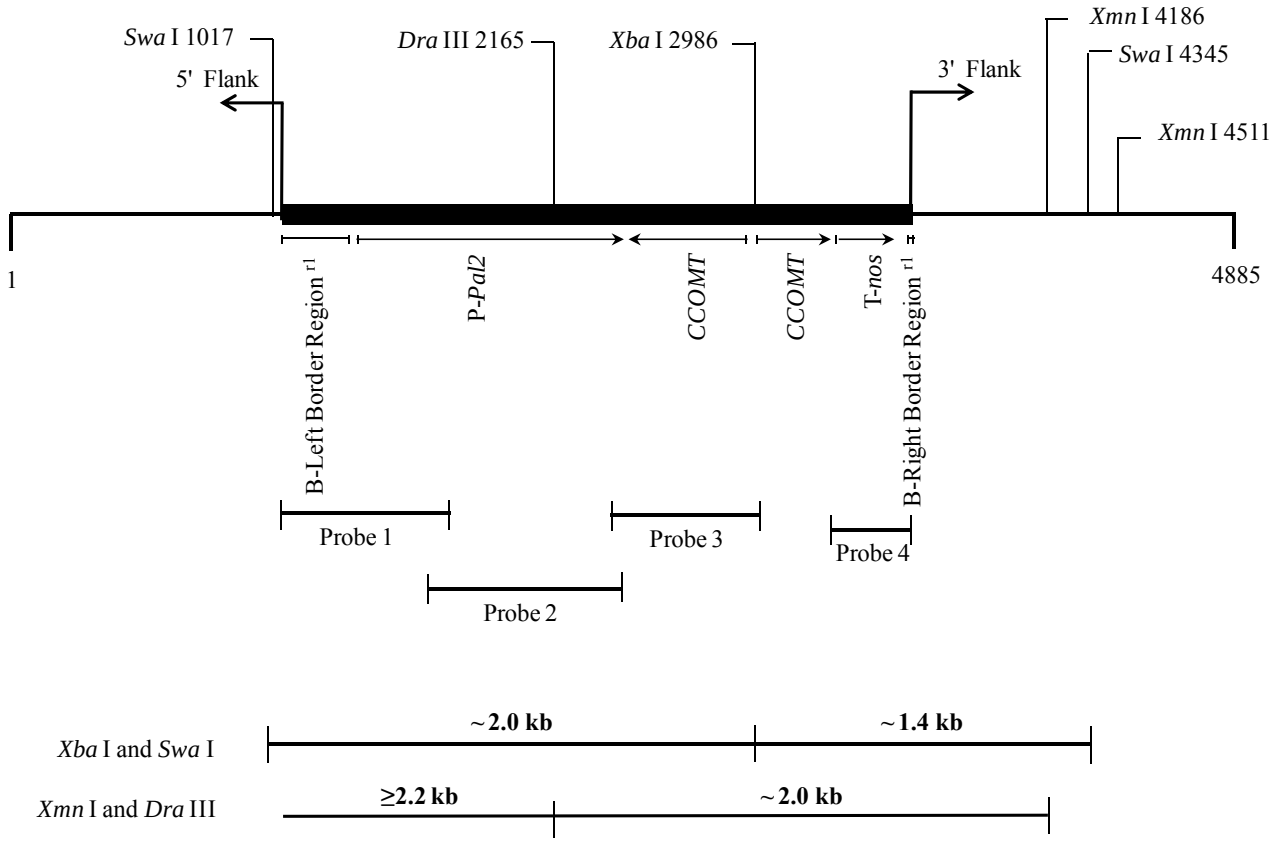


Figure IV-1. Schematic Representation of the Insert and Flanking DNA in KK179

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 IV-1.

Table IV-1. Summary Chart of the Expected DNA Segments Based on Hybridizing Probes and Restriction Enzymes Used in KK179 Analysis

Southern Blot Analysis		**T-DNA I**				**T-DNA II**	**Backbone**	
Figure		**V-2**	**V-3**	**V-4**	**V-5**	**V-6**	**V-7**	**V-8**
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 hybridization controls in Southern blot analyses when multiple probes were hybridized to the blot simultaneously

[2] '~~' indicates that this digest was not performed.

[3] '--' indicates that probe templates were not used.

[4] Not Applicable.

Table IV-2. 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 3290-2991.

IV.A. 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 P_0 generation of KK179 and the appropriate control genomic DNA samples with two sets of restriction enzymes: a combination of *Xmn* I and *Dra* III, a combination of *Xba* I and *Swa* I, and hybridized Southern blots with probes that span the T-DNA I (Figure III-1). Each restriction digest is expected to produce a specific banding pattern on the Southern blots (Table IV-1). 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 IV-1). 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 IV-1 and Table IV-1). 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 IV-1). 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 IV-1 and Table IV-1).

The Southern blots were hybridized with probes spanning the entire T-DNA I sequence (Figure III-1, 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. Alfalfa is an autotetraploid (Yang et al., 2009) and, therefore, contains multiple copies of each endogenous gene, which are randomly segregating. Southern blots hybridized with sequences specific to the *CCOMT* gene are expected to have different hybridization 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 hybridization controls. The results of this analysis are shown in Figures IV-2 through IV-8.

IV.A.1. Probe 1

Conventional control genomic DNA digested with a combination of *Xmn* I and *Dra* III (Figure IV-2, Lane 8) or with a combination of *Xba* I and *Swa* I (Figure IV-2, Lane 3 and Lane 10) and hybridized with Probe 1 (Figure III-1) showed no detectable hybridization 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 IV-2, 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 hybridized to its target sequences.

KK179 genomic DNA digested with a combination of *Xmn* I and *Dra* III and hybridized with Probe 1 (Figure III-1) produced a band at ~4.5 kb (Figure IV-2, Lane 2 and Lane 9). The ~4.5 kb band is the expected band, which represents 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 hybridized with Probe 1 produced the expected band at ~2.0 kb (Figure IV-2, Lane 4 and Lane 11). The ~2.0 kb band is the expected band, which represents the 5′ end of the inserted DNA and the adjacent DNA flanking the 5′ end of the insert. The results presented in Figure IV-2 indicate that the sequence covered by Probe 1 resides as one copy at a single detectable locus of integration in KK179.

IV.A.2. Probe 2 and Probe 4

Conventional control genomic DNA digested with a combination of *Xmn* I and *Dra* III (Figure IV-3, Lane 1 and Lane 8) or with a combination of *Xba* I and *Swa* I (Figure IV-3, Lane 3 and Lane 10) and hybridized with Probe 2 and Probe 4 (Figure III-1) showed no detectable hybridization 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 IV-3, 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 IV-1, Probe 2 and Probe 4) produced the expected bands at ~0.8 kb and ~0.7 kb, respectively (Figure IV-3, Lane 6 and Lane 7). Detection of the spiked controls indicates that the probes hybridized to their target sequences.

KK179 genomic DNA digested with a combination of *Xmn* I and *Dra* III and hybridized with Probe 2 and Probe 4 (Figure III-1) produced two bands at ~4.5 kb and ~2.0 kb (Figure IV-3, 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 hybridized with Probe 2 and Probe 4 (Figure III-1) produced two expected bands at ~2.0 kb and ~1.4 kb (Figure IV-3, Lane 4 and Lane 11). The ~2.0 kb band is the expected band, which represents 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, which represents the 3′ end of the inserted DNA and the adjacent DNA flanking the 3′ end of the insert. The results presented in Figure IV-3 indicate that the sequence covered by Probe 2 and Probe 4 resides as one copy at a single detectable locus of integration in KK179.

IV.A.3. Probe 3

Alfalfa is an autotetraploid (Yang et al., 2009) and, therefore, 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 alfalfa genome. Therefore, the random segregation of the endogenous *CCOMT* in the alfalfa genome is expected to lead to different hybridization 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 hybridization 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 hybridization 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 IV-4) or with a combination of *Xmn* I and *Dra* III (Figure IV-5) and probed with Probe 3 (Figure IV-1). As expected, different hybridization bands were present in the conventional control and conventional parental controls. All observed bands in the conventional and conventional parental controls represent hybridization with the endogenous *CCOMT* gene in the alfalfa genome.

Conventional control genomic DNA digested with *Xba* I and *Swa* I (Figure IV-4, Lane 1 and Lane 8) and hybridized with Probe 3 (Figure IV-1) displayed hybridization bands at ~7.9 kb and ~11.0 kb. Conventional parental control R2336 genomic DNA digested with *Xba* I and *Swa* I (Figure IV-4, Lane 2 and Lane 9) and hybridized with Probe 3 (Figure 1) displayed hybridization bands at ~7.9 kb and ~10.0 kb. Conventional parental control Ms208 genomic DNA digested with *Xba* I and *Swa* I (Figure IV-4, Lane 3 and Lane 10) and hybridized with Probe 3 (Figure III-1) displayed hybridization 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 hybridization bands detected in the conventional 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 IV-4, Lane 6 and Lane 7) in addition to the endogenous hybridization bands at ~7.9 kb and ~11.0 kb. Detection of the positive control indicates that the probe hybridized to its target sequences.

KK179 genomic DNA digested with *Xba* I and *Swa* I (Figure IV-4, Lane 4 and Lane 11) and hybridized with Probe 3 (Figure III-1) 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 hybridization as these bands have also been observed in either the R2336 or Ms208 conventional parental controls (Figure IV-4, Lanes 2, 3, 9, and 10). The ~1.4 kb and ~2.0 kb bands are the expected hybridization bands (Table IV-1) 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 IV-5, Lane 1 and Lane 8) and hybridized with Probe 3 (Figure III-1) displayed hybridization 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 IV-5, Lane 2 and Lane 9) and hybridized with Probe 3 (Figure III-1) displayed the hybridization 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 IV-5, Lane 3 and Lane 10) and hybridized with Probe 3 (Figure III-1) displayed the hybridization 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 hybridization 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 IV-5, Lane 6 and Lane 7) in addition to the endogenous hybridization 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 hybridized to its target sequences.

KK179 genomic DNA digested with *Xmn* I and *Dra* III (Figure IV-5, Lane 4 and Lane 11) and hybridized with Probe 3 (Figure III-1) 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 IV-5, 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 IV-4 and Figure IV-5 indicates that the sequence covered by Probe 3 resides as one copy at a single detectable locus of integration in KK179.

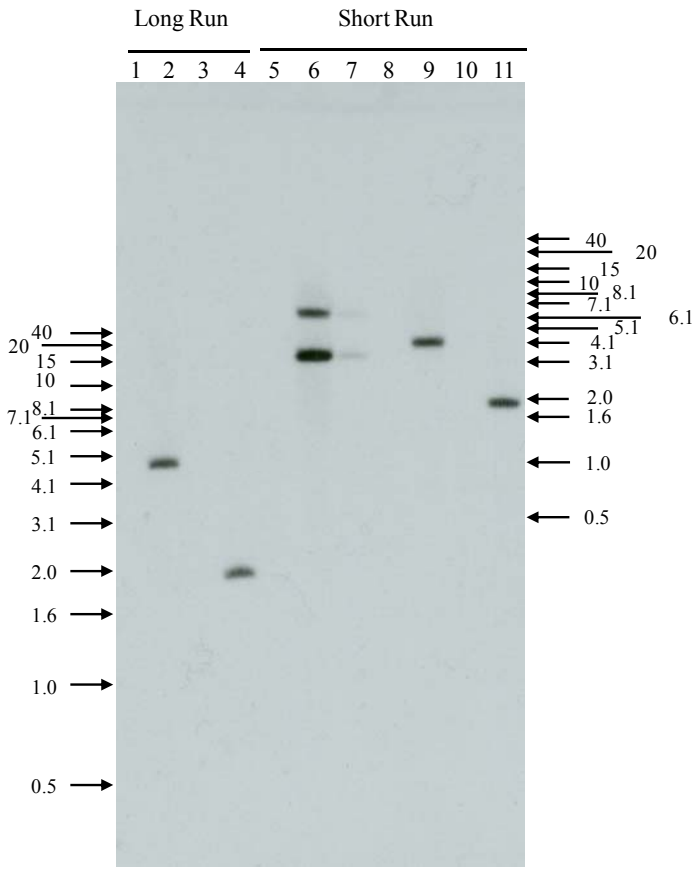


Figure IV-2. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA I in KK179: Probe 1

The blot was hybridized with one ^{32}P-labeled probe that spanned a portion of the T-DNA I sequence (Figure IV-1, Probe 1). 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.

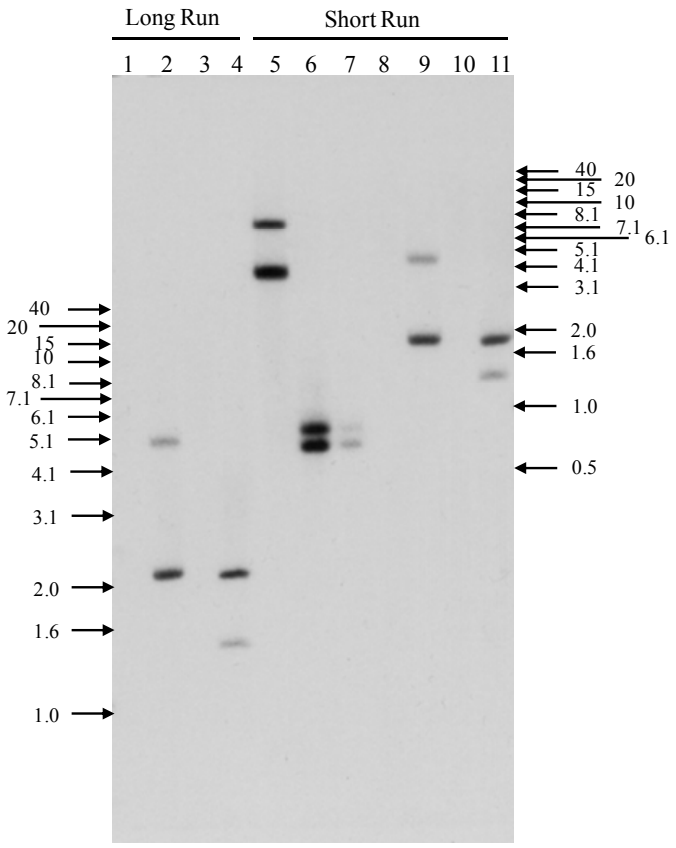


Figure IV-3. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA I in KK179: Probe 2 and Probe 4

The blot was hybridized with two ^{32}P-labeled probes that spanned a portion of the T-DNA I sequence (Figure IV-1, 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.

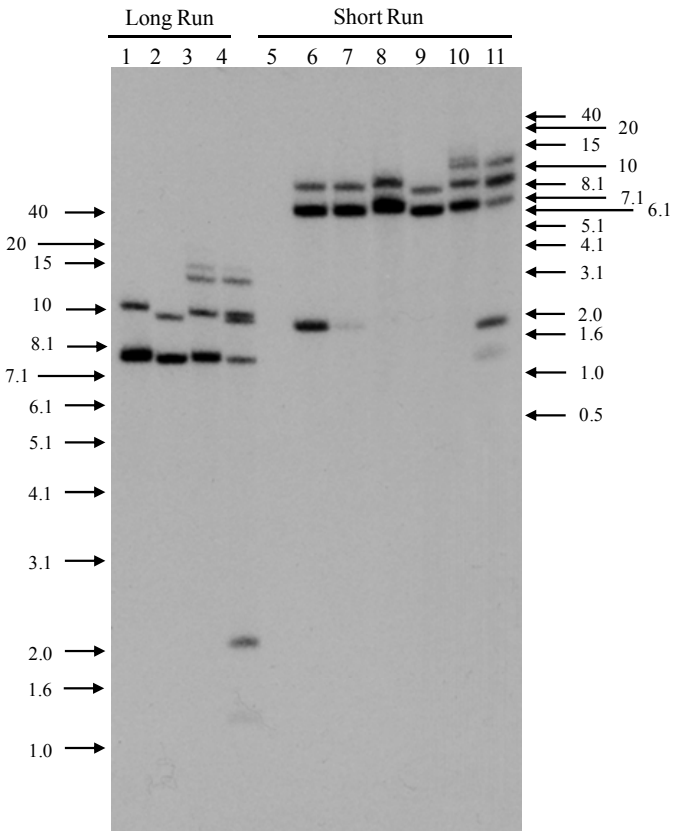


Figure IV-4. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA I in KK179: Probe 3

The blot was hybridized with one ^{32}P-labeled probe that spanned portions of the T-DNA I sequence (Figure IV-1, 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.

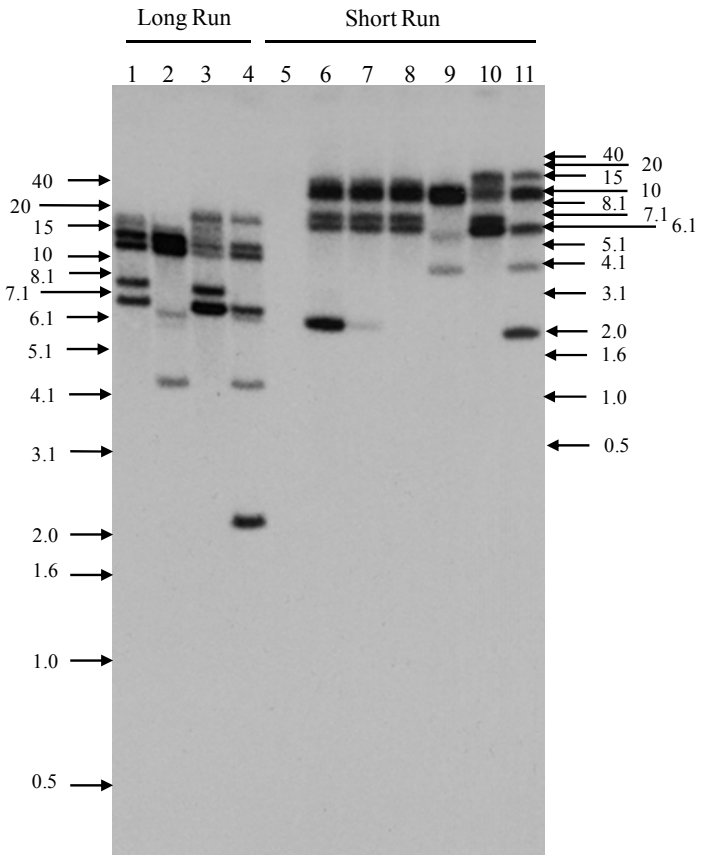


Figure IV-5. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA I in KK179: Probe 3

The blot was hybridized with one ^{32}P-labeled probe that spanned portions of the T-DNA I sequence (Figure IV-1, 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.

IV.B. 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 alfalfa 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 hybridized with a probe spanning the T-DNA II sequence, except for the border regions and some of the intervening sequences (Figure III-1, 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, hybridization 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 hybridization with Probe 5 would result in the detection of unique hybridization 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 IV-6.

IV.B.1. Probe 5

Conventional control genomic DNA digested with combination of *Xmn* I and *Dra* III (Figure IV-6, Lane 1 and Lane 8) or a combination of *Xba* I and *Swa* I (Figure IV-6, Lane 3 and Lane 10) and hybridized with Probe 5 (Figure III-1) showed no detectable hybridization 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 IV-6, Lane 6 and Lane 7). Detection of the spiked controls indicates that the probe hybridized to its target sequences.

KK179 genomic DNA digested with *Xmn* I and *Dra* III (Figure IV-6, Lane 2 and Lane 9) and hybridized with Probe 5 (Figure IV-1) 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 IV-6, Lane 4 and Lane 11) and hybridized with Probe 5 (Figure III-1) 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 hybridization 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.

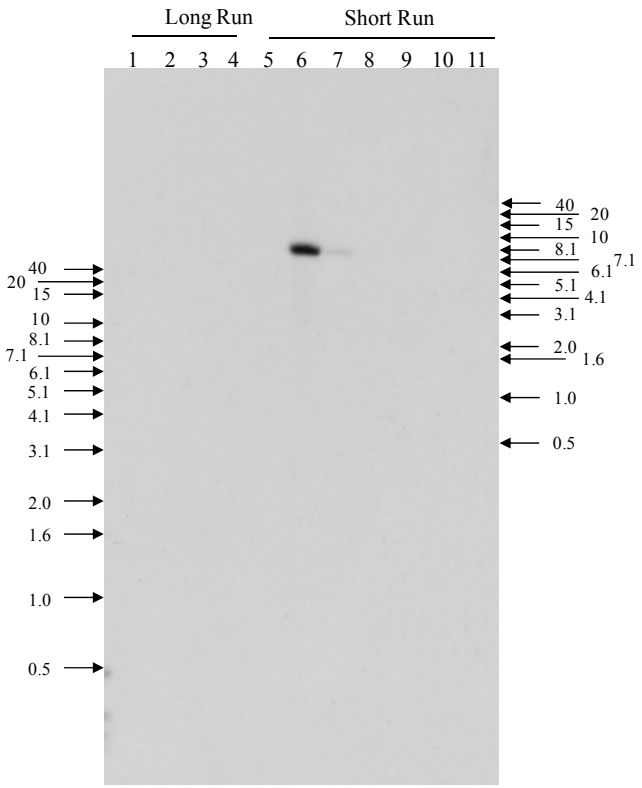


Figure IV-6. Southern Blot Analysis to Detect the Presence or Absence of T-DNA II Sequences in KK179: Probe 5

The blot was hybridized with one ^{32}P-labeled probe that spanned a portion of the T-DNA II sequence (Figure IV-1, 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.

IV.C. 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 alfalfa 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 hybridized with overlapping probes spanning the backbone sequence of PV-MSPQ12633 (Figure III-1, Probes 6, 7, 8, and 9). If backbone DNA sequences were present in KK179, then hybridizing with overlapping probes corresponding to the backbone sequence should result in the detection of hybridization bands on the Southern blot. The results of this analysis are shown in Figures IV-7 and IV-8.

IV.C.1. Backbone Probe 6 and Probe 8

Conventional control genomic DNA digested with a combination of *Xmn* I and *Dra* III (Figure IV-7, Lane 1 and Lane 8) or with a combination of *Xba* I and *Swa* I (Figure IV-7, Lane 3 and Lane 10) and hybridized with Probe 6 and Probe 8 (Figure IV-1) showed no detectable hybridization 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 IV-7, Lane 5). The ~6.9 kb band was detected because Probe 6 and Probe 8 hybridized 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 III-1).

Conventional control genomic DNA digested with a combination of *Xmn* I and *Dra* III and spiked with probe templates (Figure III-1, Probe 6 and Probe 8) generated from PV-MSPQ12633 produced the expected bands at ~1.1 kb and ~1.4 kb, respectively (Figure IV-7, Lane 6 and Lane 7). Detection of the spiked controls indicates that the probes hybridized to their target sequences.

KK179 genomic DNA digested with a combination of *Xmn* I and *Dra* III (Figure IV-7, Lane 2 and Lane 9) or with a combination of *Xba* I and *Swa* I (Figure IV-7, Lane 4 and Lane 11) and hybridized with Probe 6 and Probe 8 (Figure IV-1) 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.

IV.C.2. Backbone Probe 7 and Probe 9

Conventional control genomic DNA digested with a combination of *Xmn* I and *Dra* III (Figure IV-8, Lane 1 and Lane 8) or with a combination of *Xba* I and *Swa* I (Figure IV-8, Lane 3 and Lane 10) and hybridized with Probe 7 and Probe 9 (Figure III-1) showed no detectable hybridization 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 IV-8, 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 III-1, Probe 7 and Probe 9) produced the expected bands at ~1.6 kb and ~1.2 kb (Figure IV-8, Lane 6 and Lane 7). Detection of the spiked controls indicates that the probes hybridized to their target sequences.

KK179 genomic DNA digested with a combination of *Xmn* I and *Dra* III (Figure IV-8, Lane 2 and Lane 9) or with a combination of *Xba* I and *Swa* I (Figure IV-8, Lane 4 and Lane 11) and hybridized 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.

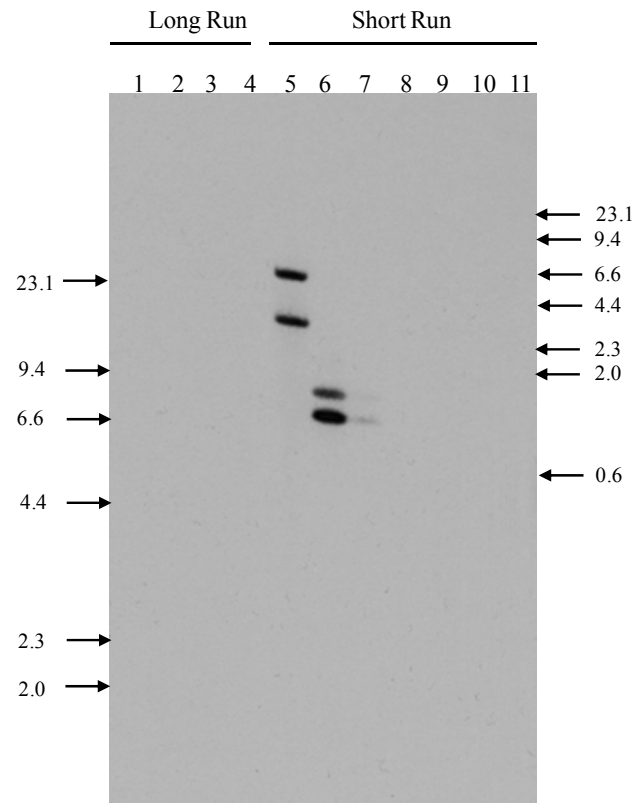


Figure IV-7. Southern Blot Analysis to Determine the Presence or Absence of PV-MSPQ12633 Backbone Sequences in KK179: Probe 6 and Probe 8

The blot was hybridized with two ^{32}P-labeled probes that spanned a portion of the PV-MSPQ12633 backbone sequence (Figure IV-1, 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.

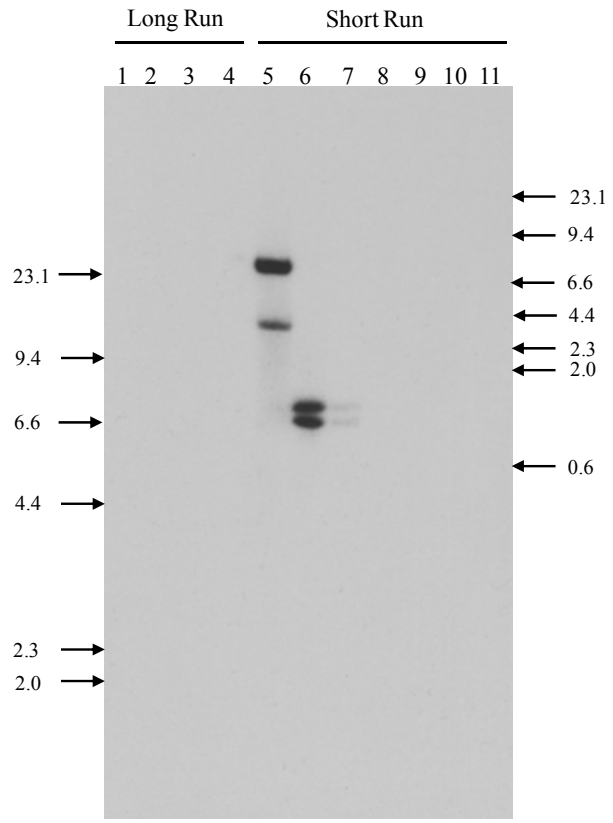


Figure IV-8. Southern Blot Analysis to Determine the Presence or Absence of PV-MSPQ12633 Backbone Sequences in KK179: Probe 7 and Probe 9

The blot was hybridized with two ^{32}P-labeled probes that spanned portions of PV-MSPQ12633 backbone sequence (Figure IV-1, 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 [~1.0 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.

IV.D. Organization and Sequence of the Insert and Adjacent Genomic DNA in KK179

PCR and sequence analyses were performed on genomic DNA extracted from KK179 and the conventional parental control R2336 to examine the organization 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 IV-9). 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 IV-2) and is identical to the corresponding T-DNA I sequence of PV-MSPQ12633 as described in Table III-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 IV-2) were also determined.

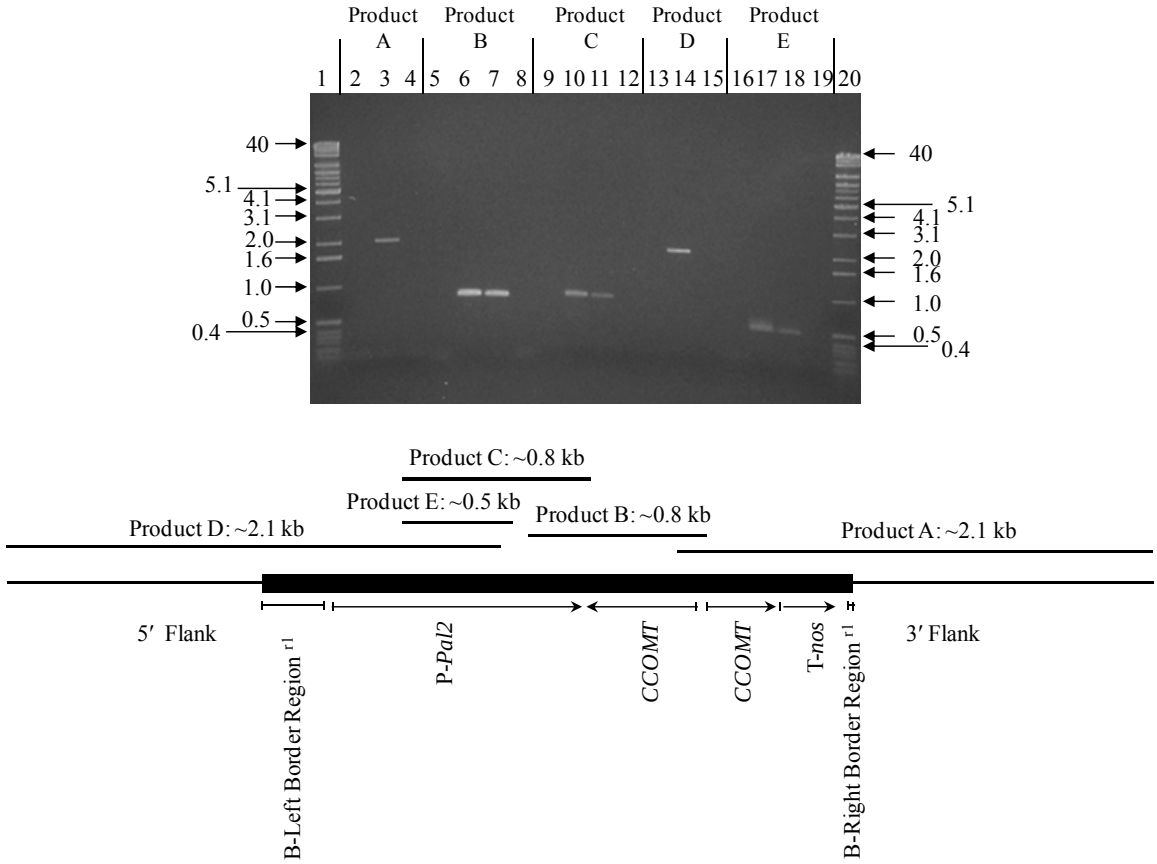


Figure IV-9. Overlapping PCR Analysis across the Insert in KK179

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 were 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.

IV.E. 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 IV-10). The amplified PCR product from the conventional parental control was subjected to DNA sequence analysis. 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 alfalfa population (Yang et al., 2009). The alignment analyses also indicated a deletion of 102 base pairs 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).

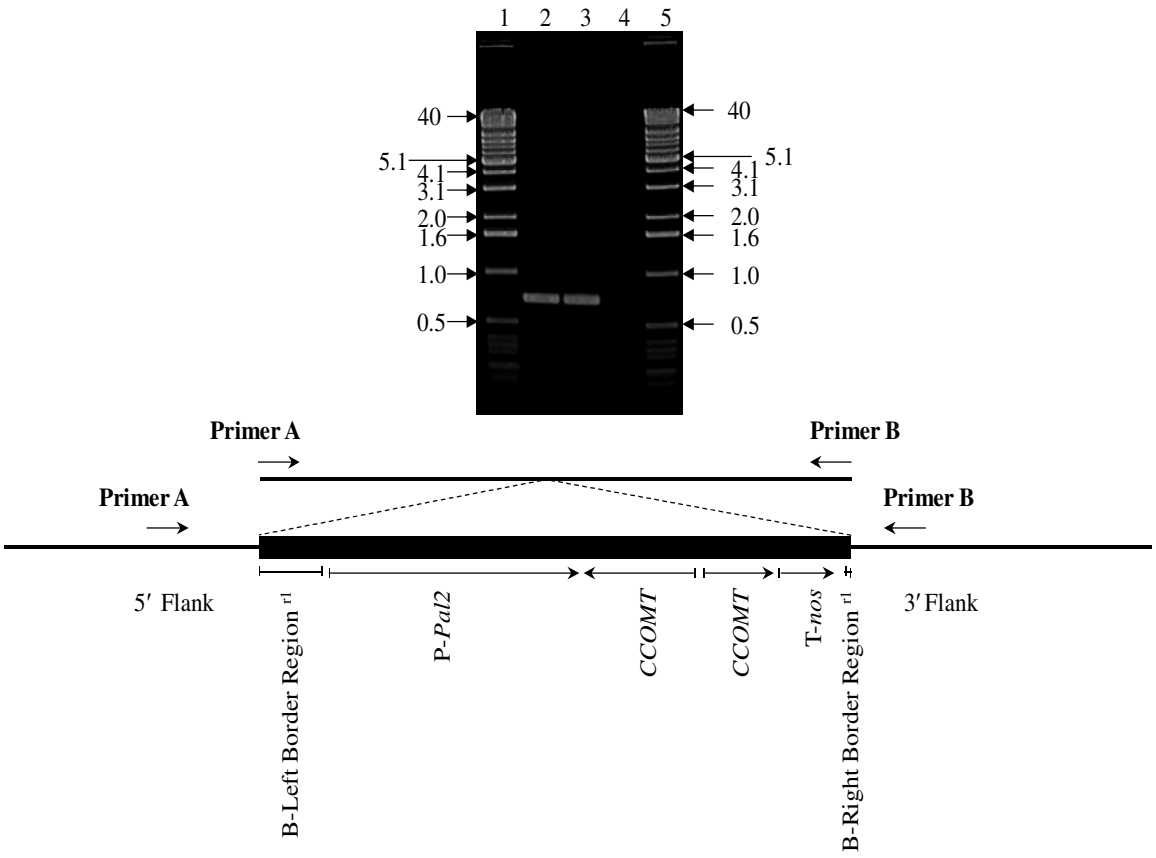


Figure IV-10. PCR Amplification of the KK179 Insertion Site in Conventional Alfalfa

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.

IV.F. 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 IV-11). Genomic DNA isolated from each of the selected generations of KK179 was digested with the restriction enzymes *Xmn* I and *Dra* III (Figure IV-12) and hybridized with Probe 2 and Probe 4 (Figure III-1). 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 IV-12.

IV.F.1. Probe 2 and Probe 4

Conventional control C_0 genomic DNA digested with *Xmn* I and *Dra* III (Figure IV-12, Lane 1) and hybridized with Probe 2 and Probe 4 (Figure III-1) showed no detectable hybridization 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 IV-12, 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 III-1, Probe 2 and Probe 4) produced the expected bands at ~0.8 kb and ~0.7 kb, respectively (Figure IV-12, Lane 3 and Lane 4). An additional faint ~1.4 kb band in the probe templates control lane (Figure IV-12, 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 hybridized to their target sequences.

KK179 DNA extracted from four generations (P_0, MBC1, MBC2, and Syn1), digested with *Xmn* I and *Dra* III (Figure IV-12, Lanes 5, 6, 7, and 8), and hybridized with Probe 2 and Probe 4 (Figure IV-1) 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.

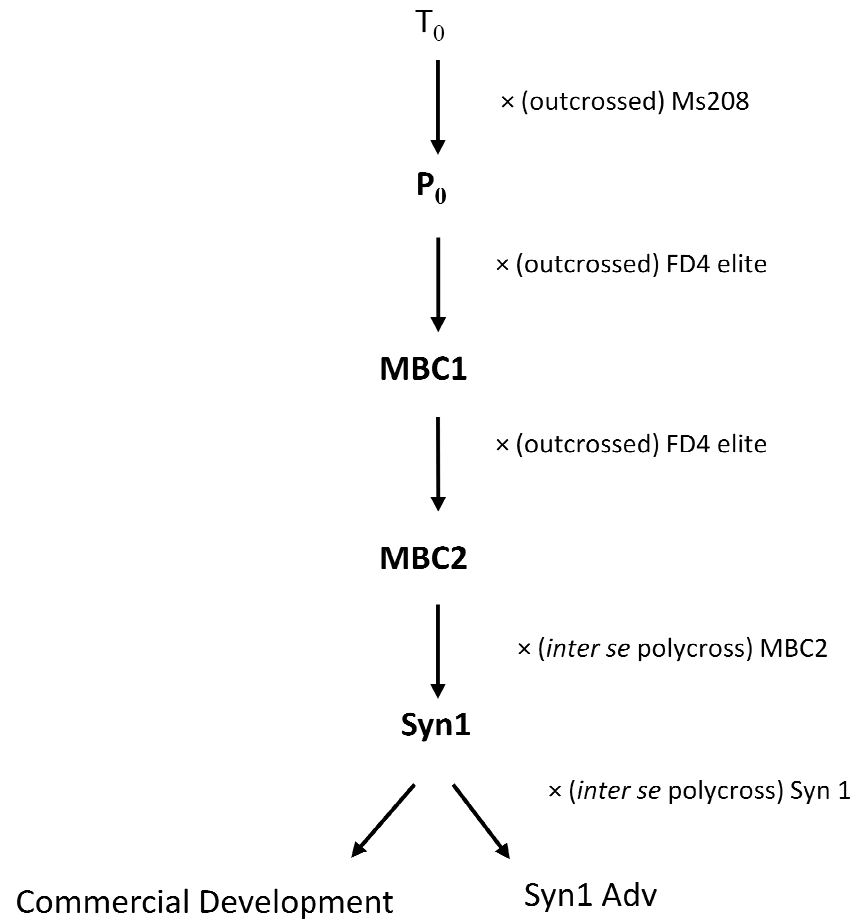


Figure IV-11. Breeding History of KK179

The P_0 generation was used for the molecular analyses of KK179 reported in Figures V-2 through V-8 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 IV-12. The Syn1 generation was used for intended changes to lignin studies described in Section I.B.3. and composition studies described in Section VI.B.

Alfalfa terminology

T_0:	the original transformed R2336 plant;
Ms208:	conventional male sterile alfalfa plant;
P_0:	the single KK179 plant selected from the progeny of T_0 × Ms208,
FD4:	10 elite alfalfa 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.

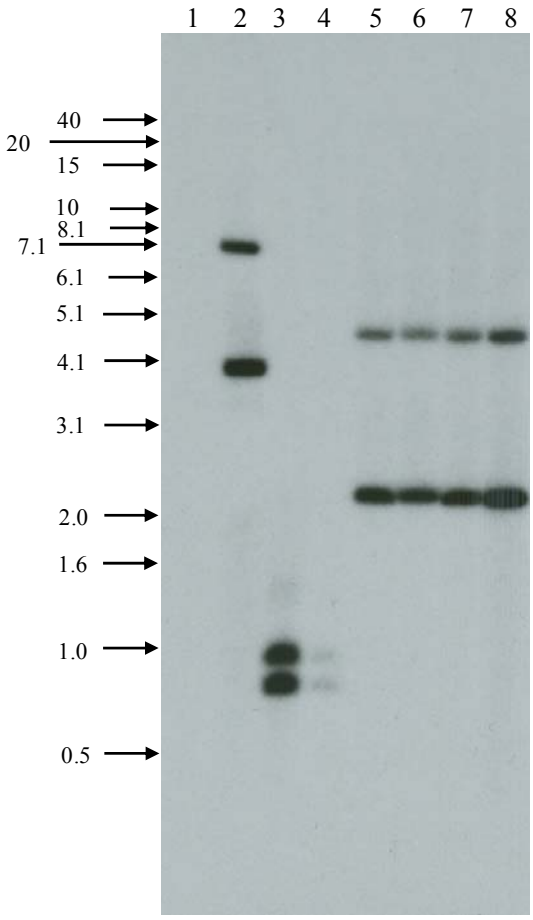


Figure IV-12. Southern Blot Analysis to Examine Insert Stability in Multiple Generations of KK179: Probe 2 and Probe 4

The blot was hybridized with two ^{32}P-labeled probes that spanned portions of the T-DNA sequence (Figure IV-1, 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 1 and Probe 2 [~1.0 genome equivalent]
4 Conventional Control (*Xmn* I and *Dra* III) spiked with Probe 1and Probe 2 [~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.

IV.G. 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 IV-13. The transformed T_0 plant was cross-pollinated to an elite male sterile alfalfa plant, Ms208, to produce F_1 seed. From the F_1 segregating population, an individual plant (designated as P_0) 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 showed 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 that showed 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 IV-13).

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; 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:

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

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 IV-3. The χ^2 values 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 alfalfa genome and is inherited according to Mendelian inheritance principles. These results are also consistent with the molecular characterization data that indicate KK179 contains a single, intact copy of the *CCOMT* suppression cassette that was inserted into the alfalfa genome at a single locus.

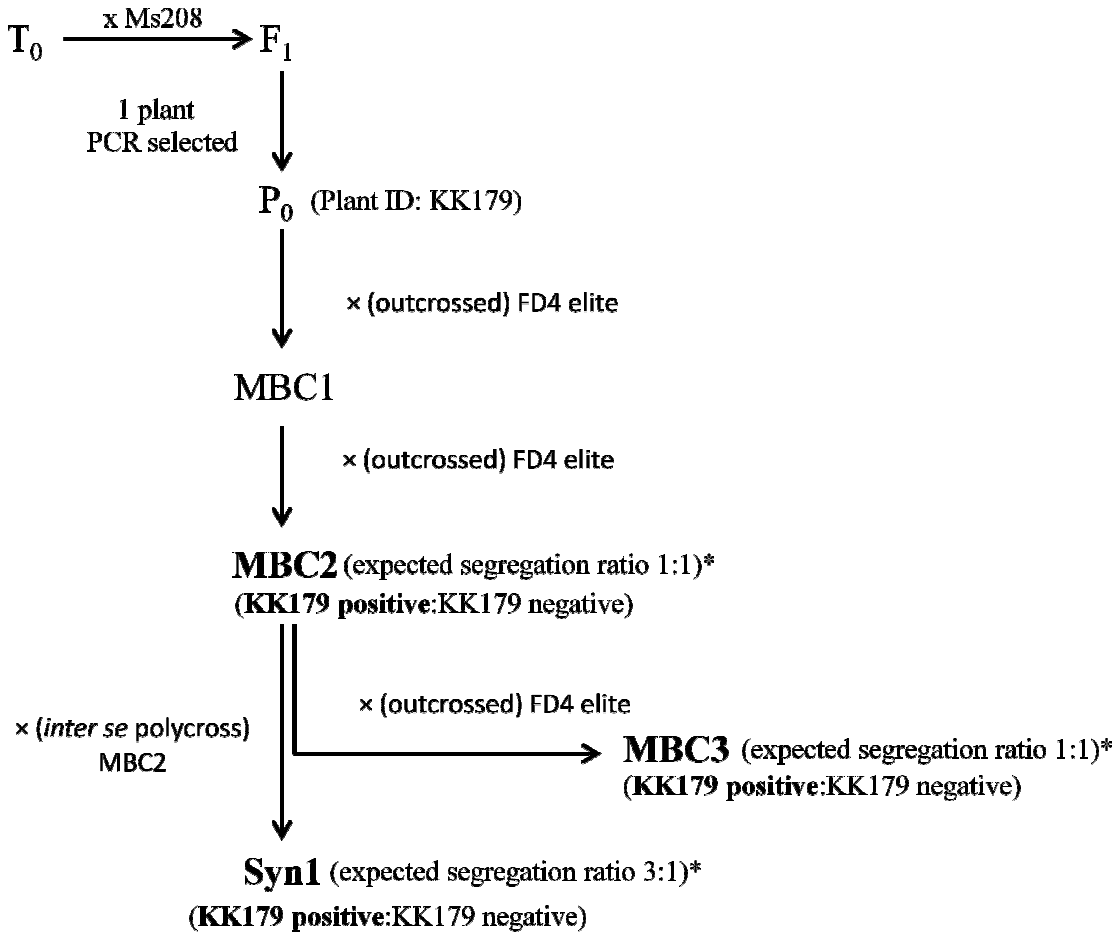


Figure IV-13. Breeding Path for Generating Segregation Data for KK179
The MBC2, MBC3, and Syn1 generations were used for analyzing the inheritance of the insert in multiple generations.

Alfalfa terminology

T_0:	the original transformed R2336 plant;
Ms208:	conventional male sterile alfalfa plant;
F1:	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 alfalfa 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 IV-3. Segregation of the KK179 Insert During Mendelian Inheritance Testing

				1:1 Segregation			
Generation	Total Plants1	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 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.

IV.H. Characterization of the Genetic Modification Summary and Conclusion

Molecular characterization of KK179 by Southern blot analyses confirmed that one copy of the *CCOMT* suppression cassette was integrated into the alfalfa genome at a single locus. No T-DNA II or backbone DNA sequence from plasmid vector PV-MSPQ12633 was detected in KK179.

PCR and DNA sequence analyses performed on KK179 and conventional parental control R2336 determined the following: the complete DNA sequence of the insert and adjacent DNA sequences in KK179; the organization of the genetic elements within the insert; the expected sequence of each element in the inserted DNA; and the 5′ and 3′ insert-to-genomic DNA junctions. The PCR and DNA sequence analysis identified a 5′ flanking G/T heterozygote and a 3′ flanking A/G heterozygote in the sequence. These two heterozygotes were most likely due to single nucleotide polymorphisms which are normal DNA sequence variations that are segregating in the autotetraploid alfalfa population (Yang et al., 2009). The PCR and DNA sequence analysis also identified a 102 base pair deletion that occurred at the insertion site in KK179.

Southern blot analysis of KK179 demonstrated that the inserted DNA has been maintained through multiple generations of breeding, thereby confirming the stability of the insert. Results from segregation analyses show heritability and stability of the insert occurred as expected across multiple generations, which corroborates the molecular insert stability analysis and establishes the genetic behavior of the T-DNA I inserted in KK179 at a single chromosomal locus.

V. CHARACTERIZATION AND SAFETY ASSESSMENT OF KK179 EXPRESSED PRODUCTS

A multistep approach is used to assess the safety of gene products introduced into plants using biotechnology. As described in Section III.C., the KK179 insert contains a *CCOMT* suppression cassette. The *CCOMT* suppression cassette encodes for dsRNA, which is highly unlikely to encode for a 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 I.A. Section V.A. describes the history of safe use of products developed using RNA-based gene suppression.

V.A. History of Safe Use of RNA-based Suppression of Endogenous *CCOMT*

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 composed of an inverted repeat sequence, suppress endogenous *CCOMT* gene via the naturally operating endogenous RNAi pathway. Double-stranded RNAs are commonly found in eukaryotes, including plants, for endogenous gene suppression and are composed of nucleic acids (Siomi and Siomi, 2009). Nucleic acids have a long history of safe consumption and are considered GRAS (Generally Recognized as Safe) by the U.S. FDA (U.S. FDA, 1992) 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 by the U.S. FDA, deregulated by USDA-APHIS, 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, 1999a; b; 2000; 2002; MOE, 2007; U.S. FDA, 1994; 1995a; b; 1997; 2008; 2009b; a; 2011; USDA-APHIS, 2012).

Analysis of KK179 DNA segments encoding dsRNA indicate that production of a protein from the dsRNA encoded by the insert in KK179 is highly unlikely. This is supported by evidence that eukaryotic dsRNA molecules are refractory to translation due to the inability of 40s ribosomal subunits to melt double-stranded regions, even ones as short as 18 nucleotides (Kozak, 1989). As a consequence, it is highly unlikely for the dsRNA produced by the transgene in KK179 to yield a translation product. Bioinformatic analyses of the KK179 DNA insert and flanking sequences provided no evidence for concern regarding safety implications of putative polypeptides. Based on this information, the inserted DNA and resulting dsRNA are considered safe and unlikely to produce a protein or polypeptide.

Based on the ubiquitous nature of the RNA-based suppression mechanism utilizing 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.

V.B. Safety Assessment of KK179 Expressed Products Summary and Conclusion

The information provided in this section address the history of safe use for RNA-based gene suppression. In Section I.B.2.2., KK179 northern blot data confirm the expected suppression of endogenous *CCOMT* RNA in alfalfa forage and root tissue. As summarized in this section, it is extremely unlikely a protein could be produced from 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 toxicity or allergenicity of dietary RNA, the RNA-based suppression technology used in KK179 poses no novel risks as a result of expressed products.

VI. COMPOSITIONAL ASSESSMENT OF KK179

Data collected on the feed and food safety of KK179 are consistent with recommendations contained in FDA's proposed rule for Premarket Biotechnology Notice (PBN) Concerning Bioengineered Foods (66FR 4706). Additionally, the data collected and the assessment approach follow the comparative safety assessment process of the Codex Plant Guidelines (Codex Alimentarius, 2009) in which the composition of grain and/or other raw agricultural commodities of the biotechnology-derived crop are compared to the appropriate conventional counterpart that has a history of safe use. Compositional assessments are also performed using the principles and analytes outlined in the OECD consensus document for alfalfa composition (OECD, 2005).

A recent review of compositional assessments conducted according to OECD guidelines, which 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). 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).

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 emphasize quantitative measurements of essential nutrients and known anti-nutrients. These quantitative measurements effectively discern any compositional changes that imply potential nutritional or safety, *e.g.,* anti-nutritional) concerns. Levels of the components in forage and/or other raw agricultural commodities of the biotechnology-derived crop product are compared to: 1) corresponding levels in a conventional comparator, a genetically similar conventional line, grown concurrently under similar field conditions; and 2) ranges generated from an evaluation of conventional commercial reference varieties grown concurrently, and from data published in the scientific literature. The comparison to data published in the literature places any potential 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.

This section provides analyses of concentrations of key nutrients, anti-nutrients, and secondary metabolites of KK179 forage compared to that of forage harvested from a conventional counterpart grown and harvested under similar conditions. In addition, conventional commercial alfalfa reference varieties were included in the composition analyses to establish a range of natural variability for each component. The production of forage for compositional analyses used field designs (randomized complete block design with four blocks) and sensitive analytical methods that allow accurate assessments of

compositional characteristics over a range of environmental conditions under which KK179 is expected to be grown.

The information provided in this section addresses relevant factors in Codex Plant Guidelines, Section 4, paragraphs 44 and 45 for compositional analyses (Codex Alimentarius, 2009).

VI.A. Compositional Equivalence of KK179 Forage to Conventional Alfalfa

Forage samples were collected from the first cutting of KK179, a conventional alfalfa control, and conventional commercial alfalfa varieties grown in a 2011 field production. The conventional control (C_0-Syn1) used as a comparator was a near-isogenic conventional alfalfa population with a genetic background similar to that of KK179. Fourteen different conventional commercial alfalfa reference varieties were included across the field production sites to provide data on the natural variability of each compositional component analyzed. The field production was conducted in typical alfalfa-growing regions at six U.S. sites: California (CAPR), Iowa (IARL), Illinois (ILCY), Kansas (KSLA), Texas (TXCL), and Wisconsin (WIDL). KK179, the conventional control and the conventional commercial varieties were planted in a randomized complete block design with four replicated plots per site and were 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 alfalfa 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 alfalfa (OECD, 2005) to compare levels of key nutrients, anti-nutrients and secondary metabolites in KK179 to levels in the conventional control. Forage samples were analyzed for the following nutrients: proximates (ash, fat, moisture, and protein), carbohydrates (determined 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-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 analyzed to evaluate the potential 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 Appendix E. Prior to compositional analysis, levels of total lignin (ADL) in forage samples from the 2011 field production were measured by Dairy One Lab as described in Section I.B.3. Dairy One Forage Lab used a semi-automated ANKOM-based methodology, described in Appendix D, as adopted by most commercial forage testing laboratories. Covance Laboratories, Inc., which conducted the compositional analyses of key nutrients, anti-nutrients, and secondary metabolites

outlined above, also included an analysis of total lignin (ADL) using a manual method described in Appendix E.

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 between KK179 and the conventional control were identified at the 5% level. Compositional data from the conventional commercial varieties were combined across all field sites to calculate a 99% tolerance interval for each compositional component to estimate the natural variability of each component in alfalfa.

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 statistically significant combined-site 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 conventional 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 alfalfa composition published in the scientific literature. Statistical summaries of nutrient, anti-nutrient, and secondary metabolites for individual sites are found in Appendix E.

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 that 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 alfalfa at the same growth stage presented in Section I.B.3, forage of KK179 is compositionally equivalent to conventional alfalfa.

VI.B. Nutrients, Anti-Nutrients, and Secondary Metabolites in KK179 Forage

The means and ranges for nutrients, anti-nutrients, and secondary metabolites from KK179 were consistent with values established from the conventional alfalfa control (Tables VI-2, VI-3, and VI-4). 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 VI-1.

The mean level of ash was significantly lower ($p<0.05$) in KK179 forage than the conventional control in the combined-site analysis (Table VI-1). The absolute difference in magnitude was 0.41% dw, which is a relative small 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 VI-5). 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, an anti-nutrient, was significantly lower ($p<0.05$) in KK179 forage than the conventional control in the combined-site analysis (Table VI-1). The absolute difference in magnitude was 16.94 ppm, which is a relative difference of -29.6%. However, the mean level of canavanine was within the 99% tolerance interval of the conventional commercial reference varieties and within the range of values found in the published literature (Table VI-5). Lower levels of canavanine would not be adverse as it is considered an anti-nutrient in leguminous plants. 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 VI-1). The absolute difference in magnitude was 110.60 ppm, which is a relatively small difference of 7.4%. The mean level of ferulic acid was within the 99% tolerance interval of the conventional commercial reference varieties and within the range of values found in the published literature (Table VI-5). 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), thus it is not unexpected that alterations in lignin content could result in alterations in ferulic acid levels. 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 I.B.3., it was numerically lower in KK179 forage compared to the conventional control in the combined-site analysis (Table VI-2). The absolute difference in magnitude was 0.32% dw, which is a relative difference of -4.89%. The use of different methods, semi-automated ANKOM-based assay by Dairy One Forage Lab and

the manual assay by Covance Lab, likely contributed to the variability in total lignin (ADL) values determined by the two laboratories. Both methods did confirm 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%.

Assessment of these compositional results supports the overall conclusion that, with the exception of the intended reduction in G lignin and total lignin as presented in Section I.B.3., forage from KK179 is compositionally equivalent to conventional alfalfa with regard to levels of nutrients, anti-nutrients, and secondary metabolites. In the case of ash and ferulic acid, the relative magnitudes of the differences 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 and within the range of values found in the published literature (Table VI-5). Therefore, these differences are not considered biologically meaningful from a feed/food safety and nutritional perspective.

Table VI-1. Summary of Differences Observed in the Combined-Site Analysis (p<0.05) of Alfalfa Forage Component Levels for KK179 vs. Conventional Control

Analytical Component (Units)[1]	KK179 Mean[2]	Control[3] Mean	Mean Difference (Test minus Control) Mean Difference (% of Control)	Mean Difference (Test minus Control) Significance (p-Value)	Test Range	Commercial Tolerance Interval[4]
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]Mean = least-square mean
[3]Control refers to the conventional alfalfa control (C_0-Syn1).
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties. Negative limits set to zero.

Table VI-2. Statistical Summary of Alfalfa Forage Nutrients for KK179 vs. Conventional Control

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (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)
Fiber (% dw)						
Acid Detergent Fiber	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 VI-2 (continued). Statistical Summary of Alfalfa Forage Nutrients for KK179 vs. Conventional Control

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (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 Fiber	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 VI-2 (continued). Statistical Summary of Alfalfa Forage Nutrients for KK179 vs. Conventional Control

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	Difference (Test minus Control) 95% Confidence Interval	Difference (Test minus Control) Significance (p-Value)	Commercial Tolerance Interval[4] (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 VI 2 (continued). Statistical Summary of Alfalfa Forage Nutrients for KK179 vs. Conventional Control

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (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 VI-2 (continued). Statistical Summary of Alfalfa Forage Nutrients for KK179 vs. Conventional Control

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (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 VI-2 (continued). Statistical Summary of Alfalfa Forage Nutrients for KK179 vs. Conventional Control

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (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]Mean (S.E.) = least-square mean (standard error)
[3]Control refers to the conventional alfalfa control (C_0-Syn1).
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties. Negative limits set to zero.

Table VI-3. Statistical Summary of Alfalfa Forage Secondary Metabolites for KK179 vs. Conventional Control

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Metabolite						
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; fw = fresh weight
[2]Mean (S.E.) = least-square mean (standard error)
[3]Control refers to the conventional alfalfa control (C_0-Syn1).
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties. Negative limits set to zero.

Table VI-4. Statistical Summary of Alfalfa Forage Anti-Nutrients for KK179 vs. Conventional Control

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
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 VI-4 (continued). Statistical Summary of Alfalfa Forage Anti-Nutrients for KK179 vs. Conventional Control

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (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]dw = dry weight; fw = fresh weight
[2]Mean (S.E.) = least-square mean (standard error)
[3]Control refers to the conventional alfalfa control (C_0-Syn1).
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties. Negative limits set to zero.

Table VI-5. Literature and OECD Ranges for Compostional Components in Alfalfa 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 fiber	23.17 - 42.59 [a]; 21.26 - 39.25 [b]	23.1 - 33.4
Neutral detergent fiber	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 VI-5 (continued). Literature and OECD Ranges for Components in Alfalfa 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

[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)

[3](OECD, 2005)

[4]NA=not available

VI.C. Compositional Assessment of KK179 Conclusion

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 alfalfa (OECD, 2005). The compositional comparisons were made by analyzing 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 alfalfa production. The compositional analysis, based on OECD consensus document for alfalfa, 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 analyzed.

Compositional analyses based on the OECD consensus document confirmed that, other than the intended reduction in G lignin and total lignin presented in Section I.B.3., there is no meaningful effect on key nutrient, anti-nutrient, and secondary metabolite components in KK179 compared to a conventional alfalfa 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 statistically significant differences between KK179 and the conventional control fall within the 99% tolerance interval determined from the conventional commercial alfalfa 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, forage of KK179 is compositionally equivalent to that of conventional alfalfa; therefore, the feed/food safety and nutritional quality of this product is comparable to conventional alfalfa.

The processing of KK179 forage into animal feed is not expected to be different from that of conventional alfalfa. 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 alfalfa 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 alfalfa.

VII. PHENOTYPIC, AGRONOMIC, AND ENVIRONMENTAL INTERACTIONS ASSESSMENT

This section provides a comparative assessment of the phenotypic, agronomic, and environmental interaction characteristics of KK179 compared to a conventional control. The data support a conclusion that KK179 is not meaningfully different from the conventional control with the exception of the trait for reduced G lignin and total lignin, and therefore is not expected to pose a plant pest risk compared with conventional alfalfa. These conclusions are based on the results of multiple evaluations from laboratory and field assessments.

Phenotypic, agronomic, and environmental interaction characteristics of KK179 were evaluated in a comparative manner to assess plant pest potential. These assessments included evaluation of seed germination characteristics, plant growth and development characteristics, observations of plant responses to abiotic stress, plant-disease and plant-arthropod interactions, pollen characteristics, flower characteristics, and plant-symbiont interactions. Results from these assessments demonstrate that KK179 does not possess 1) increased weediness characteristics; 2) increased susceptibility or tolerance to specific abiotic stressors, diseases, or arthropods; or 3) characteristics that would confer a plant pest risk compared to conventional alfalfa.

VII.A. Characteristics Measured for Assessment

In the phenotypic, agronomic, and environmental interactions assessment of KK179, data were collected to evaluate altered plant pest potential. A detailed description of the regulated article phenotype is requested as part of the petition for determination of nonregulated status in 7 CFR § 340.6, including differences from the unmodified recipient organism that would "substantiate that the regulated article is unlikely to pose a greater plant pest risk than the unmodified organism from which it was derived." As part of the characterization of KK179, data were collected to provide a detailed description of the phenotypic, agronomic, and environmental interaction characteristics of KK179. A subset of these data were included as an evaluation of specific characteristics related to weediness, *e.g.*, seed dormancy, lodging, and split pods, which is an element of the USDA-APHIS plant pest determination.

The plant characterization of KK179 encompassed seven general data categories: 1) seed germination, dormancy, and emergence; 2) vegetative growth; 3) winter survival; 4) reproductive development, including pollen, flower, and seed characteristics; 5) lodging and seed retention on the plant; 6) plant response to abiotic stress and interactions with diseases and arthropods; and 7) plant-symbiont interactions. An overview of the characteristics assessed is presented in Table VII-1.

The phenotypic, agronomic, and environmental interactions data were evaluated from a basis of familiarity (OECD, 1993) and were comprised of a combination of field, greenhouse, and laboratory studies conducted by scientists who are familiar with the production and evaluation of alfalfa. In each of these assessments, KK179 was compared to a conventional control, C_0-Syn1, which is a near-isogenic population produced via an

identical breeding path as KK179, with the exception of the intended reduction in G and total lignin compared to conventional alfalfa at the same stage of growth. In addition, multiple conventional commercial reference varieties developed through conventional breeding and selection (see Appendices F-K and Tables F-1, G-1, H-1, I-1, J-1, and K-1) were included to provide a range of comparative values for each characteristic that represent the variability in existing conventional commercial alfalfa varieties. Data collected for the various characteristics from the conventional commercial reference varieties provide context for interpreting experimental results.

Table VII-1. Phenotypic, Agronomic, and Environmental Interaction Characteristics Evaluated in North American Field Trials and Laboratory or Greenhouse Studies

Data Category	Characteristics measured (associated section where discussed)	Evaluation timing[1] (setting of evaluation)	Evaluation description (measurement endpoints)
Seed germination, dormancy, and emergence	Normal germinated (VII.C.1.)	Day 4 and 7 (20 ºC) (Laboratory)	Percentage of seed producing seedlings exhibiting normal developmental characteristics
	Abnormal germinated (VII.C.1.)	Day 7 (20 °C) (Laboratory)	Percentage of seed producing seedlings that could not be classified as normal germinated
	Germinated (VII.C.1.)	Day 4, 7 and 12 (10 °C and 30 °C) (Laboratory)	Percentage of seed that had germinated (both normally or abnormally)
	Dead (VII.C.1.)	Day 4 and 7 (20 °C) and Day 4, 7, and 12 (10 °C and 30 °C) (Laboratory)	Percentage of seed that had visibly deteriorated and become soft to the touch (also included non-viable hard and nonviable firm-swollen seed)
	Viable hard (VII.C.1.)	Day 7 (20 °C) and Day 12 (10 °C and 30 °C) (Laboratory)	Percentage of seed that did not imbibe water and remained hard to the touch (viability determined by tetrazolium test[2])
	Viable firm-swollen (VII.C.1.)	Day 7 (20 °C) and Day 12 (10 °C and 30 °C) (Laboratory)	Percentage of seed that imbibed water and were firm to the touch but did not germinate (viability determined by tetrazolium test[2])
	Seedling emergence (VII.C.2.1.)	10-21 Days after planting (DAP) (Field forage production)	Average number of emerged plants per foot
	Seedling establishment (VII.C.2.3.)	51 DAP (Field seed production)	Number of established plants in each plot
Vegetative growth	Early season vigor (VII.C.2.1.)	10-40 DAP (Field forage production)	Rated on a 1-10 scale, where: 1 = poor vigor and 10 = excellent vigor
	Seedling vigor (VII.C.2.3.)	51 DAP (Field seed production)	Rated on a 1-10 scale, where: 1 = poor vigor and 10 = excellent vigor
	Crop growth stage (VII.C.2.1.)	0-2 days before forage harvest (Field forage production)	Mean Stage by Count method, calculation based on growth stage of 35-45 stems per plot[1] (Table G-4)
	Forage yield (VII.C.2.1.)	1-10% bloom (Field forage production)	Fresh weight of forage (ton/acre) for an individual harvest
	Regrowth after forage harvest (VII.C.2.1.)	Approximately 10-15 days after a harvest (Field forage production)	Rated on a 1-10 scale, where 1 = dead and 10 = excellent vigor and growth
	Fall plant height (VII.C.2.1.)	Varied by region depending on decreasing photoperiod and temperature (Field forage production)	Distance (in) from soil surface to the uppermost node on the main stem of five representative plants

Table VII-1 (continued). Phenotypic, Agronomic, and Environmental Interaction Characteristics Evaluated in North American Field Trials and Laboratory or Greenhouse Studies

Data Category	Characteristics measured (associated section where discussed)	Evaluation timing (setting of evaluation)	Evaluation description (measurement endpoints)
Vegetative growth (cont)	Total forage yield (VII.C.2.1.)	Post-season (Field forage production)	Sum of the fresh weight of forage (ton/acre) for all harvests at a site over a season
Winter survival	Spring vigor (VII.C.2.1.)	4-6 inches of spring regrowth (Field forage production)	Rated on a 1-10 scale, where: 1 = poor vigor and 10 = excellent vigor
	Spring stand recovery (VII.C.2.1.)	4-6 inches of spring regrowth (Field forage production)	Rated on a 1-10 scale, where: 1 = 0-10% of stand survived winter and 10 = 100% of uniform stand present
	Spring stand count (VII.C.2.1.)	6-12 inches of spring regrowth (Field forage production)	Average number of stems per foot for three 1-ft segments
Reproductive development	Pollen viability (VII.C.3.)	Flowering, (Laboratory)	Percentage of viable pollen, which stain purple due to the presence of living cytoplasmic content
	Pollen morphology (VII.C.3.)	Flowering, (Laboratory)	Average diameter (μm) of 10 representative viable pollen grains
	Flowers per raceme (VII.C.4.)	Flowering, (Laboratory)	Average number of opened and unopened flowers on a raceme
	Standard petal length (VII.C.4.)	Flowering, (Laboratory)	Average length (mm) of the standard petal per 10 flowers
	Keel petal length (VII.C.4.)	Flowering, (Laboratory)	Average length (mm) of the fused keel petals per 10 flowers
	Calyx tube diameter (VII.C.4.)	Flowering, (Laboratory)	Average diameter (mm) of the calyx tube per 10 flowers
	Sexual column length (VII.C.4.)	Flowering, (Laboratory)	Average length (mm) of the sexual column, after tripping the flower, per 10 flowers
	Wing petal length (VII.C.4.)	Flowering, (Laboratory)	Average length (mm) of one wing petal per 10 flowers
	Flower color class (VII.C.4.)	Flowering, (Laboratory)	Color of completely open non-senesced flowers scored on a whole raceme basis for 10 racemes
	Gross raceme morphology (VII.C.4.)	Flowering, (Laboratory)	Classified as typical or atypical based on overall appearance for 10 racemes
	Gross flower morphology (VII.C.4.)	Flowering, (Laboratory)	Classified as typical or atypical based on overall appearance for 10 racemes
	Seed weight (VII.C.2.3.)	Post harvest (Field seed production)	Mean seed weight (mg) per 50 seed
Reproductive development (cont)	Seed per pod (VII.C.2.3.)	Post harvest (Field seed production)	Mean number of seed per 20 pods
	Seed yield (VII.C.2.3.)	At harvest (Field seed production)	Mean seed weight (g) harvested per plot

Table VII-1 (continued). Phenotypic, Agronomic, and Environmental Interaction Characteristics Evaluated in North American Field Trials and Laboratory or Greenhouse Studies

Data Category	Characteristics measured (associated section where discussed)	Evaluation timing (setting of evaluation)	Evaluation description (measurement endpoints)
Lodging and seed retention	Lodging (VII.C.2.1.)	0-2 days before forage harvest (Field forage production)	Rated on a 0-9 scale, where 0 = 0-10% erect stems (lodging susceptible) and 9 = 91-100% erect stems (lodging resistant)
	Lodging (at seed maturity; VII.C.2.3.)	At seed harvest (Field seed production)	Rated on a 0-9 scale, where 0 = 0-10% erect stems (lodging susceptible) and 9 = 91-100% erect stems (lodging resistant)
	Split pods (VII.C.2.3.)	Ripe seed pod stage, approximately five weeks after pollination (Field seed production)	Percentage of split pods per 10 racemes
Plant-environmental interactions	Plant response to abiotic stress (VII.C.2.2.)	3-5 observations per season at 0-10 days prior to forage harvest (Field forage production)	Qualitative assessment of each plot, rated on a 0-9 scale, where 0 = no symptoms and 9 = severe symptoms
	Disease damage (VII.C.2.2.)	3-5 observations per season at 0-10 days prior to forage harvest (Field forage production)	Qualitative assessment of each plot, rated on a 0-9 scale, where 0 = no symptoms and 9 = severe symptoms
	Arthropod-related damage (VII.C.2.2.)	3-5 observations per season at 0-10 days prior to forage harvest (Field forage production)	Qualitative assessment of each plot, rated on a 0-9 scale, where 0 = no symptoms and 9 = severe symptoms
	Alfalfa weevil damage (VII.C.2.2.)	3-5 observations per season at 1-9 days prior to forage harvest (Field forage production)	Specific quantitative assessment of alfalfa weevil damage from 10 areas in each plot using a 0-5 scale, where 0 = no damage and 5 = >90% of foliage with skeletonized appearance
	Potato leafhopper damage (VII.C.2.2.)	3-5 observations per season at1-9 days prior to forage harvest (Field forage production)	Specific quantitative assessment of potato leafhopper damage from 10 areas in each plot using a 0-5 scale, where 0 = no damage and 5 => 90% foliage with yellowing and puckering
	Arthropod abundance (VII.C.2.2.)	3-5 collections per season at 0-10 days prior to forage harvest (Field forage production)	Number of pest and beneficial arthropods collected per plot
Plant-symbiont interactions	Biomass (VII.C.4)	6 weeks after emergence (Greenhouse)	Nodule, root, and shoot dry weight (g)
	Total nitrogen (VII.C.4)	6 weeks after emergence (Greenhouse)	Shoot total nitrogen (%, g)
Plant-symbiont interactions	Biomass (VII.C.4)	6 weeks after emergence (Greenhouse)	Nodule, root, and shoot dry weight (g)
	Total nitrogen (VII.C.4)	6 weeks after emergence (Greenhouse)	Shoot total nitrogen (%, g)

[1]Alfalfa developmental stages were determined according to definitions provided by (Kalu and Fick, 1981).
[2]Viability of hard and firm-swollen seed were determined by a tetrazolium test (AOSA, 2007c; a).

VII.B. Interpretation of Phenotypic and Environmental Interactions Data

Plant pest risk assessments for biotechnology-dervided crops are comparative assessments, and are considered from a basis of familiarity. The concept of familiarity is based on the fact that the biotechnology-derived plant is developed from a conventional crop whose biological properties and plant pest potential are well-known. Familiarity considers the biology of the crop, the introduced trait, the receiving environment, and the interaction of these factors, and provides a basis for comparative environmental risk assessment between a biotechnology-derived plant and its conventional counterpart.

Expert knowledge and experience with conventionally bred alfalfa were the basis for selecting appropriate endpoints and estimating the range of responses that would be considered typical for alfalfa. As such, KK179 was compared to the conventional control, C_0-Syn1, in the assessment of phenotypic, agronomic, and environmental interaction characteristics. An overview of the characteristics assessed is presented in Table VII-1. A subset of the data relating to well-understood weedy characteristics, *e.g.*, seed dormancy, lodging, and split pods, was used to assess whether there was an increase in weediness of KK179 compared to conventional alfalfa. Evaluation of environmental interaction characteristics, *e.g.*, plant-abiotic stress, plant-disease, and plant-arthropod interactions, was also considered in the plant pest assessment.

Prior to analysis, the overall dataset was evaluated for possible evidence of biologically-relevant changes and unexpected plant responses. No unexpected observations or issues were identified. Based on all of the data collected, an assessment was made to determine if KK179 is expected to pose an increased plant pest risk compared to conventional alfalfa.

VII.B.1. Interpretation of Detected Differences Criteria

Comparative plant characterization data between a biotechnology-derived crop and the conventional control are interpreted in the context of contributions to increased plant pest/weed potential as assessed by USDA-APHIS. Under the framework of familiarity, characteristics for which no differences are detected support a conclusion of no increased plant pest/weed potential of the biotechnology-derived crop compared to the conventional crop. Characteristics for which differences are detected are considered in a step-wise method (Figure VII-1) or in a similar fashion. All detected differences for a characteristic are considered in the context of whether or not the difference would increase the plant pest/weed potential of the biotechnology-derived crop. Ultimately, a weight-of-evidence approach considering all characteristics and data is used for the overall risk assessment of differences and their significance. In detail, Figure VII-1 illustrates the stepwise assessment process employed:

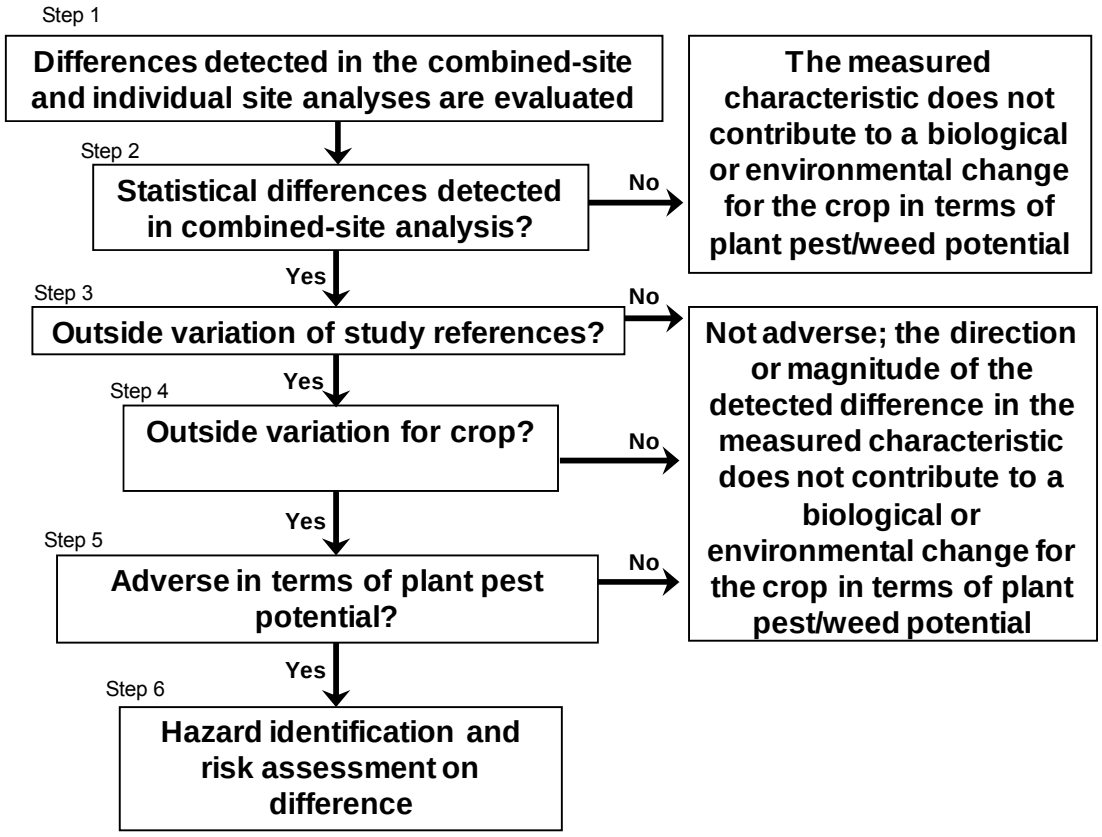


Figure VII-1. Schematic Diagram of Agronomic and Phenotypic Data Interpretation Methods

Note: A "no" answer at any step indicates that the characteristic does not contribute to a biological or environmental change for the crop in terms of plant pest potential and subsequent steps are not considered. If the answer is "yes" or "uncertain," the subsequent step is considered.

Steps 1 and 2 - Evaluate Detected Statistically Significant Differences

Data on each measured characteristic are statistically analyzed, as appropriate, within each individual site and in a combined-site analysis, in which the data are pooled among sites. All statistically significant differences are evaluated and considered in the context of a change in plant pest/weed potential. Differences detected in individual-site analyses that are not detected when data across multiple environments are pooled in the combined-site analysis are considered not biologically meaningful in terms of plant pest/weed potential and, therefore, are not further considered in subsequent steps. Any difference detected in the combined-site analysis is further assessed.

Step 3 - Evaluate Differences in the Context of Conventional Commercial Reference Varieties Included in the Study

If a difference for a characteristic is detected in the combined-site analysis across multiple environments, then the mean value of the biotechnology-derived crop for the

characteristic is assessed relative to the range of variation of the conventional commercial reference varieties included in the study, *e.g.*, reference range.

Step 4 - Evaluate Differences in the Context of the Crop

If the mean value of the characteristic for a biotechnology-derived crop is outside the variation of the conventional commercial reference varieties, the mean value of the biotechnology-derived crop is assessed relative to known values common for the crop, *e.g.*, published values.

Step 5 - Plant Pest Potential

If the mean value of the characteristic for a biotechnology-derived crop is outside the range of values common for the crop, the detected difference for the characteristic is then assessed for whether or not it is adverse in terms of plant pest/weed potential.

Step 6 - Conduct Risk Assessment on Identified Hazard

If an adverse effect (hazard) is identified, risk assessment on the difference is conducted. The risk assessment considers contributions to enhanced plant pest/weed potential of the crop itself, the impact of differences detected in other measured characteristics, and potential for and effects of trait introgression into any populations growing outside of cultivated environments or into a sexually-compatible species.

VII.B.2. Interpretation of Environmental Interactions Data

Plant responses to abiotic stress, disease damage, and arthropod damage were qualitatively assessed using a continuous scale of increasing damage severity to determine a range of responses observed across four replications. The biotechnology-derived crop and the conventional control were considered not different in plant response if the range of damage symptoms observed for the biotechnology-derived crop overlapped with the range of symptoms observed for the conventional control at a site. Any observed differences between the biotechnology-derived crop and conventional control were assessed for biological significance in the context of the range of the conventional commercial reference varieties at that site and for consistency with other observation times and sites. Differences that were not consistently observed at other observation times and sites were not considered to be biologically meaningful in terms of plant pest potential or of an effect on the environment.

Damage caused by specific arthropods (alfalfa weevil and potato leafhopper) was further assessed quantitatively, and data were analyzed within individual sites and pooled across sites in a combined-site analysis. Statistically significant differences detected between the biotechnology-derived crop and the conventional control were evaluated using the method outlined in Figure VII-1.

Pest- and beneficial-arthropod abundance was also quantitatively assessed, and data were analyzed within individual collection times and sites. Statistically significant differences between the biotechnology-derived crop and the conventional control were assessed for biological significance in the context of the range of the conventional commercial reference varieties at that site and for consistency with other observation times and sites.

Differences that were not consistently detected at other observation times and sites were not considered to be biologically meaningful in terms of plant pest potential or to have an effect on the environment..

VII.C. Comparative Assessments of the Phenotypic, Agronomic, and Environmental Interaction Characteristics of KK179

This section provides the results of comparative assessments conducted in replicated laboratory, greenhouse, and field studies to provide a detailed phenotypic, agronomic, and environmental interactions description of KK179. The KK179 characteristics evaluated in these assessments included: seed germination and dormancy characteristics (Section VII.C.1.); plant phenotypic and environmental interaction observations under field conditions of plants managed for forage production (Section VII.C.2.1. and Section VII.C.2.2); plant phenotypic observations under field conditions of plants managed for seed production (Section VII.C.2.3.); reproductive characteristics (Section VII.C.3); and plant-symbiont interactions (Section VII.C.4.). Additional details for each assessment are provided in Appendices F through K.

VII.C.1. Seed Germination and Dormancy Characteristics

USDA-APHIS considers the potential for weediness to constitute a plant pest factor (7 CFR § 340.6). Seed dormancy is an important characteristic that is often associated with plants that are considered weeds, and several dormancy mechanisms can occur in seeds (Anderson, 1996; Copeland and McDonald, 2001). Information on germination and dormancy characteristics is therefore useful when assessing a plant for increased weediness potential. To assess germination characteristics, standardized germination assays are routinely used. The Association of Official Seed Analysts (AOSA), an internationally recognized seed testing organization, recommends a temperature of 20 °C as optimal for testing the germination and dormancy characteristics of alfalfa seed (AOSA, 2007c; 2010b; a).

Seed germination and dormancy mechanisms vary among species and their genetic basis tends to be complex. While not considered a weed, alfalfa does exhibit physical dormancy, with a hard seed coat that is impermeable to water (Bass et al., 1988; Rolston, 1978). The production environment has a great impact on the seed coat of alfalfa. The percentage of hard seed may be increased by cool temperatures, high humidity, and excess available water during seed development (Bass et al., 1988; Copeland and McDonald, 2001).

A hard seed coat can be manually scarified, *i.e.*, scratched or nicked, to facilitate imbibition of water (Anderson, 1996). In fact, commercial alfalfa seed is mechanically scarified during post-harvest handling and conditioning. Since commercial seed is frequently scarified, and germination rates for certified seed are usually targeted for greater than 80%, a high percentage of hard seed and poor germination, *i.e.*, below 70%, is unlikely to be consistently observed in the field (AOSCA, 2009).

A comparative assessment of seed germination and dormancy characteristics was conducted on KK179 and the conventional control. Four conventional commercial reference varieties were also included to provide a range of germination and dormancy

characteristic values representative of commercial alfalfa varieties. Descriptions of the evaluated germination and dormancy characteristics and the timing of the evaluations for all temperature regimes are listed in Table VII-1. Additional details on the materials and experimental methods used in this evaluation are presented in Appendix F.

All starting seed were produced in the artificial environment of a greenhouse during the winter of 2010 under cool, humid conditions during seed development. Therefore, a relatively high level of hard seed would be expected among the starting seed. To account for different dormancy effects, both non-scarified and scarified seed were evaluated. Non-scarified seed were used to assess the germination and dormancy characteristics of KK179, without regard to a specific mechanism of dormancy. Scarified seed were used to assess whether any other seed dormancy effects, besides a hard seed coat, were introduced with the trait for reduced G lignin and total lignin. In addition to the AOSA-recommended temperature of 20 °C, non-scarified and scarified seed were tested at two additional temperature regimes of 10 °C and 30 °C to assess seed germination properties.

In the non-scarified treatment in the AOSA-recommended temperature regime (20 °C), no statistically significant differences (α=0.05) were detected between KK179 and the conventional control for percent abnormal germinated, dead, and viable firm-swollen seed (Table VII-2). KK179 had a higher percent normal germinated seed than the conventional control (86.0% vs. 72.5%), and the mean for KK179 for percent normal germinated seed was slightly above the range of the conventional commercial reference varieties (71.8% – 85.3%). The mean value observed for percent germinated seed is typical of alfalfa, which exhibits a wide range of germination rates across varieties (29.5% – 90.0% germination) (Weihing, 1940). Therefore, the difference in percent normal germinated seed at 20 °C is not expected to be biologically meaningful in terms of plant pest/weed potential (See Figure VII-1, Step 4, answer "no"). KK179 also had a lower percent viable hard seed than the control (13.0% vs. 25.3%) at 20 °C, although the mean for percent viable hard seed was within the reference range (12.5% – 24.4%). Therefore, the difference in percent viable hard seed at 20 °C is not expected to be biologically meaningful in terms of plant pest/weed potential (See Figure VII-1, Step 3, answer "no").

In the non-scarified treatment in both the 10 °C and 30 °C temperature regimes, no statistically significant differences were detected between KK179 and the conventional control for percent dead and viable firm-swollen seed. At 10 °C, KK179 had higher percent germinated seed than the conventional control (86.5% vs. 74.1%) and concurrently lower percent viable hard seed than the conventional control (12.8% vs. 24.7%). The means for KK179 for percent germinated seed and percent viable hard seed were within the range of conventional commercial reference varieties (75.8% – 88.8% and 11.2% – 23.0%, respectively). At 30 °C, KK179 had higher percent germinated seed than the conventional control (87.3% vs. 74.9%) and concurrently lower percent viable hard seed than the conventional control (12.5% vs. 24.8%). The means for KK179 for percent germinated seed and viable hard seed were within the reference ranges (77.5% – 89.5% and 10.5% – 21.8%, respectively). Therefore, the differences in percent germinated and viable hard seed at 10 °C and 30 °C are not expected to be biologically meaningful in terms of plant pest/weed potential (See Figure VII-1, Step 3, answer "no").

Increased germination and reduced seed dormancy, regardless of the specific mechanism of dormancy, *i.e.*, hard seed coat or other mechanism, would likely shorten the persistence of seed in the soil. Long-term persistence of seed in the soil is a characteristic common to some weed species, and for some perennial weeds, has been associated with a high percentage of dormant seed (Conn and Werdin-Pfisterer, 2010). Short-term persistence, on the other hand, has been associated with a high initial rate of germination (Hesse et al., 2007). Increased germination and reduced dormancy would reduce long-term persistence and thereby reduce the potential for weediness. Moreover, increased germination and reduced seed dormancy are common agronomic properties of domesticated crops. To this end, alfalfa seed treatments, such as varying temperature and moisture conditions, have been sought to improve germination (Bass et al., 1988; Klos and Brummer, 2000). Therefore, the differences detected for non-scarified seed are not considered to represent a meaningful increase in plant pest/weed potential.

In the scarified treatment, no statistically significant differences were detected between KK179 and the conventional control for any of the assessed characteristics in the 10 °C or 30 °C temperature regimes (Table VII-3). In the AOSA-recommended temperature regime (20 °C), KK179 had significantly higher percent normal germinated seed than the conventional control (93.5% vs. 89.8%) and this value was slightly lower than the reference range (93.8% – 98.0%). However, the mean for KK179 for percent germination of scarified seed is consistent with published germination rates of scarified alfalfa seed (93 – 98%) (Hall et al., 1993). Therefore, the difference in percent germinated seed at 20 °C is not expected to be biologically meaningful in terms of plant pest/weed potential (See Figure VII-1, Step 4, answer "no").

Scarification disrupts the hard seed coat and facilitates imbibition to allow an assessment of any other potential dormancy effects introduced with the trait. KK179 and the conventional control did not exhibit consistent differences in any of the assessed characteristics across different temperature regimes following scarification. Therefore, it is not expected that additional dormancy effects were introduced with the trait for reduced G lignin and total lignin.

While the exact nature of seed coat impermeability is not known for alfalfa, lignin is known to be present in the seed coat (Rolston, 1978); and if altered, could be hypothesized to alter the germination and dormancy characteristics of alfalfa seed. For example, a study with another legume species, *Vigna radiata* L., observed higher lignin levels in the seed coat of dormant beans compared to non-dormant beans (Rodriguez and Mendoza, 1990). Conversely, a reduction in the lignin content of the seed coat could potentially be correlated with a reduction in percent hard seed and an increase in germination rate.

The germination and dormancy characteristics evaluated were used to assess KK179 in the context of plant pest risk. The results of this assessment, particularly the observance of lower percentage hard seed and no other changes in other dormancy characteristics, support the conclusion that the introduction of the trait for reduced G lignin and total lignin is not expected to result in increased plant pest/weed potential compared to conventional alfalfa.

Table VII-2. Comparison of Germination Characteristics of Non-scarified Seed of KK179 and the Conventional Control

Temperature Regime	Germination Category	Mean %[1] (SE)		
		KK179	Control	Reference Range[2]
10 °C	Germinated	* 86.5 (0.6)	74.1 (1.7)	75.8 – 88.8
	Dead	0.3 (0.3)	0.3 (0.3)	0.0 – 0.3
	Viable firm-swollen	0.5 (0.3)	0.8 (0.5)	0.0 – 1.0
	Viable hard	* 12.8 (0.6)	24.7 (1.6)	11.2 – 23.0
20 °C	Normal germinated	* 86.0 (1.2)	72.5 (1.7)	71.8 – 85.3
(AOSA)	Abnormal germinated	0.8 (0.5)	1.8 (0.5)	1.3 – 3.2
	Dead	0.0 (0.0)	0.3 (0.3)	0.0 – 0.3
	Viable firm-swollen	0.3 (0.3)	0.3 (0.3)	0.0 – 0.3
	Viable hard	* 13.0 (1.1)	25.3 (1.7)	12.5 – 24.4
30 °C	Germinated	* 87.3 (2.4)	74.9 (1.3)	77.5 – 89.5
	Dead	0.0 (0.0)	0.0 (0.0)	0.0 – 0.5
	Viable firm-swollen	0.2 (0.2)	0.3 (0.3)	0.0 – 1.0
	Viable hard	* 12.5 (2.3)	24.8 (1.5)	10.5 – 21.8

Note: The experimental design was a two-way factorial design with four replications (n=4). The two factors assessed were material (i.e., KK179, the control, or a conventional commercial reference variety) and treatment (i.e., scarified or non-scarified).
SE = Standard Error
*Statistically significant differences detected (α=0.05) between KK179 and the conventional control using ANOVA.
[1]In some instances, the total percentage for both KK179 and the conventional control did not equal 100% due to numerical rounding of the means.
[2]Reference range is the minimum and maximum mean values observed among the four conventional commercial reference varieties.

Table VII-3. Comparison of Germination Characteristics of Scarified Seed of KK179 and the Conventional Control

Temperature Regime	Germination Category	Mean %[1] (SE)		
		KK179	Control	Reference Range[2]
10 °C	Germinated	99.5 (0.3)	99.8 (0.3)	99.0 – 100.0
	Dead	0.0 (0.0)	0.0 (0.0)	0.0 – 1.0
	Viable firm-swollen	0.0 (0.0)	0.0 (0.0)	0.0 – 0.0
	Viable hard	0.5 (0.3)	0.3 (0.3)	0.0 – 0.0
20 °C	Normal germinated	* 93.5 (1.3)	89.8 (0.6)	93.8 – 98.0
(AOSA)	Abnormal germinated	6.0 (1.3)	10.3 (0.6)	2.0 – 5.8
	Dead	0.3 (0.3)	0.0 (0.0)	0.0 – 0.0
	Viable firm-swollen	0.0 (0.0)	0.0 (0.0)	0.0 – 0.0
	Viable hard	0.3 (0.3)	0.0 (0.0)	0.0 – 0.5
30 °C	Germinated	98.8 (0.5)	99.5 (0.3)	97.3 – 99.8
	Dead	0.5 (0.3)	0.5 (0.3)	0.0 – 0.3
	Viable firm-swollen	0.5 (0.3)	0.0 (0.0)	0.0 – 2.3
	Viable hard	0.3 (0.3)	0.0 (0.0)	0.0 – 0.3

Note: The experimental design was a two-way factorial design with four replications (n=4). The two factors assessed were material (i.e., KK179, the control, or a conventional commercial reference variety) and treatment (i.e., scarified or non-scarified).
SE = Standard Error
*Statistically significant differences detected (α=0.05) between KK179 and the conventional control using ANOVA.
[1]In some instances, the total percentage for both KK179 and the conventional control did not equal 100% due to numerical rounding of the means.
[2]Reference range is the minimum and maximum mean values observed among the four conventional commercial reference varieties.

VII.C.2. Field Phenotypic, Agronomic, and Environmental Interaction Characteristics

Phenotypic, agronomic, and environmental interactions of plants managed for forage and seed production were evaluated under field conditions of forage and seed production as part of the plant characterization assessment of KK179. These data were developed to provide USDA-APHIS with a detailed description of KK179 relative to the conventional control and the conventional commercial reference varieties. According to 7 CFR § 340.6, as part of the petition to seek deregulation, a petitioner must submit "a detailed description of the phenotype of the regulated article." This information is being provided to assess whether there are phenotypic differences between KK179 and the conventional control that may impact its plant pest/weed potential. Specific characteristics that are related to weediness, *e.g.*, lodging, and split pods, were used to assess whether there is a potential increase in weediness of KK179 compared to conventional alfalfa. Environmental interactions including plant response to abiotic stress, disease damage, specific arthropod-related damage, and pest- and beneficial-arthropod abundance were also assessed as an indirect indicator of phenotypic changes to KK179 and are also considered in the plant pest assessment.

The results of the assessments of agronomic and phenotypic characteristics of KK179 managed under conditions for both forage and seed production demonstrated that the introduction of the trait for reduced G lignin and total lignin did not meaningfully alter the plant pest/weed potential of KK179 compared to conventional alfalfa. Furthermore, the lack of meaningful differences in environmental interactions also support the conclusion that the introduction of the trait is not expected to result in increased plant pest/weed potential or to have an effect on the environment for KK179 compared to conventional alfalfa.

VII.C.2.1. Field Phenotypic and Agronomic Characteristics of KK179 Managed for Forage Production

A field study was conducted, beginning in 2010 and continuing through 2012, to evaluate phenotypic and agronomic characteristics of KK179 compared to the conventional control when managed under the agronomic practices of forage production. The selected sites reflected the diverse range of environmental and agronomic conditions representative of commercial alfalfa forage production areas in North America. At each site, all plots of KK179, the conventional control, and the conventional commercial reference varieties were uniformly managed for forage production, in order to assess whether the introduction of the trait for reduced G lignin and total lignin altered phenotypic and agronomic characteristics of KK179 compared to the conventional control.

Phenotypic and agronomic data were collected from field trials at 10 sites within U.S. and Canadian alfalfa production regions (Table VII-4). The experimental design at each site was a randomized complete block with four replications. At the nine U.S. sites, KK179, the conventional control, and four conventional commercial reference varieties were evaluated. At the Canadian site, KK179, the conventional control, and five conventional

commercial reference varieties were evaluated. A total of twelve unique conventional commercial reference varieties were evaluated among the 10 sites (Table G-1).

The trials at each site were managed according to standard, local agronomic practices for forage production in order to harvest forage at a growth stage of 1-10% bloom. The total number of cuttings harvested per growing season at a site depends on numerous factors including agro-ecological environment, choice of variety, harvest schedule, and weather. The number of cuttings per growing season at each site is listed in Table G-3.

This study was conducted over two years, from spring 2010 through spring 2012. The first year was considered to be from planting in 2010 through the winter survival assessment in the spring of 2011. The second year was considered to be from the first cutting of 2011 through the winter survival assessment in the spring of 2012. For each year, assessments were made within each crop growth cycle, *i.e.*, the period of forage growth between cuttings, and at each cutting.

Descriptions of the evaluated phenotypic characteristics and the timing of the evaluations are listed in Table VII-1. The materials, methods, details concerning the timing of phenotypic assessments, and detailed results of the individual-site data comparisons are presented and discussed in Appendix G. The results of the combined-site and combined-year analyses are summarized below. The results of the agronomic and phenotypic assessment demonstrate that there were no unexpected changes in the phenotype of KK179 compared to the conventional control when managed under the agronomic practices for forage production. Thus, the introduction of the trait is not expected to result in increased plant pest/weed potential of KK179 compared to conventional alfalfa.

Table VII-4. Field Phenotypic Evaluation Sites for KK179 during 2010-2012

Site Code	County, State/Province, Country
CADV	Yolo County, CA, U.S.A.
CANA	Branchton, Ontario, Canada
IABN	Boone County, IA, U.S.A.
IDNP	Canyon County, ID, U.S.A.
ILCL	Clinton County, IL, U.S.A.
KSLA	Pawnee County, KS, U.S.A.
NYNR	Wayne County, NY, U.S.A.
PACO	Lancaster County, PA, U.S.A.
WATC	Walla Walla County, WA, U.S.A.
WIWS	La Crosse County, WI, U.S.A.

In the combined-site analysis of the phenotypic characteristics assessed in the first year (2010-2011), no statistically significant differences (5% level of significance) were detected between KK179 and the conventional control for any of the assessed characteristics, including seedling emergence, early season vigor; lodging, crop growth stage, or regrowth after cutting for cutting #1; lodging, crop growth stage, forage yield or regrowth after cutting for cutting #2; lodging, crop growth stage, or forage yield for cutting #3; fall plant height, total forage yield; spring vigor, spring stand recovery, or spring stand count (Table VII-5). A fourth growth period and cutting occurred only at two sites (CADV, KSLA), therefore, a combined-site analysis could not be conducted for the following phenotypic characteristics: regrowth after cutting #3, lodging #4, crop growth stage #4, and forage yield for cutting #4.

In the combined-site analysis of the phenotypic characteristics assessed in the second year (2011-2012), no statistically significant differences were detected between KK179 and the conventional control for any of the assessed characteristics, including lodging, crop growth stage, forage yield or regrowth after cutting for cuttings #1, #2, #3, or #4; lodging, crop growth stage, or forage yield for cutting #5; fall plant height, total forage yield; spring vigor, spring stand recovery, or spring stand count (Table VII-6).

An additional, combined-year analysis was conducted for the characteristics measured in both growing seasons, which were fall plant height, total forage yield, spring vigor, spring stand recovery, and spring stand count. No statistically significant differences were detected between KK179 and the conventional control for any of the assessed characteristics in the combined-year analysis (Table VII-7).

Lodging was a characteristic used to specifically assess the potential weediness of KK179. No differences were observed between KK179 and the conventional control for lodging within any crop growth cycle assessed over the two years.

Based on the assessed phenotypic and agronomic characteristics within each individual year and across years, the results demonstrate that there were no unexpected changes in phenotype indicative of increased plant pest/weed potential of KK179 compared to the conventional control (See Figure VII-1, Step 2, answer “no”).

Table VII-5. Combined-Site Comparison of KK179 to the Conventional Control for Phenotypic and Agronomic Characteristics in the First Year (2010-2011) of Forage Production

	KK179	Control	Reference Range[1]	
Phenotypic Characteristic (units)	Mean (SE)	Mean (SE)	Minimum	Maximum
Seedling emergence (# plants/foot)	19.5 (1.51)	18.5 (1.41)	12.0	27.1
Early season vigor (1-10 rating)	4.8 (0.33)	5.1 (0.38)	3.3	7.0
Cutting #1				
Lodging (0-9 rating)	8.5 (0.19)	8.4 (0.24)	7.6	9.0
Crop growth stage (stage number 0-6)	2.8 (0.12)	2.7 (0.14)	1.9	3.2
Regrowth after cutting (1-10 rating)	9.1 (0.16)	8.7 (0.19)	6.8	8.9
Cutting #2				
Lodging (0-9 rating)	8.2 (0.19)	8.3 (0.22)	7.5	9.0
Crop growth stage (stage number 0-6)	3.1 (0.14)	3.0 (0.15)	1.2	3.7
Forage yield (fresh weight ton/acre)	7.0 (0.46)	6.6 (0.42)	4.5	8.7
Regrowth after cutting (1-10 rating)	8.8 (0.22)	8.7 (0.22)	6.4	8.4
Cutting #3				
Lodging (0-9 rating)	8.7 (0.10)	8.6 (0.11)	8.4	9.0
Crop growth stage (stage number 0-6)	2.1 (0.15)	2.2 (0.13)	1.5	2.8
Forage yield (fresh weight ton/acre)	4.7 (0.38)	4.8 (0.40)	2.8	6.9
Regrowth after cutting (1-10 rating)	† 8.0 (0.42)	8.1 (0.23)	6.5	7.4
Cutting #4				
Lodging (0-9 rating)	† 7.4 (0.68)	7.0 (0.78)	5.8	9.0
Crop growth stage (stage number 0-6)	† 2.1 (0.04)	2.2 (0.04)	2.0	2.2
Forage yield (fresh weight ton/acre)	† 4.1 (0.21)	3.4 (0.36)	3.7	4.4

Table VII-5. Combined-Site Comparison of KK179 to the Conventional Control for Phenotypic and Agronomic Characteristics in the First Year (2010-2011) of Forage Production (continued)

	KK179	Control	Reference Range[1]	
Phenotypic Characteristic (units)	Mean (SE)	Mean (SE)	Minimum	Maximum
Fall plant height (in)	5.5 (0.42)	5.6 (0.44)	2.8	6.8
Total forage yield (fresh weight ton/acre)	12.6 (0.85)	12.0 (0.88)	8.0	17.8
Spring vigor (1-10 rating)	7.8 (0.16)	7.9 (0.25)	5.8	7.2
Spring stand recovery (1-10 rating)	8.4 (0.23)	8.6 (0.23)	7.2	10.0
Spring stand count (# stems/foot)	62.9 (3.53)	63.4 (3.85)	42.9	73.1

Note: The experimental design was a randomized complete block with four replications. Means based on n=40 for all characteristics except as follows: for early season vigor and lodging at cutting #1, n=36 for both KK179 and the conventional control; for crop growth stage at cutting #1, n=35 for both KK179 and the conventional control; for regrowth after cutting #1, n=36 for both KK179 and the conventional control; for lodging at cutting #2, n=36 for both KK179 and the conventional control; for crop growth stage at cutting #2, n=35 for both KK179 and the conventional control; for crop growth stage at cutting #3, n=39 for KK179 and n=37 for the conventional control; for regrowth after cutting #3 and lodging at cutting #4, n=8 for both KK179 and the conventional control; for crop growth stage at cutting #4, n=6 for both KK179 and the conventional control; for forage yield #4, n=8 for KK179 and n=7 for the conventional control; for total forage yield, n=39 for the conventional control; for spring vigor, spring stand recovery, and spring stand count, n=36 for both KK179 and the conventional control.
SE = Standard Error
No statistically significant differences were detected between KK179 and the conventional control (α=0.05) using ANOVA.
† No statistical comparisons were made because data were collected at only two sites.
[1]Reference range is the minimum and maximum mean values among the conventional commercial reference varieties.

Table VII-6. Combined-Site Comparison of KK179 to the Conventional Control for Phenotypic and Agronomic Characteristics in the Second Year (2011-2012) of Forage Production

	KK179	Control	Reference Range[1]	
Phenotypic Characteristic (units)	Mean (SE)	Mean (SE)	Minimum	Maximum
Cutting #1				
Lodging (0-9 rating)	7.1 (0.32)	7.0 (0.36)	6.2	8.9
Crop growth stage (stage number 0-6)	2.9 (0.13)	2.8 (0.14)	2.6	3.7
Forage yield (fresh weight ton/acre)	12.1 (0.88)	12.3 (0.87)	8.7	13.7
Regrowth after cutting (1-10 rating)	8.8 (0.13)	9.1 (0.13)	6.3	8.9
Cutting #2				
Lodging (0-9 rating)	7.7 (0.29)	7.5 (0.33)	7.0	9.0
Crop growth stage (stage number 0-6)	3.3 (0.16)	3.4 (0.16)	2.7	3.9
Forage yield (fresh weight ton/acre)	9.7 (0.63)	9.8 (0.65)	6.1	12.6
Regrowth after cutting (1-10 rating)	9.0 (0.13)	9.1 (0.16)	6.8	9.4
Cutting #3				
Lodging (0-9 rating)	7.2 (0.32)	7.5 (0.28)	7.2	8.8
Crop growth stage (stage number 0-6)	3.8 (0.13)	3.8 (0.14)	3.2	4.1
Forage yield (fresh weight ton/acre)	8.3 (0.61)	8.2 (0.58)	4.8	10.3
Regrowth after cutting (1-10 rating)	8.8 (0.22)	9.0 (0.22)	6.3	9.2
Cutting #4				
Lodging (0-9 rating)	7.9 (0.29)	8.1 (0.26)	6.9	9.0
Crop growth stage (stage number 0-6)	2.8 (0.10)	3.0 (0.12)	2.2	3.4
Forage yield (fresh weight ton/acre)	5.4 (0.44)	5.5 (0.43)	2.5	6.8
Regrowth after cutting (1-10 rating)	8.4 (0.29)	8.5 (0.31)	6.3	9.0
Cutting #5				
Lodging (0-9 rating)	8.3 (0.30)	8.3 (0.31)	7.5	9.0
Crop growth stage (stage number 0-6)	1.7 (0.24)	1.8 (0.25)	1.0	3.2
Forage yield (fresh weight ton/acre)	4.6 (0.49)	4.4 (0.41)	1.4	4.9

Table VII-6 (continued). Combined-Site Comparison of KK179 to the Conventional Control for Phenotypic and Agronomic Characteristics in the Second Year (2011-2012) of Forage Production

	KK179	Control	Reference Range[1]	
Phenotypic Characteristic (units)	Mean (SE)	Mean (SE)	Minimum	Maximum
Fall plant height (in)	6.2 (0.47)	6.6 (0.51)	3.1	8.0
Total forage yield (fresh weight ton/acre)	36.6 (2.28)	37.2 (2.28)	23.1	44.5
Spring vigor (1-10 rating)	7.7 (0.28)	7.3 (0.32)	6.0	7.6
Spring stand recovery (1-10 rating)	8.1 (0.21)	8.5 (0.17)	7.2	9.3
Spring stand count (# stems/foot)	54.6 (4.46)	51.9 (4.31)	39.5	71.0

Note: The experimental design was a randomized complete block with four replications. Means based on n=40 for all characteristics except as follows: for lodging at cutting #1, n=36 for both KK179 and the conventional control; for crop growth stage at cutting #1, n=30 for KK179 and n=29 for the conventional control; for forage yield at cutting #1, n=39 for the conventional control; for crop growth stage at cutting #2, n=26 for KK179 and n=28 for the conventional control; for forage yield at cutting #2, n=36 for both KK179 and the conventional control; for crop growth stage at cutting #3, n=34 for both KK179 and the conventional control; for forage yield at cutting #3, n=39 for both KK179 and the conventional control; for crop growth stage at cutting #4, n=31 for KK179 and n=35 for the conventional control; for regrowth after cutting #4, n=20 for both KK179 and the conventional control; for lodging at cutting #5, n=20 for both KK179 and the conventional control; for crop growth stage at cutting #5, n=16 for both KK179 and the conventional control; for forage yield at cutting #5, n=20 for both KK179 and the conventional control; for fall plant height, n=32 for both KK179 and the conventional control; for total forage yield, n=39 for both KK179 and the conventional control; and for spring vigor, spring stand recovery, and spring stand count, n=39 for KK179.
SE = Standard Error
No statistically significant differences were detected between KK179 and the conventional control (α=0.05) using ANOVA.
[1]Reference range is the minimum and maximum mean values among the conventional commercial reference varieties.

Table VII-7. Combined-Site and Combined-Year Comparison of KK179 to the Conventional Control for Phenotypic and Agronomic Characteristics during Forage Production

	KK179	Control	Reference Range[1]	
Phenotypic Characteristic (units)	Mean (SE)	Mean (SE)	Minimum	Maximum
Fall plant height (in)	5.8 (0.31)	6.0 (0.34)	3.3	6.6
Total forage yield (fresh weight ton/acre)	24.5 (1.81)	24.6 (1.88)	16.2	31.0
Spring vigor (1-10 rating)	7.8 (0.17)	7.6 (0.21)	6.0	7.4
Spring stand recovery(1-10 rating)	8.3 (0.15)	8.5 (0.14)	7.3	9.6
Spring stand count (# stems/foot)	58.6 (2.89)	57.4 (2.97)	44.2	71.9

Note: The experimental design was a randomized complete block with four replications. Means based on n=80 for all characteristics except as follows: for fall plant height, n=72 for both KK179 and the conventional control; for total forage yield, n=79 for KK179 and n=78 for the conventional control; for spring vigor, spring stand recovery, and spring stand count, n=75 for KK179 and n=76 for the conventional control.
SE = Standard Error
No statistically significant differences were detected between KK179 and the conventional control (α=0.05) using ANOVA.
[1]Reference range is the minimum and maximum mean values among the conventional commercial reference varieties.

VII.C.2.2. Environmental Interaction Characteristics of KK179 Managed for Forage Production

USDA-APHIS considers the environmental interactions of the biotechnology-derived crop compared to its conventional control to determine the potential for increased plant pest characteristics. Evaluations of environmental interactions were conducted as part of the plant characterization of KK179 managed under forage production conditions. In the 2010-2012 field trials conducted for evaluation of phenotypic and agronomic characteristics of KK179, data were also collected on plant response to abiotic stressors, (*e.g.*, drought, wind, nutrient deficiencies), disease damage, arthropod-related damage, and arthropod abundance (Tables VII-8 through VII-12 and Tables G-8 through G-18). These data were used as part of the environmental analysis (Section IX) to assess plant pest potential and provide an indication of potential effects of KK179 on non-target organisms (NTOs) compared to the conventional control. In addition, multiple conventional commercial reference varieties were included in the analysis to establish a range of natural variability for each assessed characteristic. The results of the field evaluations showed that the trait for reduced G lignin and total lignin did not unexpectedly alter the assessed environmental interactions of KK179 compared to the conventional control. The lack of significant biological differences in plant responses to abiotic stress, disease damage, arthropod-related damage, alfalfa weevil damage, potato leafhopper damage, and pest- and beneficial-arthropod abundance support the conclusion that the introduction of the trait for reduced G lignin and total lignin is not expected to result in increased plant pest potential or to have an effect on the environment for KK179 compared to conventional alfalfa.

VII.C.2.2.1. Qualitative Environmental Interactions Assessment

KK179 was compared to the conventional control for qualitative environmental interactions in 2010 and 2011. Qualitative assessments were conducted at 10 sites and included plant responses to abiotic stressors, disease damage, and arthropod damage. The number of observation times at a site depended on the number of cuttings at that site. Assessments were conducted three to four times during the 2010 growing season and four to five times during the 2011 growing season.

Plant responses to abiotic stressors, disease damage, and arthropod damage were assessed at natural levels, *i.e.*, no artificial infestation or imposed abiotic stress; therefore these levels typically varied between observations at a site and among sites. Plant responses to abiotic stress, disease damage, and arthropod damage data were collected from each plot using a $0 - 9$ scale of increasing severity of observed damage for each stressor. This scale was utilized to allow for the evaluation of the wide variety of potential abiotic stressor, disease damage, and arthropod damage symptoms potentially occurring across the season and across sites. Due to the non-specific nature of the scale used, the data were not statistically analyzed but rather were placed into one of the following categories: none (0), slight (1-3), moderate (4-6), or severe (7-9) and then expressed as a range of responses observed across the four replications at a site (e.g, none to slight). For a particular stressor, all comparisons of the range of responses for KK179 to the range of responses for the conventional control across all observation times and sites are reported.

Descriptions of the evaluated environmental interactions characteristics and the timing of the evaluations are listed in Table VII-1. The materials, methods, additional details concerning the qualitative environmental interactions assessments, and detailed results of the qualitative data comparisons are presented and discussed in Appendix G (Tables G-8 through G-10 and G-14 through G-18).

In 2010, no differences in the range of responses were observed between KK179 and the conventional control for any of the 93 comparisons of plant response to abiotic stressors, including drought, flood, frost, hail, heat, nutrient deficiency, soil compaction, and wind (Table VII-8 and Table G-8). Additionally, no differences in the range of responses were observed between KK179 and the conventional control for any of the 93 comparisons for plant damage caused by diseases, including Anthracnose, bacterial wilt, black stem, damping-off, downy mildew, Fusarium wilt, leaf spots, root rot, Sclerotinia crown and stem rot, stem nematode, and Verticillium wilt (Table VII-8 and Table G-9). Finally, no differences in the range of responses were observed between KK179 and the conventional control for any of the 96 comparisons for plant damage caused by arthropods, including alfalfa caterpillar, alfalfa weevil, aphid, armyworm, blister beetle, cutworm, grasshopper, meadow spittlebug, plant bug, potato leafhopper, spider mite, and thrips (Table VII-8 and Table G-10).

In 2011, no differences in the range of responses were observed between KK179 and the conventional control for any of the 129 comparisons of plant response to abiotic stressors, including drought, frost, hail, heat, heaving, nutrient deficiency, soil compaction, wet soil, wind, and winter injury kill (Table VII-9 and Table G-14). Additionally, no differences in the range of responses were observed between KK179 and the conventional control for any of the 129 comparisons for plant damage caused by diseases, including Anthracnose, bacterial wilt, black stem, crown rot, downy mildew, Fusarium wilt, leaf spots, root rot, Sclerotinia crown and stem rot, and Verticillium wilt (Tables VII-9 and G-15). Finally, no differences in the range of responses were observed between KK179 and the conventional control for any of the 129 comparisons for plant damage caused by arthropods, including alfalfa caterpillar, alfalfa leafminer, alfalfa weevil, aphid, armyworm, bean leaf beetle, blister beetle, cutworm, grasshoppers, green cloverworm, Japanese beetle, Lygus bug, meadow spittlebug, plant bug, potato leafhopper, southern corn rootworm beetle, spider mite, and thrips (Table VII-9 and Table G-16).

The lack of differences observed between KK179 and the conventional control for plant responses to abiotic stressors, disease damage, and arthropod-related damage in multiple environments across the U.S. and Canada supports the conclusion that the introduction of the trait for reduced G lignin and total lignin is not expected to cause a biologically meaningful change in terms of plant pest/weed potential or to have an effect on the environment for KK179 compared to the conventional control (See Section VII.B.2.) .

Table VII-8. Summary of Qualitative Environmental Interactions Assessments in the First Year (2010)

Stressor	Number of observations across all sites in 2010	Number of observations with no differences between KK179 and the conventional control across all sites in 2010[1]
Abiotic stressors	93	93
Disease damage	93	93
Arthropod-related damage	96	96
Total	282	282

Note: The experimental design was a randomized complete block with four replications. Observations were made during each crop growth cycle, prior to harvest.
No differences were observed between KK179 and the conventional control during any observation for damage caused by any of the assessed stressors.
[1]KK179 and the conventional control were considered different in susceptibility or tolerance if the range of injury symptoms across four replications did not overlap between KK179 and the conventional control.

Table VII-9. Summary of Qualitative Environmental Interactions Assessments in the Second Year (2011)

Stressor	Number of observations across all sites in 2011	Number of observations with no differences between KK179 and the conventional control across all sites in 2011[1]
Abiotic stressors	129	129
Disease damage	129	129
Arthropod-related damage	129	129
Total	387	387

Note: The experimental design was a randomized complete block with four replications. Observations were made during each crop growth cycle, prior to harvest.
No differences were observed between KK179 and the conventional control during any observation for damage caused by any of the assessed stressors.
[1]KK179 and the conventional control were considered different in susceptibility or tolerance if the range of injury symptoms across four replications did not overlap between KK179 and the conventional control.

VII.C.2.2.2. Quantitative Environmental Interactions Assessment

Quantitative arthropod assessments were conducted at three sites (ILCL, KSLA, NYNR) and included alfalfa weevil damage, potato leafhopper damage, and pest- and beneficial-arthropod abundance. Each quantitative assessment occurred three to four times in 2010 and four to five times in 2011 based on the number of cuttings at each site. Alfalfa weevil and potato leafhopper damage were assessed in each plot from 10 non-systematically selected 6 × 6 inch areas using an arthropod-specific 0–5 rating scale of increasing severity. Areas were evaluated because individual alfalfa plants in direct seeded plots are very difficult to separate.

Damage data were collected for the same two arthropod species at the three sites in 2010 and in 2011; Both an individual-site analysis and a combined-site analysis were conducted, in which the data were pooled among the three sites for each single year separately. Descriptions of the evaluated environmental interactions characteristics and the timing of the evaluations are listed in Table VII-1. The materials, methods, additional details concerning the specific arthropod damage assessments, and detailed results of the individual-site data comparisons are presented and discussed in Appendix G (Tables G-11 and G-17). The results of the combined-site analysis are summarized below.

In the combined-site analyses in both 2010 and in 2011, no statistically significant differences were detected between KK179 and the conventional control for any observations of plant damage caused by alfalfa weevil or potato leafhopper (Tables VII-10 and VII-11). Considering the lack of differences, in both years, the results indicate there was no biological difference in alfalfa weevil or potato leafhopper damage that would contribute to increased plant pest/weed of KK179 compared to the conventional control (See Figure VII-1, Step 2, answer “no”).

Pest- and beneficial-arthropod abundance data were also collected at the three sites in 2010 and in 2011. Variations in temporal activity and geographical distribution of arthropod taxa occur between sites, therefore, only individual-site analyses were conducted for arthropod abundance data in each year. Additional details of the arthropod abundance assessments and detailed results of the individual-site data comparisons are provided in Appendix G (Tables G-12, G-13, G-18, and G-19). The results of these analyses are summarized below and in Table VII-12.

In 2010, a total of 69 comparisons were made between KK179 and the conventional control for arthropod abundance involving the following pest- and beneficial-arthropods: aphid, alfalfa weevil, alfalfa looper, false chinch bug, green cloverworm, garden webworm, thrips, damsel bug, ladybird beetle, parasitic wasps, and lacewing. No statistically significant differences were detected between KK179 and the conventional control for 65 out of 69 comparisons, including 39 pest-arthropod comparisons and 30 beneficial-arthropod comparisons (Tables G-12 and G-13). Of the four differences detected, there were two statistically significant differences (α=0.05) in pest-arthropod abundance and two statistically significant differences in beneficial-arthropod abundance.

In the assessment of pest arthropod-abundance, KK179 had significantly lower abundance of potato leafhopper in collection #1 at ILCL (152.3 vs. 329.0) and KK179 had significantly higher abundance of Lygus bug than the conventional control in collection #2 at KSLA (1.3 vs. 0.0; Table G-12). The mean abundance values of potato leafhopper in collection #1 at ILCL and of Lygus bug for collection #2 at KSLA were outside the respective range of the conventional commercial reference varieties for that site and collection time (178.8 – 256.8 and 0.0 – 0.3, respectively). However, the differences for these taxa were not consistently detected across collection times or sites. Thus, the detected differences in arthropod abundance were not considered to be biologically meaningful in terms of plant pest/weed potential or potential to have an effect on the environment for KK179 compared to the conventional control.

In the assessment of beneficial-arthropod abundance, KK179 had significantly lower abundance of spiders than the conventional control in collection #2 at ILCL (0.0 vs. 1.5) and at KSLA (0.0 vs.1.3; Table G-13). The mean abundance values of KK179 were within the respective range of the conventional commercial reference varieties for that site and collection time. In addition, the difference in abundance of spiders was not consistently detected across collection times and sites. Thus, the detected differences were not considered to be biologically meaningful in terms of plant pest/weed potential of KK179 compared to the conventional control (See Section VII.B.2.).

In 2011, a total of 83 comparisons were made between KK179 and the conventional control for arthropod abundance involving the following pest- and beneficial-arthropods: aphid, armyworm, false chinch bug, blister beetle, green cloverworm, Lygus bug, meadow spittlebug, potato leafhopper, thrips, soybean looper, spiders, ladybird beetle, lacewing, nabids, and chalcid wasps. No statistically significant differences were detected between KK179 and the conventional control for 82 out of 83 comparisons, including 50 pest-arthropod comparisons and 33 beneficial-arthropod comparisons (Tables G-18 and G-19).

In the assessment of pest-arthropod abundance, KK179 had a significantly lower abundance of alfalfa weevils compared to the conventional control for collection #1 at ILCL (0.3 vs. 7.0; Table G-18). The mean abundance value of KK179 was within the range of the conventional commercial reference varieties for that site and collection time. Thus, the detected difference was not considered to be biologically meaningful in terms of plant pest/weed potential or potential to have an effect on the environment for KK179 compared to the conventional control (See Section VII.B.2.).

Table VII-10. Combined-Site Comparison of Potato Leafhopper and Alfalfa Weevil Damage to KK179 Compared to the Conventional Control in the First Year (2010)

		Damage (0-10 rating scale)			
		KK179	Control	Reference Range[1]	
Arthropod	Observation	Mean (SE)	Mean (SE)	Minimum	Maximum
Alfalfa weevil (*Hypera postica*)	1	0.1 (0.04)	0.2 (0.07)	0.0	0.7
	2	0.2 (0.07)	0.2 (0.11)	0.0	0.9
	3	0.0 (0.02)	0.1 (0.03)	0.0	0.3
	4	† 0.0 (0.00)	0.0 (0.00)	0.0	0.0
Potato leafhopper (*Empoasca fabae*)	1	0.4 (0.09)	0.5 (0.11)	0.0	0.9
	2	0.2 (0.11)	0.3 (0.11)	0.0	0.5
	3	0.0 (0.03)	0.0 (0.01)	0.0	0.1
	4	† 0.0 (0.00)	0.0 (0.00)	0.0	0.0

Note: The experimental design was a randomized complete block with four replications. Means based on n=12 except for Observation #4, in which n=4 for both KK179 and the conventional control.
SE = Standard Error
No statistically significant differences were detected between KK179 and the conventional control (α=0.05) using ANOVA.
† No statistical comparisons were made due to lack of variability in the data.
[1]Reference range is the minimum and maximum mean values among the four conventional commercial reference varieties.

Table VII-11. Combined-Site Comparison of Potato Leafhopper and Alfalfa Weevil Damage to KK179 Compared to the Conventional Control in the Second Year (2011)

		Damage (0-10 rating scale)			
		KK179	Control	Reference Range[1]	
Arthropod	Observation	Mean (SE)	Mean (SE)	Minimum	Maximum
Alfalfa weevil (*Hypera postica*)	1	1.2 (0.47)	0.9 (0.38)	0.0	2.3
	2	0.2 (0.09)	0.2 (0.10)	0.0	0.6
	3	0.2 (0.08)	0.2 (0.08)	0.0	0.6
	4	0.0 (0.02)	0.0 (0.02)	0.0	0.2
	5	† 0.0 (0.00)	0.0 (0.00)	0.0	0.0
Potato leafhopper (*Empoasca fabae*)	1	0.8 (0.31)	0.6 (0.23)	0.0	1.5
	2	0.1 (0.06)	0.1 (0.05)	0.0	0.4
	3	0.1 (0.05)	0.1 (0.06)	0.0	0.3
	4	0.7 (0.27)	0.8 (0.27)	0.0	1.4
	5	† 0.0 (0.00)	0.0 (0.00)	0.0	0.0

Note: The experimental design was a randomized complete block with four replications. Means based on n=12 except for Observation #5, in which n=4 for both KK179 and the conventional control.
SE = Standard Error
No statistically significant differences were detected between KK179 and the conventional control (α=0.05) using ANOVA.
† No statistical comparisons were made due to lack of variability in the data.
[1]Reference range is the minimum and maximum mean values observed among the four conventional commercial reference varieties.

Table VII-12. Summary of Arthropod Abundance Assessments and Detected Differences in 2010 and 2011

Summary of Statistical Comparisons[1]

Arthropod Abundance Assessment	Number of sites	Number of comparisons across sites	Number of comparisons where no differences were detected
2010			
Pest	3	39	37
Beneficial	3	30	28
2011			
Pest	3	50	49
Beneficial	3	33	33

Summary of Detected Differences[2]

Arthropod	Site	Collection Number	Within reference range?	Consistently detected across collections or sites?
2010				
Potato leafhopper	ILCL	1	No	No
Lygus bug	KSLA	2	No	No
Spiders	ILCL	2	Yes	No
	KSLA	2	Yes	No
2011				
Alfalfa weevil	ILCL	1	Yes	No
–	–	–	–	–

[1]Quantitative arthropod abundance assessments were statistically analyzed at α=0.05 using ANOVA.
[2]Five statistically significant differences were detected. These differences are further assessed following Section VII.B.2.
– Indicates no differences were detected.

VII.C.2.3. Field Phenotypic and Agronomic Characteristics of KK179 Managed for Seed Production

Alfalfa seed production requires different environmental conditions and agronomic practices than alfalfa grown for forage. Alfalfa seed production is limited globally to more arid regions, and more than 85% of all seed production occurs in the five U.S. Western states of California, Idaho, Washington, Nevada, and Oregon (Mueller, 2008; Rincker et al., 1988). Seed production fields are typically planted in rows and are seeded at a much lower density than forage production fields (Mueller, 2008). Thinner stands help maximize seed yield, while dense stands help maximize forage yield. As a result of this agronomic practice, plants in seed production fields tend to have an increased number of stems per plant and an increased number of pods per stem (Mueller, 2008). Additionally, agronomic and phenotypic characteristics at reproductive growth stages may only be assessed in seed production fields, as forage is typically cut between late vegetative and early bloom stages.

A seed production study was established in 2010 in Canyon County, Idaho, which represents an environment that is appropriate for commercial alfalfa seed production in North America. The experimental design was a randomized complete block with six replications. All plots of KK179, the conventional control, and seven conventional commercial reference varieties were uniformly managed under the agronomic practices for seed production, in order to assess whether the introduction of the trait for reduced G lignin and total lignin altered the phenotypic and agronomic characteristics of KK179 compared to the conventional control.

Descriptions of the evaluated phenotypic characteristics and the timing of the evaluations during seed production are listed in Table VII-13. The details of the materials and experimental methods used in this evaluation are presented in Appendix H.

No statistically significant differences (α=0.05) were detected between KK179 and the conventional control for seedling establishment, seedling vigor, seed weight, seed per pod, or seed yield (Table VII-13). Additionally, no statistically significant differences were detected between KK179 and the conventional control for lodging or split pods, both characteristics related to weediness. Thus, the results demonstrate that there were no unexpected changes in phenotype indicative of increased plant pest/weed potential or potential to have an effect on the environment for KK179 compared to the conventional control (See Figure VII-1, Step 1, answer “no”).

The results of the agronomic and phenotypic assessment demonstrate that there were no unexpected changes in the phenotype of KK179 compared to the conventional control when managed under the agronomic practices for seed production. Thus, the introduction of the trait for reduced G lignin and total lignin is not expected to result in increased plant pest/weed potential of KK179 compared to conventional alfalfa.

Table VII-13. Comparison of KK179 to the Conventional Control for Phenotypic and Agronomic Characteristics during Seed Production

	Mean (SE)		Reference Range[1]	
Characteristic (units)	KK179	Control	Minimum	Maximum
Seedling establishment (# plants/plot)	282.3 (35.72)	307.3 (26.53)	206.7	328.5
Seedling vigor (1-10)	7.3 (0.49)	7.3 (0.49)	5.8	7.5
Lodging (0-9)	6.0 (0.37)	5.7 (0.49)	5.3	7.0
Seed weight (mg)	2.5 (0.05)	2.5 (0.07)	2.3	2.6
Seed per pod (# seed/pod)	4.9 (0.40)	4.7 (0.34)	4.7	6.0
Split pods (%)	0.1 (0.14)	0.1 (0.13)	0.0	0.5
Seed yield (g/plot)	622.8 (27.14)	772.7 (89.71)	627.0	867.7

Note: The experimental design was a randomized complete block design with six replications (n=6).
SE = Standard Error
No significant differences were detected between KK179 and the conventional control (α=0.05) using ANOVA.
[1]Reference range is the minimum and maximum mean values observed among the four conventional commercial reference varieties.

VII.C.3. Reproductive Characteristics

USDA-APHIS considers the potential for gene flow and introgression of the biotechnology-derived trait into other alfalfa varieties and wild relatives to assess the potential for increased weedy or invasive characteristics of the receiving species. Information on reproductive characteristics including pollen morphology and viability and flower morphology are pertinent to this assessment, and therefore were assessed for KK179. In addition, characterizations of pollen and flowers produced by KK179 and the conventional control are relevant to the plant pest risk assessment because they add to the detailed description of the phenotype of KK179 compared to the conventional control.

VII.C.3.1. Pollen

The viability and morphology of pollen collected from KK179 compared to that of the conventional control was also assessed. KK179, the conventional control, and four conventional commercial reference varieties were grown under similar agronomic conditions in a greenhouse. The trial was arranged in a randomized complete block design with four replications, with 10 plants of each material per replication. Once all plants across the replications reached the flowering stage, flowers were collected and pollen extracted and stained for assessment.

Descriptions of the evaluated viability and morphology characteristics and the timing of the evaluations are listed in Table VII-1. The details of the materials and experimental methods used in this evaluation are presented in Appendix I.

No statistically significant differences (α=0.05) were detected between KK179 and the conventional control for percent viable pollen or pollen grain diameter (Table VII-14). Furthermore, no visual differences in general pollen morphology were observed between KK179 and the conventional control (Figure I-1).

The pollen characterization data contribute to the detailed phenotypic description of KK179 compared to the conventional control. Based on the assessed characteristics, the results support a conclusion that neither pollen viability nor morphology of KK179 were altered compared to conventional alfalfa.

Table VII-14. Pollen Characteristics of KK179 Compared to the Conventional Control

	KK179	Control	Reference Range[1]	
Pollen Characteristic	Mean (SE)	Mean (SE)	Minimum	Maximum
Viability[2] (%)	89.2 (2.72)	88.4 (1.06)	86.0	89.9
Diameter[3] (μm)	32.6 (0.63)	33.2 (0.45)	32.5	33.7

Note: The experimental design was a randomized complete block design with four replications (n=4).
SE = Standard Error
No significant differences were detected between KK179 and the conventional control (α=0.05) using ANOVA.
[1]Reference range is the minimum and maximum mean values observed among the four conventional commercial reference varieties.
[2]Evaluated from five sub-samples per replication for four replications.
[3]Recorded from ten representative viable pollen grains per replication.

VII.C.3.2. Flowers

The purpose of this evaluation was to assess the morphology of flowers collected from KK179 compared to those of the conventional control. Flowers were collected from KK179, the conventional control, and four conventional commercial reference varieties grown under similar agronomic conditions in a Wisconsin field trial. The field trial was arranged in a randomized complete block design with four replications. Flowers were collected from the field once all plots had reached approximately 10% bloom.

Descriptions of the evaluated phenotypic characteristics and the timing of the evaluations are listed in Table VII-1. The details of the materials and experimental methods used in this evaluation are presented in Appendix J.

No statistically significant differences (α=0.05) were detected between KK179 and the conventional control for any of the assessed flower characteristics including number of flowers per raceme, standard petal length, keel petal length, calyx tube diameter, sexual column length, and wing petal length (Table VII-15). Furthermore, no visual differences in flower color class, gross raceme morphology, or gross flower morphology were observed between KK179 and the conventional control (Figures J-2 and J-3).

The flower characterization data contribute to the detailed phenotypic description of KK179 compared to the conventional control. Based on the assessed characteristics, the results support a conclusion that flower morphology of KK179 was not altered compared to conventional alfalfa.

Table VII-15. Flower Characteristics of KK179 Compared to the Conventional Control

	Mean (SE)[1]		Reference Range[2]	
Characteristic (units)	KK179	Control	Minimum	Maximum
Flowers per raceme (#)	15.28 (0.71)	15.45 (0.67)	10.35	17.03
Standard petal length (mm)	10.60 (0.07)	10.43 (0.10)	10.33	10.80
Keel petal length (mm)	7.63 (0.15)	7.33 (0.13)	7.28	7.43
Calyx tube diameter (mm)	1.91 (0.02)	1.93 (0.05)	1.83	1.95
Sexual column length (mm)	7.50 (0.09)	7.58 (0.11)	7.33	7.45
Wing petal length (mm)	8.60 (0.14)	8.53 (0.12)	8.20	8.45
Flower color class[3]	Variegated dark purple	Variegated dark purple	Light purple	Variegated dark purple
Gross raceme morphology	Typical	Typical	Typical	Typical
Gross flower morphology	Typical	Typical	Typical	Typical

Note: The experimental design was a randomized complete block design with four replications.
SE = Standard Error
No significant differences in flowers per raceme, standard petal length, keel petal length, calyx tube diameter, sexual column length, or wing petal length were detected between KK179 and the control (α=0.05) using ANOVA. No visual differences in flower color class, gross raceme morphology, or gross flower morphology were observed between KK179 and the conventional control.
[1]Means based on n=4. Flower color class, gross raceme morphology, and gross flower morphology data were not subjected to statistical analysis.
[2]The reference range for flower characteristics statistically analyzed is the minimum and maximum mean values from among the conventional commercial reference varieties. The reference range for those characteristics that were not statistically analyzed consists of the minimum and maximum observed ratings from among the conventional commercial reference varieties.
[3]Flower color classification (USDA-ARS, 1972).

VII.C.4. Symbiont Interactions

As part of the plant pest risk assessment, USDA-APHIS considers the impact of the biotechnology-derived crop on plant pest potential and the environment as well as on agricultural or cultivation practices compared to its conventional counterpart. Potential changes in the symbiotic relationship between alfalfa and members of the bacterial families, *Rhizobiaceae* and *Bradyrhizobiaceae*, which inhabit the rhizosphere, could effect plant pest potential or agronomic practices, ***i.e.***, the need to add additional nitrogen to sustain alfalfa production. In alfalfa, atmospheric nitrogen is fixed into organic nitrogen through a symbiotic association with the bacterium *Sinorhizobium meliloti,* which can contribute up to 64% of alfalfa's nitrogen requirement (Lanyon and Griffith, 1988). Thus, the purpose of this evaluation was to assess whether the introduction of the trait for reduced G lignin and total lignin altered the symbiotic interaction of KK179 with *Sinorhizobium meliloti* compared to that of the conventional control.

KK179, the conventional control, and six conventional commercial reference varieties were grown under similar agronomic conditions in a greenhouse. Descriptions of the evaluated phenotypic characteristics are listed in Table VII-16. The details of the materials and experimental methods used are presented in Appendix K.

No statistically significant differences (α=0.05) were detected between KK179 and the conventional control for each measured parameter, including dry weight of nodules, root material, shoot material, shoot percent total nitrogen, and shoot total nitrogen (Table VII-16).

Based on the assessed characteristics, the lack of statistical differences between KK179 and the conventional control indicate that introduction of the trait for reduced G lignin and total lignin did not alter the symbiotic relationship between *S. meliloti* and KK179 compared to that of conventional alfalfa. Thus, the results demonstrate that there were no unexpected changes indicative of increased plant pest/weed potential or potential to have an effect on the environment for KK179 compared to the conventional control.

Table VII-16. Symbiont Interaction Assessment of KK179 and the Conventional Control

	Mean (SE)		Reference Range[1]	
Measurements	KK179	Control	Minimum	Maximum
Nodule Dry Wt (g)	0.23 (0.02)	0.24 (0.02)	0.19	0.25
Root Dry Wt (g)	3.01 (0.41)	3.06 (0.46)	3.08	3.41
Shoot Dry Wt (g)	5.22 (0.58)	5.11 (0.61)	4.98	6.35
Shoot Percent Total Nitrogen (% dwt)	3.49 (0.03)	3.46 (0.08)	3.17	3.53
Shoot Total Nitrogen (g)	0.18 (0.02)	0.18 (0.02)	0.16	0.21

Note: The experimental design was a randomized complete block design with 10 replications (n=10 for KK179 and n=8 for the conventional control).
SE = Standard Error
No statistically significant differences were detected between KK179 and the conventional control (α=0.05) using ANOVA.
[1]Reference range is the minimum and maximum mean values observed among the six conventional commercial reference varieties.

VII.D. Conclusions for Phenotypic, Agronomic, and Environmental Interactions Evaluations

Comparative plant characterization data between a biotechnology-derived crop and the conventional control are interpreted in the context of contributions to increased plant pest/weed potential as assessed by USDA-APHIS. Under the framework of familiarity, characteristics for which no differences are detected support a conclusion of no increased plant pest/weed potential of the biotechnology-derived crop compared to the conventional crop. Ultimately, a weight-of-evidence approach that considers all characteristics and data is used for the overall risk assessment of differences and their significance.

An extensive and robust set of agronomic, phenotypic, and environmental interactions data, including specific weedy characteristics, were used to assess whether the introduction of the trait for reduced G lignin and total lignin altered the plant pest potential of KK179 compared to the conventional control, considered within the context of the variation among the conventional commercial reference varieties. These assessments included seven general data categories: 1) seed germination, dormancy, and emergence; 2) vegetative growth; 3) winter survival; 4) reproductive development including pollen, flower, and seed characteristics; 5) lodging and seed retention on the plant; 6) plant response to abiotic stress and interactions with diseases and arthropods; and 7) plant-symbiont interactions. Within these data categories, data relevant to understanding specific characteristics associated with weediness were also assessed to determine whether there was a potential increase in weediness of KK179 compared to conventional alfalfa.

Results from the phenotypic, agronomic, and environmental interactions assessments comparing KK179 and the conventional control demonstrate that KK179 does not possess: 1) increased weediness characteristics; 2) increased susceptibility or tolerance to specific abiotic stress, diseases, or arthropods; or 3) characteristics that would confer a plant pest risk compared to conventional alfalfa. Therefore, based on the results of multiple assessments discussed above and presented in the appendices, the weight of evidence indicates that KK179 is not meaningfully different from conventional alfalfa with the exception of the trait for reduced G lignin and total lignin and is not expected to pose a plant pest/weed risk compared to conventional alfalfa.

VIII. U.S. AGRONOMIC PRACTICES

VIII.A. Introduction

As part of the plant pest assessment required by 7 CFR § 340.6(c)(4), impacts of deregulation on agricultural and cultivation practices must be considered. This section provides a summary of current agronomic practices in the U.S. for producing alfalfa and is included in this petition as a baseline to assess possible impacts to agricultural practices due to the cultivation of KK179. Discussions include the importance of alfalfa as a crop; alfalfa production; seed production; plant growth and development; practices for the establishment, management, and termination of alfalfa stands; pest management practices; alfalfa rotational crops; and volunteer and feral alfalfa management. Information on U.S. alfalfa agronomic practices can be found in the published literature and in USDA-APHIS Environmental Impact Statement for Roundup Ready alfalfa (Section III and Appendices G, H, and J) (USDA-APHIS, 2010). The overview presented here in Section VIII collectively discusses the agricultural practices from those sources.

Information presented in Section VII demonstrated that KK179 is no more susceptible to diseases or pests than conventional alfalfa. Additionally, data presented in Section VII show that KK179 is not expected to pose a plant pest risk compared to conventional alfalfa. Thus, there are no changes to the inputs needed for KK179, and no specific impacts to most of the agronomic practices employed for the production of alfalfa. Where there is potential impact on agronomic practices from the deregulation of KK179, discussion delineating the scope and magnitude of those impacts is provided.

Alfalfa (*Medicago sativa* L.) is grown for forage, grazing, and seed production. Alfalfa is one of the most important forage crops in the U.S. and ranks fourth on the list of most widely grown crops by acreage, behind corn, soybean, and wheat (USDA-APHIS, 2010). In terms of value, alfalfa is ranked fourth among agricultural crops. More than 20 million acres of alfalfa forage production have been grown annually since 1950 (USDA-NASS, 2011b). Alfalfa grows in a wide range of soils and climates as evidenced by production in all continental states, Alaska and Hawaii (USDA-APHIS, 2010). Although alfalfa production practices vary by region due to climatic differences, some practices are similar among growing regions. Alfalfa production has two distinct growing phases: the establishment phase and the production phase associated with established stands (USDA-APHIS, 2010). Proper seedbed preparation, correct variety selection, optimum planting dates, optimum plant population, and good integrated pest management practices are important for optimizing the yield potential and economic returns of alfalfa.

Established mechanical and chemical measures are available to control volunteer alfalfa, *i.e.*, alfalfa plants that carry over from an established stand or seed that have germinated and emerged unintentionally in a subsequent rotational crop. Mechanical and chemical methods are also effective in removing or taking out old, thinning stands when rotating to other crops. Due to the lack of differences between KK179 and conventional alfalfa in phenotypic, agronomic, and environmental interaction assessments presented in Section VII, KK179 is not meaningfully different in its weediness potential. Therefore,

introduction of KK179 in the alfalfa production system would have a negligible impact on managing KK179 volunteer plants in rotational crops such as corn, soybean, sorghum, and wheat. The numerous control measures that are effective on conventional volunteer alfalfa plants will continue to be effective on volunteer KK179 plants should they arise.

As shown in Sections VI and VII, with the exception of the trait for reduced G lignin and total lignin, no biologically meaningful phenotypic, compositional, or environmental interaction differences between KK179 and conventional alfalfa have been observed. Therefore, it is not anticipated that commercialization of KK179 in the U.S. would have a notable impact on current alfalfa cultivation practices.

VIII.B. Overview of U.S. Alfalfa Production

VIII.B.1. Alfalfa Forage Production

Alfalfa is grown today on all continents (except Antarctica) in some capacity, but primarily in temperate climates. Alfalfa was introduced into the United States through a number of channels, first by Spanish missionaries from Mexico into Texas and the Southwest region, and later into the British colonies during the first half of the 18th century. The first known plantings of alfalfa in the U.S. were in Georgia in 1736. The use of alfalfa spread slowly until it was introduced into California during the Gold Rush of 1851, after which it dispersed rapidly throughout the nation (Janick et al., 1974).

Alfalfa is grown for forage, grazing, and seed production. Alfalfa is generally regarded as the "queen of forages" because of its high protein content and highly digestible fiber for ruminants and horses (USDA-APHIS, 2010). The highest quality alfalfa hay is generally used for dairy cows. For instance, dairy farms consume between 75 to 85% of the alfalfa hay production in California (USDA-APHIS, 2010). Another 10 to 15% is consumed by horses. Alfalfa hay that is lower in protein and higher in fiber is fed to beef cattle, horses, heifers, and non-lactating dairy cows. Alfalfa 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 alfalfa in the vegetative state is practiced sometimes for dormant-season alfalfa 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 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. Alfalfa plants are also used for a variety of non-agricultural purposes, including rehabilitation of over-grazed rangelands, erosion-control projects in interior forests, treatment of compacted soils, re-vegetation of areas damaged by wildfire, and erosion reduction in mined soils (USDA-APHIS, 2010).

Of the 79 million acres grown worldwide, approximately 70% is produced in the United States, Russia, and Argentina (Klonsky et al., 2008). In 2009, when U.S. production was valued at approximately $8 billion, U.S. exports of alfalfa hay were valued (average

prices × volumes) at approximately $354 million and imports at $3.8 million, resulting in a U.S. consumption value of $7.6 billion (USDA-APHIS, 2010). Pacific Rim nations, including Japan, Korea, and Taiwan, are major importers of alfalfa hay and cubes (compressed bales of hay) from the western U.S. (Klonsky et al., 2008).

Alfalfa is a deep-rooted and short-lived perennial plant with a long growing season. Alfalfa grows from early spring until late fall or early winter. Growth begins when the average temperature reaches 50°F and continues until a killing freeze occurs. It is adapted to a wide range of climatic and soil conditions. Deep, medium- to coarse-textured soils with adequate water are ideal (Kansas State University, 1998). Fine-textured soils are usually difficult to manage. In deep, well-aerated soil, roots may extend 8 to 12 feet deep (Kansas State University, 1998). Because of its deep taproot alfalfa 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, using in excess of 40 inches of water during the season (Kansas State University, 1998). During the summer months, the water use is 6 to 7 acre-inches per ton of forage. The water requirements for alfalfa are greater than the annual rainfall in the Great Plains and Western regions of the U.S. Approximately 31% of the total U.S. alfalfa hay acreage was irrigated in 2007 (USDA-NASS, 2007). Excess moisture is conducive to development of root and crown diseases, and shallow water tables limit root growth (Kansas State University, 1998).

Alfalfa is grown for forage in all continental states, Alaska and Hawaii. The distribution of alfalfa grown as forage across the U.S. is presented in Figure VIII-1. Alfalfa hay acreage peaked in the mid-1950s and 60s at approximately 30 million acres, and has slowly declined during the past 40 years to the 2010 level of approximately 20 million acres (USDA-NASS, 2011d). Currently, the harvested acres of alfalfa hay represent approximately 33% of the harvested acres for all types of hay (USDA-NASS, 2011d). Approximately 20 to 24 million acres of alfalfa hay have been harvested annually over the past 10 years (Table VIII-1). Approximately 2.5 to 3.3 million acres (12-13% of the harvested acres) are seeded annually for new alfalfa stands (Table VIII-1). Annual production has ranged from 68 to 82 million tons of hay. Average yields have remained fairly constant at 3.19 to 3.47 tons per acre over that same period. The annual value of production has ranged from $6.7 to $10.7 billion (due to most alfalfa being fed to livestock on-farm, the value is an estimate based on multiplying average prices with production volumes and does not correspond to actual sales). Thus, alfalfa has been and continues to be an important U.S. crop.

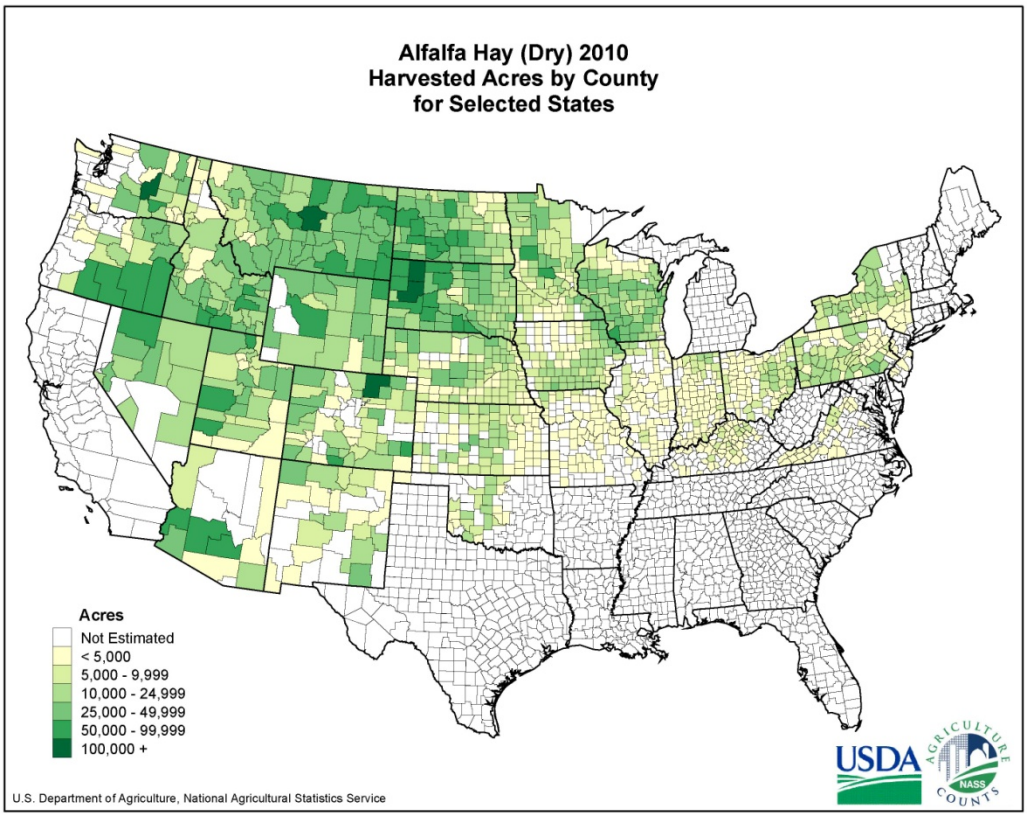


Figure VIII-1. Geographic distribution of alfalfa hay harvested acres in the U.S.
Source: (USDA-NASS, 2010).

Table VIII-1. Alfalfa Hay Production in the U.S. from 2000 to 2010[1]

Year	Seeded Alfalfa Acres (000)	Harvested Acres (000)	Production (000 tons)	Yield (tons/acre)	Value of Production[2] ($000)
2000	3,065	23,463	81,520	3.47	6,812,286
2001	3,260	23,952	80,354	3.35	7,533,401
2002	3,282	22,923	73,014	3.19	7,137,469
2003	3,119	23,527	76,098	3.23	6,707,172
2004	2,793	21,697	75,375	3.47	6,961,519
2005	3,290	22,359	75,610	3.38	7,290,854
2006	3,184	21,138	70,548	3.34	7,519,232
2007	2,828	21,126	69,880	3.31	8,855,044
2008	2,699	21,060	70,180	3.33	10,747,161
2009	2,665	21,247	71,072	3.35	7,941,539
2010	2,545	19,966	67,971	3.40	7,728,468

[1] Source: (USDA-NASS, 2011c)
[2] The value is an estimate based on multiplying average prices with production volumes and does not correspond to actual sales.

For purposes of this agronomic practices discussion, alfalfa production (alfalfa and alfalfa mixtures) is divided into six major alfalfa growing regions: North Central region (IA, MN, ND, SD, WI), East Central region (AR, DE, IL, IN, KY, MD, MI, MO, New England states, NJ, NY, NC, OH, PA, TN, VI, WV), Plains region (KS, NE, OK, TX), Intermountain region (CO, MT, UT, WY), Pacific Northwest region (ID, NV, OR, WA), and Southwest region (AZ, CA, NM) (Table VIII-2). Approximately 20 million acres of alfalfa hay and 2 million acres of alfalfa haylage were harvested in 2010. The largest acreage of alfalfa (hay and haylage) is grown in the North Central region (8.2 million acres) followed by the East Central region (4.2 million acres). The North and East Central regions grow 56% of the total alfalfa acreage. The states with the largest acreage are South Dakota (2.2 million), Wisconsin (2.2 million), Montana (2.0 million), North Dakota (1.6 million), and Minnesota (1.3 million), which represent 42% of the total U.S. acreage. The highest yields were reported in the Pacific Northwest and Southwest regions with 4.6 and 6.8 tons per acre, respectively. The yields in the other regions range from 2.8 to 3.9 tons per acre. The U.S. produced 79 million tons of alfalfa hay and haylage in 2010. Over 50 percent was produced in the North and East Central regions and 32% in the 5-state area of the North Central region alone. California produced the largest amount of alfalfa hay (6,549,000 tons), but Wisconsin produced the largest amount of hay and haylage combined (8,846,000 tons). The value of the alfalfa production in the U.S. totaled $7.7 billion in 2010 at an average price of $118 per ton.

Table VIII-2. Alfalfa and Alfalfa Mixtures Forage (Hay and Haylage) Production by State in 2010[1].

Region/State	Harvest Acres (000)	Average Yield (tons/acre)	Production (000 tons)	Value of Hay Production[2] ($000)
North Central Region				
Iowa	910	3.6	3,233	347,072
Minnesota	1,315	3.7	4,916	435,600
North Dakota	1,560	2.3	3,588	218,868
South Dakota	2,185	2.4	5,245	417,960
Wisconsin	2,200	4.0	8,846	399,620
Totals	**8,170**	**3.2**[4]	**25,828**	**1,819,120**
East Central Region				
Arkansas	10	3.5	35	4,760
Delaware	5	3.4	17	3,247
Illinois	360	3.9	1,418	174,420
Indiana	300	3.6	1,080	150,120
Kentucky	230	2.8	644	88,228
Maryland	40	3.0	120	22,920
Michigan	950	3.4	3,249	226,800
Missouri	250	2.9	731	90,720
New England States[3]	96	3.5	339	17,966
New Jersey	20	2.9	58	8,352
New York	740	3.2	2,391	110,250
North Carolina	5	3.2	16	2,480
Ohio	420	3.6	1,508	205,920
Pennsylvania	650	3.2	2,089	191,100
Tennessee	15	3.4	51	9,129
Virginia	80	2.3	184	31,280
West Virginia	20	2.6	52	8,060
Region Totals	**4,191**	**3.3**[4]	**13,982**	**1,350,162**

Table VIII-2 (continued). Alfalfa and Alfalfa Mixtures Forage (Hay and Haylage) Production by State in 2010[1].

Region/State	Harvest Acres (000)	Average Yield (tons/acre)	Production (000 tons)	Value of Production[2] ($000)
Plains Region				
Kansas	665	3.8	2,536	279,110
Nebraska	895	4.2	3,714	284,622
Oklahoma	310	3.3	1,023	142,197
Texas	130	4.8	625	109,800
Region Totals	**2,000**	**3.9**[4]	**7,898**	**815,729**
Intermountain Region				
Colorado	820	3.5	2,870	367,360
Montana	1,950	2.3	4,485	354,315
Utah	540	4.0	2,160	228,960
Wyoming	620	2.6	1,612	149,916
Region Totals	**3,930**	**2.8**[4]	**11,127**	**1,100,551**
Pacific Northwest Region				
Idaho	1,150	4.5	5,208	602,742
Nevada	280	4.3	1,204	151,704
Oregon	415	4.3	1,785	267,750
Washington	465	5.0	2,329	299,250
Region Totals	**2,310**	**4.6**[4]	**10,526**	**1,321,446**
Southwest Region				
Arizona	280	8.2	2,296	296,184
California	960	6.8	6,549	841,092
New Mexico	229	5.2	1,182	184,184
Region Totals	**1,469**	**6.8**[4]	**10,027**	**1,321,460**
U.S. Totals	**22,070**	**3.6**[4]	**79,388**	**7,728,468**

[1] Source: (USDA-NASS, 2011c)

[2] The value is an estimate based on multiplying average prices with production volumes and does not correspond to actual sales. Excludes the value of haylage.

[3] New England States include Connecticut, Maine, Massachusetts, New Hampshire, Rhode Island, and Vermont.

[4] Average yield of Regional and U.S. Totals are calculated by dividing the corresponding Production (000 tons) by Harvest Acres (000) values.

In the past, alfalfa was viewed in some regions as a low-value rotation or pasture crop and used primarily as a supplement to other higher value row and specialty crops (Klonsky et al., 2008). However, alfalfa has become a valuable and profitable crop that competes successfully with many higher-value specialty crops, especially in the high production areas of California. Dairy producers are the most important consumers or purchasers of alfalfa hay (Putnam et al., 2008c; USDA-APHIS, 2010). While the dairy market utilizes alfalfa with quality categories ranging from low to supreme quality grades, high producing dairy cows require high-quality hay (supreme and premium grades) to maximize milk production. The horse industry is also an important market for high-quality alfalfa and alfalfa-grass hay mixtures. The beef, sheep, and goat markets are another important market for alfalfa, but these producers typically put less emphasis on high-quality hay. Medium- and lower-quality hay are frequently acceptable for non-lactating dairy cows and meat-producing livestock. Alfalfa is also used for processed feeds and alfalfa pellets for pets and rabbits. Unlike many other regions, the majority of the alfalfa hay produced in the North and East Central regions is produced and consumed on the same farm (USDA-APHIS, 2010). Whereas in the western regions of the U.S., over 95 percent of the alfalfa is sold as a hay product on the open market (Putnam et al., 2008c).

Alfalfa hay quality is defined by a number of nutritional traits including acid detergent fiber (i.e., cellulose and lignin content), neutral detergent fiber (i.e., lignin, cellulose and hemi-cellulose content), crude protein, total digestible nutrients, and relative feed value (Putnam et al., 2008b; USDA-APHIS, 2010). Certain visual or sensory observations are also important to assess hay quality, such as the presence of weeds, molds, or anti-palatability factors like poor texture, evidence of heating, or unpleasant odor. These factors can significantly affect nutritional value and overall forage quality. The USDA's Agricultural Marketing Service (USDA-AMS) uses the grades of supreme, premium, good, fair, and utility to regularly report average prices for alfalfa in various states (USDA-APHIS, 2010). Prices vary significantly by quality grade and region. Prices paid for supreme grade alfalfa hay averaged $160.63/ton, while fair grade alfalfa averaged $116.48/ton in California (Hanford-Corcoran-Tulare-Visalia counties) in 2010 (USDA-AMS, 2010).

Alfalfa producers can implement one of several different strategies or a combination of the following strategies to market alfalfa: 1) the low-cost production strategy, 2) product-quality differentiation or niche-marketing strategy, and 3) provision of additional service strategy (Klonsky et al., 2008). Producers utilizing the low-cost strategy produce maximum yields, control costs, and compete with other growers primarily on price. Product-quality differentiation or niche-marketing strategy targets certain markets such as the dairy industry to get top dollar for a consistently high-quality alfalfa product. Differentiation can include designations such as certified weed-free hay, certified organic hay, special cubed alfalfa containing grasses for horses, and low-potassium hay. The additional service strategy involves providing special service for the customer such as making hay available to the customer on demand, meeting specific baling requirements, developing favorable payment or delivery contracts, offering quality warrantees, or allowing customers to harvest the alfalfa themselves for silage.

Managing input costs is a major component to the profitability of producing an alfalfa crop. Since alfalfa is a perennial crop and harvested over several years, the analysis of production costs for alfalfa are generally presented in two parts: 1) costs associated with stand establishment, and 2) the annual costs during the production years. Table VIII-3 provides a summary of the variable and fixed costs for producing non-irrigated, conventional alfalfa in the North Central region based on the Ag Decision Maker spreadsheet by Iowa State University (Johanns, 2012). Assuming two cuttings and two tons yield per acre from a spring seeding, variable and fixed costs were $268.70 and $199.00 per acre, respectively, during the establishment year, with a total cost of $467.70 per acre. There was a net return of $31.30 per acre over variable costs, but a negative net return of $167.70 over total costs. Assuming three cuttings, four tons yield per acre per year, and three years of production after establishment year, variable and fixed costs were $225.30 and $189.50 per acre, respectively, during the production years with a total annual cost of $414.80 per acre. Average net return over variable costs during the production years was $374.70 per acre per year. Average net return over total costs was $185.20 per acre per year. When assessing costs and gross returns for both establishment and production (four years) net return per year averaged $289.75 per acre above variable costs and $97.88 above total costs.

Similar cost studies were conducted by the University of California on irrigated conventional alfalfa in the San Joaquin Valley in 2008 (Mueller et al., 2008a). These data are presented in Table VIII-4. In these studies, the fall-seeded alfalfa was assumed to produce a total of nine cuttings yielding eight tons of hay and three green tons (one-ton hay equivalent) of haylage per acre. Operating costs and total overhead costs were $385 and $375 per acre ($760 total), respectively, for establishing alfalfa. Annual operating costs and total overhead costs were $708 and $654 per acre ($1,362 total), respectively, during the production years. Net return above operating costs was $912 per acre per year. Net return above total costs was $258 per acre per year which includes the total cash costs of stand establishment ($478 per acre) amortized over the three-year stand life. In contrast to non-irrigated alfalfa grown in the North Central region, the gross returns are much higher with irrigated alfalfa but the cost of production is substantially higher, mainly due to the cost of irrigation.

Table VIII-3. Costs and Returns for Establishment and Production of Non-Irrigated Conventional Alfalfa in the North Central U.S.[1]

Operations/Cost Item	Establishment Year Cost/Acre ($)	Production Years Cost/Acre ($)
Gross Returns:		
Hay ($150/ton)	300	600
Total Gross Returns	**300**	**600**
Variable Costs:		
Preharvest Machinery	11.80	1.20
Fertilizer	121.50	192.00
Lime	29.00	-
Seed	72.00	-
Herbicide	13.10	-
Harvest	21.30	32.10
Total Operating Costs	**268.70**	**225.30**
Fixed Costs:		
Preharvest Machinery	15.80	1.60
Labor	55.00	44.00
Land (cash rent equivalent)	100.00	100.00
Harvest	28.20	43.90
Total Fixed Costs	**199.00**	**189.50**
Total Costs	**467.70**	**414.80**
Net Return Above Variable Costs	**31.30**	**374.70**
Net Return Above Total Costs	**-167.70**	**185.20**
Average Annual Net Return Above Variable Costs Over 4 Years		**289.75**
Average Annual Net Return Above Total Costs Over 4 Years		**97.88**

[1] Source: (Johanns, 2012).

Assumptions: 300 acres of alfalfa; three years hay production after establishment year; two cuttings and 2 tons/acre in establishment year; three cuttings and 4 tons/acre in production years; no irrigation.

Table VIII-4. Costs and Returns for Establishment and Production of Irrigated Conventional Alfalfa in the San Joaquin Valley, California. [1]

Operations or Cost Item	Establishment Year Cost/Acre ($)	Production Years Cost/Acre ($)
Gross Returns:		
Hay (8 tons)	-	1,480
Silage (3 tons) [2]	-	140
Total Gross Returns	-	1,620
Operating Costs:		
Land preparation	123	-
Fertilizer	67	41
Seed and seeding	109	-
Irrigation	26	182
Weed control	48	98
Insect control	-	43
Harvest		322
Other cost items (truck & ATV)	6	12
Interest on capital @ 6.75%	8	-11
Total Operating Costs	**385**	**708**
Cash Overhead Costs:		
Insurance	3	3
Taxes	60	76
Office Expenses	24	30
Investment Repairs	6	13
Total Cash Overhead Costs	**93**	**122**
Total Cash Costs	**478**	**830**
Non-Cash Overhead Costs (Capital Recovery):		
Land	247	308
Irrigation System	19	38
Building, Fuel, and Equipment	16	13
Alfalfa Establishment		173
Total Non-Cash Overhead Costs	**282**	**532**
Total Cost/Acre	**760**	**1362**
Net Return Above Operating Costs		**912**
Net Return Above Total Costs	**-**	**258**

[1]Source: (Mueller et al., 2008a). Supporting information: 1200 acre farm with 300 acres of alfalfa; fall seeding; 3-year stand life for alfalfa; irrigation - 8 acre-inches of water used for establishment and 4.5 acre-feet in 10 irrigations for production; harvest cost for two cuttings of haylage and seven cuttings of hay, buyer of haylage paid harvest costs.

[2] Haylage price equals market price less harvest cost (assumes buyer harvests haylage) multiplied by percent haylage dry matter divided by percent hay dry matter ($185-$45) × (30% DM/90% DM) = $46.67/ton.

VIII.B.2. Alfalfa Seed Production

Standardized seed production practices are responsible for maintaining high-quality seed stocks, an essential basis for U.S. agriculture. By the early 20th century, agronomists learned how to develop specific plant varieties with desirable traits. In the U.S., state agricultural experiment stations developed many seed varieties that were distributed to growers for use. Seed was saved by growers and later sold to neighbors; however, the desirable traits of the varieties often were lost through random genetic changes and contamination with other crop and weed seed (Sundstrom et al., 2002). The value of seed quality (including genetic purity, vigor, and presence of weed seed, seed-borne diseases, and inert materials, such as dirt) was quickly identified as a major factor impacting crop yields. States developed seed laws and certification agencies to ensure that purchasers who received certified seed could be assured that the seed met established seed quality standards (Bradford, 2006). The federal government passed the U.S. Federal Seed Act of 1939 to recognize seed certification and the establishment of official certifying agencies. Regulations first adopted in 1969 under the Federal Seed Act recognize land history, field isolation, and varietal purity standards for foundation, registered, and certified seed. Under international agreements such as the Organisation for Economic Co-operation and Development (OECD), the U.S. and other countries mutually recognize minimum seed quality standards (Bradford, 2006). The Association of Official Seed Certifying Agencies (AOSCA) represents state and private seed certification organizations in the U.S., and includes international member countries in North and South America, Australia, and New Zealand.

Alfalfa seed is separated into four seed classes: 1) breeder, 2) foundation, 3) registered, and 4) certified (AOSCA, 2011). Breeder seed is seed that is directly controlled by the originating or sponsoring plant breeding organization or firm. Foundation seed is first-generation seed increased from breeder seed and is handled in a manner designed to maintain specific levels of varietal purity and identity. Registered seed is the progeny of foundation seed that is handled to maintain satisfactory varietal purity and identity. Certified seed is the progeny of breeder, foundation or registered seed, and is typically two generations removed from foundation seed. While not all alfalfa seed sold to growers is officially certified, commercial alfalfa seed sold and planted for typical alfalfa production is produced predominately to meet or exceed certified seed standards. This section of the petition will provide a broad overview of the practices used in producing certified seed.

Alfalfa seed breeders and producers have put in place practical measures to assure the quality and genetic purity of alfalfa varietal seed for commercial planting. The need for such systems arose from the recognition that the quality of improved varieties quickly deteriorated in the absence of monitoring for quality and genetic purity (CAST, 2007). Seed certification programs were initiated in the early 1900s in the U.S. to preserve the genetic identity and variety purity of seed. There are special land requirements, seed stock eligibility requirements, field inspections, and seed labeling standards for seed certification. Seed certification services are available through various state agencies affiliated with AOSCA. Large seed producers implement their own seed quality assurance programs that go beyond mandated quality standards. However, large seed

producers often will utilize the services of state certifying agencies as a third party source to perform certain field inspections and audits.

In contrast to the broad geographic distribution for forage production, most commercial alfalfa seed production is highly concentrated in the irrigated regions of the western U.S. (Table VIII-5). The arid climate of the western U.S. provides a warm, dry production and harvest season to maximize seed yield and quality. Over 121,000 acres of alfalfa seed were harvested in 2007, producing approximately 62 million pounds of seed with an average yield of approximately 510 pounds per acre. Historically, California has been and remains the largest producer of alfalfa seed, but acreage has declined in recent years due to economics, environmental constraints, and regulatory issues (USDA-APHIS, 2010). Ninety-five percent of the seed produced in California is of non-dormant varieties (FD 7-10) while the Pacific Northwest produces seed of semidormant (FD 5-6) and dormant (FD 2-4) varieties (Mueller, 2008). Approximately 42 million and 8.5 million pounds of certified and non-certified alfalfa seed, respectively, were exported from the U.S. in 2011 (WASGA, 2011). The largest quantities of seed went to Saudi Arabia, Mexico, Argentina, and Canada.

Table VIII-5. Alfalfa Seed Production in the U.S.[1]

State	Farms	Seed Harvested (Acres)	Seed Harvested (lbs)
California	114	36,625	19,083,458
Washington	82	17,127	10,860,608
Idaho	92	12,788	9,346,709
Wyoming	62	10,548	5,915,816
Nevada	19	6,498	4,237,101
Montana	80	10,338	3,729,635
Oregon	32	4,959	3,183,375
Utah	54	3,803	2,077,813
Arizona	53	5,206	1,902,669
South Dakota	47	6,014	428,447
Oklahoma	29	2,004	281,121
Texas	24	546	79,885
Minnesota	17	611	63,461
Missouri	19	699	40,540
North Dakota	6	(D)	34,784
New Mexico	15	310	29,907
Kansas	5	342	22,430
Nebraska	29	545	21,216
Michigan	10	(D)	15,610
New York	3	27	6,180
Iowa	5	(D)	(D)
Ohio	1	(D)	(D)
Colorado	8	1,815	(D)
Undisclosed total	-	962	587,405
U.S. Total	**806**	**121,467**	**61,948,170**

(D) = Data withheld to avoid disclosing data for individual farms.
[1]Source: (USDA-APHIS, 2010).

California is credited with 31% of the seed production, Washington with 17%, and Idaho with 15% (USDA-APHIS, 2010). Over 60% of the seed production is concentrated in those three states, and if Arizona, Nevada, Oregon, Wyoming, Montana, and Utah are included, the total seed production among these nine states collectively represents over 95% of alfalfa seed production. The U.S. alfalfa seed growers compete with seed-producers in Canada, Australia, and other countries where comparative production costs are significantly lower (USDA-APHIS, 2010). The demand for alfalfa seed is driven by the demand for seed to establish new stands of alfalfa forage, with a minor amount used as field seed stock or for human consumption. It is estimated that 2.5 percent or less of the seed produced is used for human consumption as alfalfa sprouts (USDA-APHIS, 2010). Alfalfa seed is not consumed as a grain and, therefore, is not used directly as a feed or food product (USDA-APHIS, 2010).

Seed production is limited by the alfalfa plant's genetics and pollination characteristics. Most alfalfa plants exhibit various forms of genetic self-incompatibility or self-sterility and will not successfully self-pollinate (Viands et al., 1988). Alfalfa is adversely affected by inbreeding, *i.e.*, self-fertilized plants commonly demonstrate a dramatic reduction in seed yield potential (Rumbaugh et al., 1988). Inbreeding depression may be because of the loss of heterosis and/or accumulation and unmasking of deleterious recessive alleles that occur as a result of self-pollination and/or pollination among close relatives. Therefore, commercial alfalfa breeding programs are structured to avoid significant inbreeding and the resulting negative effects of inbreeding depression (Rumbaugh et al., 1988).

Alfalfa varieties are primarily bred for forage yield (vegetative production), forage quality, longevity, and adaptation to a broad geographic area. A typical alfalfa variety may have ten to 200 parent plants that were initially crossed in isolation to form the breeder generation seed (Figure VIII-2). The breeder seed of commercial alfalfa varieties is produced by the random intercrossing (open pollination) of all parent plants. An alfalfa variety is maintained through multiple seed generations beyond breeder seed via the open pollination of their progeny in isolation from other alfalfa varieties or pollen sources. Plant varieties bred in this way are called synthetic varieties (Rumbaugh et al., 1988).

Individual plants within a synthetic variety are genotypically and phenotypically heterogeneous, *i.e.*, no two individuals within the variety are exactly alike. Synthetic alfalfa varieties are closed populations that segregate, within a defined range, for most morphological traits and naturally occurring genetic markers. Because alfalfa varieties are segregating heterogeneous populations, alfalfa varieties are routinely described in terms appropriate to populations (mean or % trait expression). For example, alfalfa variety registration agencies require that the pest resistance of a variety be described as the mean percent of plants that express the segregating trait when the population is tested under standardized conditions.

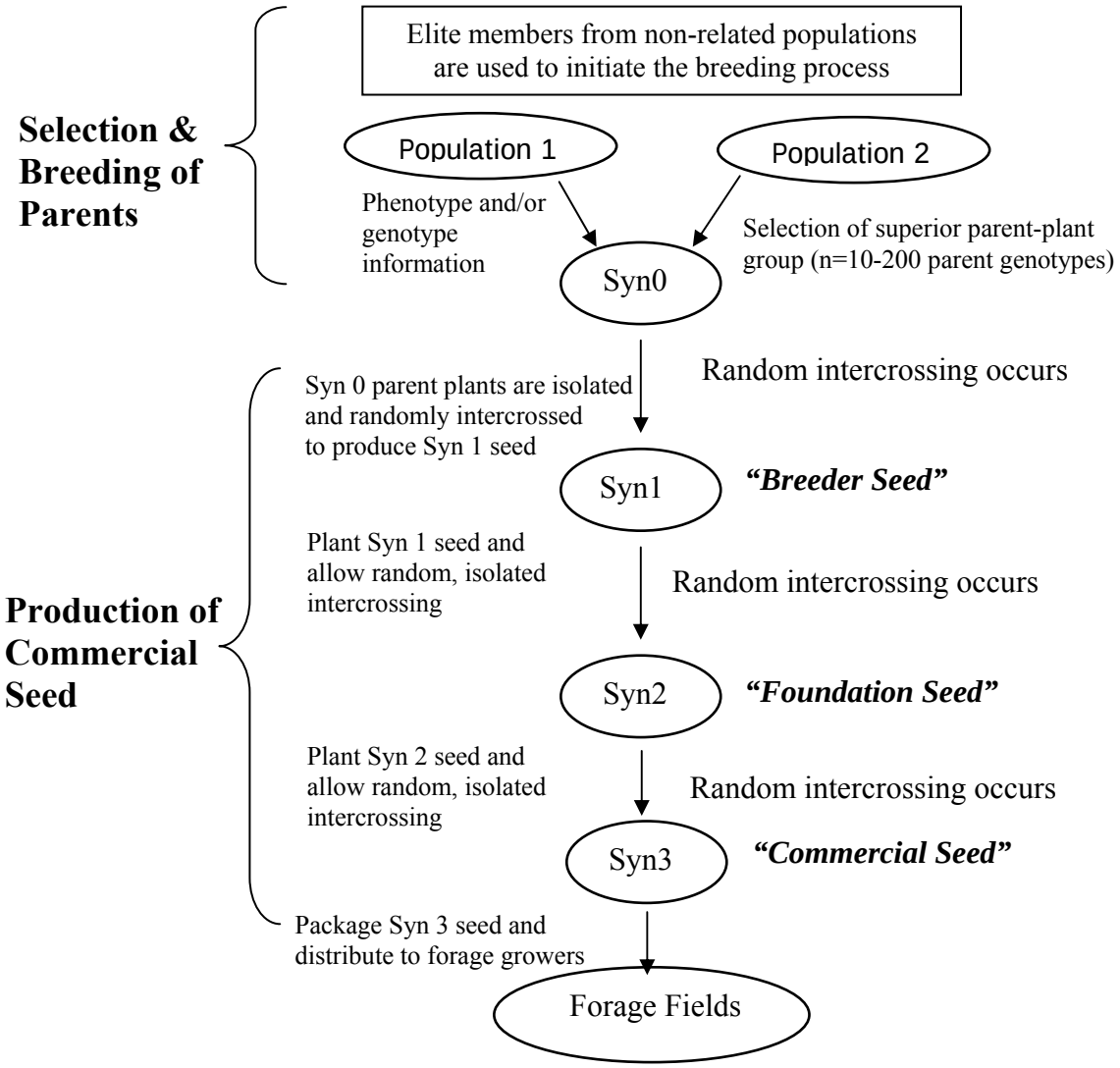


Figure VIII-2. Commercial synthetic alfalfa variety breeding schematic

As illustrated in Figure VIII-2, commercial seed of alfalfa varieties is commonly produced according to the following sequence: 1) A set of superior alfalfa plants (usually 10-200 genotypes, also known as Syn0 parents) are identified by an alfalfa breeder for use as parent plants for a new variety. The Syn0 parents are randomly intercrossed to produce the first synthetic generation of seed (Syn1 seed); 2) Syn2 generation seed is produced from a random, isolated intercross of Syn1 plants; and -3) Syn3 seed is produced from a random, isolated intercross of Syn2 plants. Breeder, foundation, registered, and certified seed classes are defined at the discretion of the plant breeder during the variety registration process. Breeder and foundation seed are typically Syn1 and Syn2 generations, respectively. Commercial seed (registered and certified) is typically Syn3 generation.

Commercial variety testing and registration by alfalfa breeders typically use Syn1 and Syn2 generation seed to establish variety testing and evaluation experiments. The U.S. alfalfa variety registration and review process is based on data from tests initiated with Syn1 generation seed although other Syn generation test data are also recognized.

Alfalfa breeding and development of varieties with new quality and agronomic characteristics are conducted by both public and private research programs. Alfalfa varieties are the result of germplasm development, enhancement, and the use of standard breeding techniques typical for cross-pollinated crops.

Alfalfa seed is produced by a number of companies that produce and sell seed, such as Forage Genetics International, Pioneer Hi-Bred, Dairyland Seed, and Cal/West Seeds. Seed companies in turn contract with growers to produce the needed amount of alfalfa seed on their acreage. To produce the seed, seed companies identify top growers then monitor and inspect seed fields throughout the growing season. Seed companies have processing facilities to clean, condition, and bag the harvested seed as well as monitor and inspect all the processes at the plant.

The entire seed production process at the majority of the seed companies operate using International Organization for Standardization (ISO) certification standards, and therefore include internal and external audits (ISO, 2012). ISO standards ensure desirable characteristics of seeds and services, such as quality, safety, reliability, and efficiency. The ISO standards represent an international consensus on good management practices with the aim of ensuring that the organization can consistently deliver excellent products or services. The standards not only must meet customers' and applicable seed regulatory requirements, but must aim to enhance customer satisfaction and achieve continual improvement of its performance in pursuit of these objectives (ISO, 2012).

While many of the field operations and management practices for producing alfalfa seed are similar to normal alfalfa forage production, there are significant differences. Special attention is needed in certain areas to optimize yields and to produce seed with high quality, high germination rates, and high genetic purity. Fields selected must not have grown alfalfa on the land for a four-, three-, and one-year period prior to stand establishment for foundation, registered, and certified seed, respectively (AOSCA, 2011). A two-year restriction applies to certified seed produced in northern and central regions following production of varieties adapted to the southern region. This restriction is to limit the spontaneous germination and sprouting of alfalfa seeds left in the seed bank. In addition, the land must be free of volunteer alfalfa plants the year prior to seed production (AOSCA, 2011).

Successful seed production begins with proper stand establishment. The majority of the dedicated alfalfa seed production fields, *i.e.*, not including common or catch-crop seed, are established according to the terms of a two- or three-year seed company contract, on a variety basis. The company supplies the seed stock, *e.g.*, foundation seed, to the seed producer and genetic source variety of the seed is documented (USDA-APHIS, 2010). Depending upon the relative market prices for forage versus common or variety not stated (VNS) alfalfa seed, a minor proportion of the U.S. alfalfa seed crop is produced

without contract as a catch-crop, where solid-seeded hay fields are allowed to set and ripen seed instead of being cut for forage. This practice occurs sporadically in the Plains, Pacific Northwest, and Desert Southwest. "Catch-crop" seed is typically produced with lower management and inputs, the genetic purity of the seed stock is often unspecified or unknown, and the resultant seed quality is highly variable and cannot be certified as to cultivar or variety identity (USDA-APHIS, 2010).

Alfalfa grown for the dedicated purpose of commercial seed production is always established alone without a companion crop (Hower et al., 1999). Recommended planting dates are similar to forage production. Fall establishment predominates (58%) because first year seed yields in fall-established stands are greater than for spring seedings (Hower et al., 1999; Mueller, 2008). Alfalfa stands dedicated to seed production are planted in rows with spacings of 30 to 40 inches and are usually on beds to facilitate furrow irrigation and early cultivation for weed control (Mueller, 2008). The plant density required to optimize seed yields depends on row spacing and soil type, which influence growth and final size of the alfalfa plant. Successful stands have been established with seeding rates as low as 0.5 to 0.75 pounds per acre (Mueller, 2008). Although, some seed producers may use slightly higher seeding rates and mechanically thin stands after emergence. Higher seed yields with thinner stands are attributed to improved water use efficiency, pest control, and pollination.

After stand establishment, fields are clipped in the spring. The clipback serves several purposes: 1) it encourages plants to come into bloom uniformly and to synchronize bloom with the period in the season when pollinators are most active, 2) it removes growth that has over-wintered and cleans up the field for herbicide applications, and 3) it also provide a cultural method for controlling the alfalfa seed chalcid (*Bruchophagus roddi*) insect pest (Mueller, 2008).

Field inspections are vital to ensure the alfalfa seed meets seed certification requirements, ISO certification standards, regulatory standards, and licensing agreement standards. Field inspections are conducted on seed production fields throughout the growing season to visually evaluate variety purity, ensure alfalfa plants are developing properly, and fields are maintained free of weeds, insects, and diseases. The fields are also mapped to ensure the seed field has the minimum isolation requirement as a physical barrier. Foundation seed fields must be isolated from alfalfa of different varieties by 900 feet, while certified fields must be isolated by 165 feet (USDA-APHIS, 2010). However, the ten percent rule is followed for certified seed fields, where if ten percent or less of the certified field is in the 165 foot isolation zone, the entire field is considered certified, but if more than ten percent is in the isolation zone, then that part of the field must be separated and not harvested as certified seed. Some states and seed producers may have a stricter isolation requirement. Volunteer plants may be a cause for rejection or reclassification of a seed field (AOSCA, 2011).

Weed management is critical in alfalfa seed production because weed competition can directly reduce seed yields and weeds produce abundant seed which can impact the strict purity standards required for certified seed. Since alfalfa for seed is often grown in wide rows, there is greater potential for weed invasion. All available weed control methods,

including herbicides, mechanical cultivation, and hand weeding are used to eliminate weeds including volunteer alfalfa during the growing season (Mueller, 2008). Herbicide use on alfalfa seed acres averaged 78% from 1988 to 1992 (Hower et al., 1999) and, undoubtedly, has increased in recent years. The separation of weed seeds from alfalfa seeds after harvesting is costly, so controlling the weeds in the fields is a more desirable method of seed quality control than post-harvest screening and separation (USDA-APHIS, 2010). Dodder (*Cuscuta* spp.), a parasitic weed that lives off alfalfa plants, is a particularly troublesome weed because there is zero tolerance for dodder seed in certified alfalfa seed (Canevari et al., 2008). Registered herbicides for control of dodder must be applied prior to emergence and attachment to a host plant to be effective.

Insect pests can have an impact on alfalfa seed yields and quality. The major insect pests in alfalfa include lygus bugs (*Lygus* spp.), spider mites (*Tetrancychus* spp.), and alfalfa seed chalcid (*Bruchophagus roddi*) (Mueller, 2008). Lygus bugs are among the most significant pest species in California because they occur throughout the season, moving between crops, and they are the most difficult insect pest to manage in alfalfa seed fields. They feed on the reproductive parts of alfalfa plants causing premature drop of buds and flowers, seed deformation, and reduced seed viability. Seed producers monitor insect pest and beneficial insect populations throughout the growing season and implement appropriate insect management strategies. Multiple pesticide applications are usually required during the bloom and pollination period. Applicators must exercise special precautions and follow special label instructions to protect bee colonies that are present to facilitate the pollination necessary for seed production.

Alfalfa flowers require tripping and cross-pollination for maximum seed yields. Three types of pollinators are used in seed production: honey bees (*Apis mellifera* L.), alfalfa leafcutting bees (*Megachile rotundata*), and alkali bees (*Nomia melanderi*) (Mueller, 2008; USDA-APHIS, 2010). Alfalfa leafcutting bees are utilized in the Pacific Northwest region and, to a limited extent, in California. Alfalfa leafcutting bees are more efficient than honey bees, but often are more expensive and require greater management. Alkali bees are used only in certain areas of Washington. Bee colonies are usually placed around seed fields when the alfalfa plants are between the one-third to one-half bloom stage. The pollination process generally takes 30 days and it takes an additional 20 to 25 days for the plants to produce mature seed (Mueller, 2008). Costs associated with pollination can be $160 per acre, or 25 percent of the total seed production costs (USDA-APHIS, 2010).

Once alfalfa plants reach maturity, they must be dried before harvest to efficiently separate the seed from the pod and other plant material. This is accomplished by cutting and windrow curing or chemically desiccating the standing crop. Windrow curing can begin once the majority of the seed is mature (two-thirds or three-fourths of the pods are dark brown in color) and harvesting can begin when the plant reaches 12 to 18% moisture (Mueller, 2008). As an alternate curing method, desiccants can be applied to the fields 7 to 10 days prior to harvest; the standing crop is then directly harvested using a combine. To minimize seed losses and obtain high-quality seed, harvest machinery adjustments and operations must match the field and crop conditions. Seed is typically harvested once per year.

Harvested seed is conditioned to remove soil, weed seeds, and other debris. Separating machines utilizing the differences in physical characteristics of alfalfa seeds and the non-seed fractions complete the cleaning process. Seed scarification (mechanical or chemical) is performed on seed lots that contain high percentages of hard seed to improve germination (Mueller, 2008). All seed lots are tested for purity, germination, and noxious weed seed content before bagging and sale.

Commercial certified alfalfa seed must meet state and federal seed standards and seed bag tag labeling requirements. AOSCA standards for certified alfalfa seed are as follows: 99% pure seed (minimum), 1% inert matter (maximum), 0.5% weed seed (maximum), 1.0% other crop seeds (maximum) – 2.0% other varieties (maximum) and 0.05% other kinds (maximum), and 80% germination (minimum) (AOSCA, 2011). The alfalfa industry historically sets a higher minimum germination rate for labeling purposes. State seed certification standards vary slightly from state to state and can be more restrictive than the seed standards of AOSCA.

The phenotypic, agronomic, and environmental interaction assessments presented in Section VII have demonstrated that KK179 is not meaningfully different from conventional alfalfa. Therefore, if deregulated, KK179 seed would be produced in the same manner as certified alfalfa seed, such that it will meet all state and federal seed standards and labeling requirements.

VIII.C. Alfalfa Forage Production Management Considerations

VIII.C.1. Pre-Season

Production decisions regarding crop rotation, tillage system, soil fertility, and variety selection need to be made well in advance of planting the alfalfa crop. Many of the decisions in this area are made prior to or immediately after harvest of the previous crop. Rotations with other crops should be an integral part of a farm management program. Alfalfa provides soil improvement and other benefits to subsequent crops (Mueller and Teuber, 2008). Alfalfa is commonly rotated with wheat, corn, cotton, sugar beets, and vegetables. Alfalfa assimilates nitrogen through nitrogen fixation and can provide levels of nitrogen ranging from 40 to 60 pounds per acre to the crop that follows alfalfa in the rotation (Orloff, 2008). Vegetable growers realize the added value from alfalfa in the rotation by improving water infiltration, soil fertility, and reduced weed pressure following alfalfa. Planting alfalfa after alfalfa is not recommended because of autotoxicity (Mueller et al., 2008b; Undersander et al., 2011). Autotoxicity occurs because alfalfa plants from the previous stand produce chemical inhibitors that can reduce germination and growth of new alfalfa seedlings. Production costs, relative rate of return, and current market conditions will dictate which crops to rotate with alfalfa.

Profitable alfalfa production begins with the selection of the proper sites or fields to plant alfalfa. Sites that provide adequate rooting depth, nutrition, aeration, and water, and have no salinity or alkalinity problems, can produce good forage yields with good management. Alfalfa is adaptable to a wide range of soil types, but requires a well-drained soil for optimum production (Orloff, 2008). Therefore, fields should be selected

that have good surface drainage and have soils with good internal drainage and lack subsoil impediments (Orloff, 2008; Undersander et al., 2011). Wet soils create conditions suitable for diseases that may kill seedlings, reduce forage yield, and kill established plants. Of major importance in the western alfalfa growing regions, is selecting sites that have an adequate supply of quality water available for season-long irrigation. In the arid Southwest region, irrigation supplies between 70 and 100% of the total crop water requirement (Hanson et al., 2008). Site selection should also take into account the previous crop and any soil residual herbicides that may preclude planting alfalfa.

Tillage has been an integral part of production agriculture and is synonymous with seedbed preparation. The purposes of primary preplant tillage are to incorporate residue from the previous crop, reduce wheel traffic compaction from the previous season, improve water filtration and soil aeration, control weeds, loosen the soil for root penetration, and provide a suitable environment for the planting and germination of the seed (Hake et al., 1996; Mueller et al., 2008b). Proper field preparation before planting is critical because the alfalfa stand will be intensively managed and harvested for three to five years, or longer in some areas. Deep tillage with moldboard and disc plows, rippers, and chisels can increase infiltration rate, fractures stratified layers, mixes the soil profile, and reduces bulk density and soil strength (Mueller et al., 2008b). The cost of deep tillage must be weighed against the potential benefit to determine whether it is economically feasible. Land leveling is an important step in the western irrigated regions because water must flow evenly over the ground surface in flood irrigation systems. Due to the high potential for erosion on slopes using conventional and deep tillage systems, there is a great deal of interest in reduced-tillage alfalfa establishment in the North and East Central regions (Undersander et al., 2011). Reduced tillage may involve chisel plowing instead of moldboard plowing or a single pass with a secondary tillage tool. Crop residue management is an important consideration in practicing reduced-tillage seeding. No-till seeders are recommended in reduced tillage and no-tillage systems with residue levels above 35% (Undersander et al., 2011). Cultipacker seeders, which broadcast the seed on the soil surface and then press the seed into the soil with rollers, have been used extensively in conventional tillage systems because they provide consistently even seed depth placement and good seed-soil contact. However, cultipacker seeders do not perform well in residue levels above 35% (Undersander et al., 2011). Weed control is more difficult in reduced-tillage systems because there is limited to no tillage to decrease weed populations. Perennial weeds may also be more of a problem with the lack of deep tillage. Still, there is some interest in alfalfa establishment with no tillage because of the high potential for soil erosion on slopes with conventional tillage. Special attention must be given to soil fertility and pH in no-till alfalfa establishment. Fertilizer and lime requirements should be incorporated into the soil well in advance of the alfalfa establishment year in the cropping system.

Maintaining optimum crop nutrition and proper lime management is critical in achieving high yields and quality in alfalfa. Proper fertilization allows for good stand establishment and promotes early growth, increases yield and quality, improves winter-hardiness and stand persistence, improves the ability of alfalfa to compete with weeds, and strengthens disease and insect resistance (Undersander et al., 2011). Seventeen elements are essential

in varying amounts for plant growth (Meyer et al., 2008). Carbon, hydrogen, and oxygen come from the water or air. The other 14 elements are obtained from either the soil or fixation of atmospheric nitrogen by bacteria in root nodules of alfalfa. Cobalt is essential to legumes for nitrogen fixation. Soil testing is the most convenient and economical method of evaluating the fertility levels of a soil and accurately assessing nutrient and liming requirements. However, plant tissue testing at the 10% bloom stage is the most precise method of determining nutrients needs, especially for sulfur, boron, and molybdenum. The nutrients that are most commonly in short supply for alfalfa production are phosphorus, followed by potassium, sulfur, molybdenum, and boron. Adequate levels of nitrogen are almost always provided by symbiotic nitrogen-fixing bacteria (*e.g.*, *Rhizobium meliloti*). The most common causes of nitrogen deficiency are poor inoculation and nodule formation. Alfalfa seed should be inoculated when planting into fields without a history of alfalfa production. Small amounts of nitrogen fertilizer (25 to 30 pounds per acre) have been shown to enhance establishment and first year yields when direct-seeding on low organic matter, coarse-textured soils (Undersander et al., 2011).

Maximum nutrient availability for most crops occurs when pH values are between 6.0 and 7.0 (Orloff, 2008). Higher pH values of 6.7 and 6.9 are recommended for alfalfa for maximum yields and to favor activity of nitrogen-fixing *Rhizobium* bacteria (Undersander et al., 2011). Soils with pH values above 7.5 are not recommended for alfalfa (Orloff, 2008). Lime should be applied during the production years of rotational crops following alfalfa in the rotation sequence to allow time for the lime to react with the soil and be mixed into the soil with tillage prior to replanting the next alfalfa crop.

Variety selection is an important preseason decision in alfalfa production that can affect crop yield, crop quality, and pest management. Since alfalfa is a perennial crop, growers must stay with their choice of variety for several years. Variety selection is a real challenge since there are over 250 alfalfa varieties to choose from and new varieties become available each year. Alfalfa varieties are diverse populations of plants having multiple genotypes rather than uniform genetic strains (Putnam et al., 2008a). Alfalfa is a polyploid having four complete sets of chromosomes which means that the offspring of alfalfa crosses are much more diverse than most crop species. This genetic diversity enables alfalfa varieties to be well adapted over a wide range of environments, and to resist a wide range of insects, diseases, and nematodes to a greater degree than any other crop. Variety selections should weigh the importance of yield potential, stand persistence, fall dormancy, winter-hardiness, disease resistance, and forage quality (Undersander et al., 2011).

Yield potential is typically the most important economic factor with regard to variety selection. Growers are advised to look for the top yielding varieties in university variety trials which are grown in a site with as similar a soil type and climate to their farm as possible (Undersander et al., 2011). They should look for top yields over several sites because soils vary on the farm and weather conditions change from year to year. Persistence of stands in northern locations depends primarily on the winter-hardiness of the variety, while persistence in southern locations depends more on pest resistance. Winter-hardiness is a measure (1 to 6 scale, 1 being the most hardy) of the alfalfa plant's

ability to survive the winter without injury. Fall dormancy measures the degree of growth (plant height) of alfalfa in the fall. Varieties with fall dormancy (FD) ratings of 1 to 4 are considered dormant, 5 to 7 semi-dormant, and 8 to 11 non-dormant (Putnam et al., 2008a). Growers should choose less dormant varieties that meet their winter survival requirement. These varieties will green up earlier in the spring and recover more quickly between cuttings to give higher total yields for the season. Plant breeders have successfully incorporated resistance to certain diseases, nematodes, and insects into alfalfa varieties over the past 40 years (Putnam et al., 2008a). Growers need to determine the most significant diseases, nematodes, and insect pests in their region to decide which variety has the best pest-resistance package. Although agronomic practices such as cutting schedule and weed control influence forage quality to a greater degree than variety selection, varieties should also be assessed on forage quality by evaluating ratings on digestibility, intake, and relative feed value (Undersander et al., 2011). Since the introduction of alfalfa varieties with the biotechnology-enhanced glyphosate-tolerant trait (Roundup Ready), growers also need to weigh the weed management benefits and costs this trait will provide to their alfalfa production program.

VIII.C.2. Planting and Stand Establishment

Stand establishment is a critical step in alfalfa production that can impact profitability for many years. Good seedling establishment results in dense, vigorous stands that produce high-quality, high yielding alfalfa throughout the life of the stand. Alfalfa seed germinates best at soil temperatures from 65 to 85°F (Mueller et al., 2008b). Alfalfa will germinate in approximately six days when the soil temperature is 40°F, but will only take two days at 65°F. Alfalfa seed contains a portion of hard seed which is highly resistant to water penetration and germination of hard seed can be delayed many weeks or months after the majority of the seed germinates (Mueller and Teuber, 2008). Seed lots containing more than 10% hard seed may need to be scarified to improve germination. Research at the University of California indicates that optimum root growth during the first month of seedling growth occurs between 69 to 76°F, depending on dormancy class. Shoot growth is optimum at temperatures ranging from 72 to 76°F (Mueller et al., 2008b). Alfalfa stops growing when the air temperature drops below 42-34°F.

Alfalfa can be established successfully in either the spring or in the late summer and fall. In the U.S., 70% of alfalfa acres are spring-seeded and the remaining 30% are planted during late summer and early fall (Hower et al., 1999). Spring seeding is preferred in the northern states of the North and East Central regions, while late summer and early fall seeding is preferred in all the remaining regions of the U.S. (Mueller et al., 2008b; Undersander et al., 2011). Temperature, soil moisture, and length of growing season are important factors that impact seed germination and stand establishment and ultimately determine which planting time is most successful and provide the highest alfalfa yields for a given area. Spring seeding begins as soon as the potential for damage from spring frosts is over which is normally April and May in the northern areas of the North and East Central regions (Undersander et al., 2011). Although alfalfa is very tolerant of cold temperatures at emergence, seedlings at the second trifoliate leaf stage become more susceptible to cold injury and may be killed by four or more hours at or below 26°F (Undersander et al., 2011). Fall seeding of alfalfa requires at least six weeks of growth

after germination to survive the winter in the Central regions. In the Imperial Valley of California, fall seeding of alfalfa begins following the harvest of the summer crop and with adequate soil moisture which is September to early October (Mueller et al., 2008b). Pre-irrigation or overhead irrigation after seeding is often needed for successful stand establishment in the arid West. Planting date research in Central Valley of California has shown fall seeding can result in 20 to 30% higher yields in the establishment year compared to spring seeding (Mueller et al., 2008b).

Seeding alfalfa with a companion crop (or nurse crop) such as annual ryegrass, oats, spring barley, and rye is often practiced with spring seeding to help control erosion on steep slopes, reduce seedling damage from wind erosion on sandy soils, and reduce weed competition during alfalfa establishment (Undersander et al., 2011; USDA-APHIS, 2010). Companion crops also provide additional forage or grain, and the straw can be harvested for livestock bedding. Fall seeding of alfalfa with a companion crop is seldom practiced because of limited soil moisture and competition with alfalfa. Small-grain companion crops grown to mature grain can damage alfalfa either by competition or by lodging, which smothers the alfalfa seedlings. Direct-seeding alfalfa (seeding without a companion crop) can produce up to two extra cuttings of alfalfa and produce higher quality forage in the seeding year (Undersander et al., 2011). However, total forage production may be less than that of companion-crop seeding. An advanced seeding technique can be used with companion crops to obtain the benefits of direct seeding while controlling erosion (Undersander et al., 2011; USDA-APHIS, 2010). Alfalfa is seeded with a companion crop to obtain the benefits of early weed control and erosion control. However, the companion crop is killed with a postemergence herbicide at between four and six inches in height to avoid competition with the alfalfa crop.

Alfalfa forage may be grown in pure stands or mixed with various other forage species (*e.g.*, cool-season grass mixtures, with or without other legumes, such as forage peas, birdsfoot trefoil [*Lotus corniculatus* L.], or clover [*Trifolium* L. spp.]). The use of mixed stands is widespread in the eastern and southern regions of the U.S., where pure-stand alfalfa production is challenged by climate and/or soil-type. In general, species mixtures compete with alfalfa plants for available nutrients, light, space, and moisture, and also limit the market value and alfalfa yield (*i.e.*, percent alfalfa composition or tonnage). Therefore, an increasing number of forage fields are direct (solo) seeded to exploit the benefits of pure-stand alfalfa.

Alfalfa is a small-seeded crop (220,000 seeds per pound) and is recommended to be seeded into a fine, firm seedbed that provides good seed-to-soil contact (Mueller et al., 2008b). Seeding rates should be between 12 and 15 pounds per acre in the North and East Central regions (Undersander et al., 2011). For irrigated alfalfa, slightly higher seeding rates of 15 to 20 pounds per acre are recommended for drilled seeding and 20 to 25 pounds per acre for broadcast seeding (Mueller et al., 2008b). Poor seedbed conditions, poor seeding depth control, insufficient or excessive moisture, poor seed germination, seedling diseases, and inclement weather are factors that reduce alfalfa emergence and stand density (Mueller et al., 2008b). Under normal conditions in Wisconsin, only about 60% of the seeds germinate and nearly 60 to 80% of the seedlings die the first year (Undersander et al., 2011). Seed should be inoculated with strains of

alfalfa *Rhizobium* bacteria when seeding into soils without a recent history (within the past 10 years) of alfalfa (Mueller et al., 2008b). Within four weeks after germination, *Rhizobium* bacteria create nodules on alfalfa roots, allowing the bacteria to fix adequate nitrogen for the life of the alfalfa stand. Seed treatment with a fungicide (metalaxyl) is also recommended to protect the seed from seedling diseases (Mueller et al., 2008b).

Following the emergence of the cotyledons, the alfalfa plant produces the first unifoliolate leaf and the seedling stem continues to produce alternately arranged trifoliolate or mutifoliolate leaves as well as secondary stems until it develops into a mature plant (Mueller and Teuber, 2008). Unique to early alfalfa development is contractile growth or the formation of the crown during stand establishment. The crown provides protection of the growing points from desiccation, cold, or mechanical damage. Harvesting and other farming practices that might disrupt the formation of the crown should be delayed until this process is complete. When the plant is 8 to 12 inches in height, the alfalfa plant has produced sufficient energy to maintain growth, and root reserves are replenished in preparation for the next forage harvest or for winter survival.

VIII.C.3. Mid- to Late-Season

Alfalfa development is divided into four categories and ten growth stages – vegetative stages, bud stages, flowering stages, and seed development stages (Mueller and Teuber, 2008). There are three vegetative stages (Stage 0, 1, and 2), distinguished by stem length. Flowering stages are early flower (Stage 3) and late flower (Stage 4), and are distinguished by one or more nodes with open flowers. Seed development stages are early seed pod (Stage 7), late seed pod (Stage 8), and ripe seed pod (Stage 9), distinguished by the number of nodes with green pods or nodes with mostly brown mature seed pods. With the growth and development of the alfalfa plant, the proportion of leaves and stems changes. Stems lengthen and become more fibrous, increasing their total proportion in the forage. Overall forage quality declines because there is no concomitant increase in leaf percentage, and the forage quality of leaves is higher than stems. The stems of alfalfa plants develop through vegetative, bud, flower, and seed stages. Numerous stems at various stages of development are typically found on one plant. Therefore, a "Mean Stage of Development" method is utilized to more accurately relate relative maturity to forage quality in a field. The Mean Stage method involves examining individual stems and classifying them according to the staging system outlined in a detailed protocol defined by Kalu and Fick (1981).

Alfalfa fields should be monitored routinely for insect pests and foliar diseases throughout the growing season. Integrated pest management programs involving chemical and cultural methods can significantly reduce insect losses in alfalfa. These programs include correct insect identification, use of economic threshold values, careful monitoring and sampling of insects, and implementing control strategies that minimize effects to natural enemies and other non-target species (Summers et al., 2008). Accelerating or delaying cutting schedules is also an important cultural method of managing some insect pests. Despite the use of cultural control methods, insect pests can reach economic threshold levels requiring the use of insecticides. Unlike many field

crops, economic threshold values with alfalfa forage production must take into consideration both yield and quality losses.

Management practices need to maximize forage yield while achieving a level of quality that meets the nutritional requirement for the intended use or market. The maturity of alfalfa plants at harvest has the greatest impact on forage quality. Prior to flowering, forage yield generally increases faster than quality declines. However, during the flowering period, reduction in quality is very rapid due to increased fiber (cellulose and lignin) concentration in the stems.

VIII.C.4. Harvest

Harvest management decisions are critical to forage yield, forage quality, and to the profitability of an alfalfa crop. Identifying the best harvesting or cutting schedule is one of the most difficult management decisions because it involves market considerations in addition to several agronomic factors (Orloff and Putnam, 2008) Several cutting strategies can be considered or implemented including harvesting on a calendar basis, certain growth stage, growing-degree day models, numerical staging, and Predictive Equations for Alfalfa Quality (PEAQ) (Orloff and Putnam, 2008) Each strategy has advantages and disadvantages, as well as different degrees of flexibility, that growers must weigh to determine which strategy is most suitable for their farming operation. A combination of cutting strategies may be the best strategy for some farming operations that supply hay to more than one market.

Deciding when to cut alfalfa forage is challenging because plant maturity affects yield and quality differently and can affect the life and vigor of the stand. Alfalfa yield and forage quality are almost always inversely related within a growth cycle. Alfalfa yield can double from the pre-bud to full-bloom stage and generally reaches maximum yield at about the 50% bloom stage and then levels off (Orloff and Putnam, 2008). Alfalfa harvested at an immature growth stage (short interval between cuttings) results in relatively low yield but high forage quality (Orloff and Putnam, 2008). Conversely, the cutting of alfalfa at a mature growth stage (long interval between cuttings) results in high yield but low quality forage. The growth stage to cut alfalfa should reflect the intended use of the hay. The value of alfalfa forage and hay varies considerably by the level of quality or quality grade. In addition to the visual appearance of alfalfa hay, alfalfa hay quality is defined by a number of nutritional traits including acid detergent fiber (i.e., cellulose and lignin content), neutral detergent fiber (i.e., lignin, cellulose and hemi-cellulose content), crude protein, total digestible nutrients, and relative feed value (Putnam et al., 2008b). Alfalfa hay intended for the dairy market must be cut early (late-bud stage at the latest) (Putnam et al., 2008b). Beef cows and horses usually are fed lower quality alfalfa that can be cut later, at 10 to 30% bloom, to maximize forage yields.

Repeatedly cutting alfalfa plants at immature growth stages (pre-bud to bud) shortens stand life because it does not allow sufficient time for the alfalfa plants to replenish root reserves (Orloff and Putnam, 2008). Stand loss can lead to the invasion of weeds that compete for available resources (*e.g.*, water, nutrients, light, and space) and negatively

impact forage quality. Additionally, cutting schedules influence the number of harvests possible in a year and influence seasonal yield and costs.

More cuttings per year do not necessarily equate to higher total production per year. University of California studies have shown the opposite to be true (Orloff and Putnam, 2008). nine to ten times at pre-bud (harvest interval of 21 days) resulted in a 3-year average yield of 7.5 tons per acre in the Central Valley. In comparison, harvesting at full bloom (harvest interval of 37 days) with a total of five to six cuttings per year yielded an average of 11.6 tons per acre. Most California growers harvest during early to late-bud stage to obtain forage with lower lignin content, because most of their alfalfa production is targeted for the dairy industry (Orloff and Putnam, 2008). The most common cutting schedule in the Low Desert and Mediterranean regions of California is 28 days and as often as 21-24 days where high quality is the objective (Orloff and Putnam, 2008). To produce high quality hay in the North Central region, a 28- to 33-day interval between the first and second cutting is recommended and a 30- to 35-day harvest interval is recommended for the remaining cuttings (Undersander et al., 2011).

Pest management decisions may impact cutting schedules. Harvesting early can provide effective management of alfalfa weevil in the spring and worm management in the summer (Summers et al., 2008). Also, longer harvest intervals can enable the alfalfa crop to compete more vigorously with weeds.

When cutting alfalfa, university alfalfa specialists recommend leaving no more than 2-4 inches of stubble height (Orloff and Putnam, 2008). Studies have shown that cutting above two inches results in a yield reduction of 0.5 ton per acre per year per inch of additional cutting height. However, raising the cutting height does increase forage quality.

High alfalfa forage yields and stand life depend on minimizing winter injury, particularly in the northern states of the North and East Central regions. Fall management of alfalfa involves assessing the risk of winter injury and the need for additional forage. Snow cover, temperature, and moisture are several uncontrollable environmental factors that impact the risk of winter injury (Undersander et al., 2011). Extended periods of cool temperatures are required in the fall for alfalfa to develop resistance to cold temperatures. Winter-hardy alfalfa varieties can be injured or killed by two weeks or more of temperatures below 5 to 15°F. Snow cover of 6 inches or more can protect alfalfa plants from these low temperatures. Warm fall weather (40°F or higher) and midwinter thaws cause alfalfa to break dormancy and have less resistance to freezing temperatures. However, growers are able to utilize several management options to reduce the risk of winter injury. Maintaining good soil fertility is very important to maintaining productive stands and good winter survival (Undersander et al., 2011). Cuttings should be avoided during the critical fall period 6 weeks before the first killing frost, especially when previous harvest intervals are 35 days or less. This allows plants to enter the winter with higher root carbohydrates. Late-fall cuttings (October) should be made at a height of 6 inches to catch snow and insulate the soil. The decision to make a late-fall cutting needs to weigh the risk of winter injury against the need for additional forage.

Harvesting hay is a four step process: 1) cutting the forage, 2) raking the partially cured hay into windrows, 3) baling the dry hay, and 4) storing the hay (Orloff and Mueller, 2008). The moisture content of alfalfa growing in the field is generally about 80% (Undersander et al., 2011). Soluble sugars and proteins are dissolved in the forage liquid. When forage is dried to hay before being baled, water in the forage evaporates, resulting in a higher concentration of nutrients in the remaining liquid where cell growth and enzyme activity are restricted. The objective during the harvesting process is to accelerate the drying rate and minimize losses in dry matter. The drying rate, mechanical handling of the forage, and the moisture content at baling all affect the quality of the hay. Rapid drying is important to minimize quality losses caused by bleaching, respiration, leaf loss, and rain damage (Orloff and Mueller, 2008). Weather conditions can make harvesting hay a real challenge. Rainy weather causes delays in harvest which increases the neutral detergent fiber (NDF) and acid detergent fiber (ADF) and decreases digestibility and crude protein of the hay (Undersander et al., 2011). In addition, rain on hay before baling leaches soluble nutrients (protein and carbohydrates). The following management practices are recommended for harvesting hay to accelerate drying and minimize losses: 1) cut the forage early in the day, 2) rake into a wide swath or windrow, 3) rake at 40 to 50% moisture, 4) bale hay at 18 to 20% moisture content, and 5) store hay under cover (Undersander et al., 2011).

Mechanical conditioning or crimping the alfalfa during the cutting operation is a widely accepted practice to accelerate curing (Orloff and Mueller, 2008). The purpose of crimping is to facilitate water loss from the stems, making the drying rate of stems more similar with that of the leaves. This can hasten the drying process by as much as 30% (Orloff and Mueller, 2008). Chemical conditioning or use of drying agents can also be used to speed the drying process. Drying agents such as potassium carbonate or sodium carbonate sprayed on the forage at cutting can shorten drying time by 5 to 24 hours (Undersander et al., 2011). Drying agents are most effective when the weather is warm and sunny. They provide little to no benefit during poor curing conditions. If rain falls on treated hay, the hay reabsorbs water more readily than untreated hay. For these reasons and others, university alfalfa specialists do not believe drying agents are cost effective.

Preservatives are used to a limited degree to allow storage of alfalfa hay baled at moisture contents higher than would ordinarily be considered safe. Hay baled and stored at too high a moisture content without a preservative is subject to problems with mold, discoloration, and even spontaneous combustion (Orloff and Mueller, 2008). Hay preservatives are organic acids, primarily propionic acid or propionic-acetic acid blends that prevent mold growth and heating losses by lowering the pH and retarding the growth of microorganisms that cause hay spoilage. Preservatives are used by some growers in the North Central region but are not considered cost effective in the western states (Orloff and Mueller, 2008).

VIII.D. Management of Insect Pests

Many insects are present in alfalfa, but fewer than 20 cause injury, and fewer insects are considered serious pests (Summers et al., 2008). However, those injury-causing insect

pests can cause substantial yield and quality losses in alfalfa when present in high numbers. Alfalfa weevil (*Hypera postica*) and Egyptian alfalfa weevil (*Hypera brunneipennis*) routinely cause damage annually in established alfalfa throughout the U.S. Damage and yield losses are more sporadic and less frequent with other insect pests. Integrated pest management programs involving chemical and cultural methods can significantly reduce insect losses in alfalfa. These programs include correct insect identification, use of economic threshold values, careful monitoring and sampling of insects, and implementing control strategies that minimize effects to natural enemies and other nontarget species (Summers et al., 2008). Selection of resistant varieties is an effective element in the management of several alfalfa pests. However, even with highly resistant varieties only a minimum of 50% of the plant population are resistant (Summers et al., 2008). Accelerating or delaying cutting schedules is also an important cultural method of managing some insect pests. In irrigated alfalfa areas, border-strips are utilized to serve as refuge for natural enemies and to retain lygus bugs in the alfalfa where they do little harm and keeps them out of neighboring crops (Summers et al., 2008). Despite the use of cultural control methods, insect pests can reach economic threshold levels requiring the use of insecticides. Unlike many field crops, economic threshold values with alfalfa forage production must take into consideration both yield and quality losses.

As indicated previously, alfalfa weevil is one of the most serious pests in alfalfa. The larvae feed on the terminal buds and upper leaves and under severe infestations can completely defoliate the plant. Additionally, the adult weevils feed on alfalfa stems. Damage is usually greatest on the first cutting, although second and third generations of weevil larvae can cause damage in subsequent harvests (Kansas State University, 1998; Summers et al., 2008). Both the larvae and adult forms can suppress yields by delaying regrowth. Insecticides and early harvest are the main control options for alfalfa weevil. Varieties with sufficient resistance to heavy infestations are currently not available. General biological control agents are not effective, although several parasitic wasps are effective in some growing regions to suppress weevil population buildup (Kansas State University, 1998). However, an alfalfa weevil-specific fungus aids in biological control (Summers et al., 2008).

Among species of aphid, several are important insect pests that can damage alfalfa, including pea aphid (*Acyrthosiphon pisum*), blue alfalfa aphid (*A. kondoi*), spotted alfalfa aphid (*Therioaphis maculate*), and cowpea aphid (*Aphis craccivora*) (Summers et al., 2008). Some Lepidoptera insects are also important pests, including armyworm (*Spodoptera exigua*, *S. praefica*), alfalfa caterpillar (*Colias eurytheme*), and leafhoppers (*Empoasca* spp.) (Summers et al., 2008). Of occasional importance are cutworms (*Agrotic subterranea*, *Peridroma sausia*), alfalfa webworm (*Loxostege cereralis*), alfalfa looper (*Autographa californica*), clover root curculio (*Sitona hispidulus*), ground mealybug (*Rhizoecus kondonis*), spider mites (*Tetranychus* spp.), silverleaf whitefly (*Bermisia argentifolii*), grasshoppers (*Melanoplus* spp. and *Trimerotropis spp*.), Three-cornered alfalfa hopper (*Spissistilus festinus*), blister beetles (*Epicauta* spp., *Lytta* spp., *Tegrodera* spp.), and thrips (*Frankliniella* spp., *Caliothrips phaseoli*) (Summers et al., 2008).

The phenotypic, agronomic, and environmental interaction assessments presented in Section VII have demonstrated that KK179 is not meaningfully different from conventional alfalfa. Therefore, if deregulated, KK179 would require the same management practices for insect pests as conventional alfalfa.

VIII.E. Management of Diseases and Other Pests

Diseases can kill alfalfa seedlings, reduce stand life, cause yield reduction, and reduce the feeding value of the forage (Frate and Davis, 2008; Undersander et al., 2011). The occurrence and severity of diseases depends on environmental conditions, soil type, and crop management (Undersander et al., 2011). Temperature and moisture are the environmental factors affecting the occurrence and severity of diseases. Pathogens that cause alfalfa diseases include fungi, bacteria, viruses, and nematodes. Selecting disease-resistant varieties is one of the most effective methods to managing many of the alfalfa diseases. Other integrated strategies and techniques utilized to manage alfalfa diseases include irrigation management, planting methods, promotion of crop vigor, manipulation of cutting schedules, canopy management, and crop rotation (Frate and Davis, 2008).

The first threat to alfalfa comes from the seedling diseases or damping-off caused by soil-borne fungi, including *Pythium* spp. *Rhizoctonia* spp., and *Phytophora* spp. (Frate and Davis, 2008). Their development is favored by excessive soil moisture, compacted or poorly drained soils, and adverse temperatures for seedling growth. Seedling diseases can be devastating to the long-term productivity and life of a new alfalfa seeding. Cultural and chemical control measures are generally effective in managing seedling diseases.

Foliar diseases reduce photosynthesis and defoliate alfalfa plants subsequently decreasing yield and forage quality (Frate and Davis, 2008). Some important foliar diseases include common leaf spot (*Pseudopeziza medicaginis*), downy mildew (*Peronospora trifoliorum*), spring and summer blackstem (*Phoma medicaginis* and *Cercospora medicaginis*), Stagonospora leaf spot (*Stagonospora meliloti*), Stemphylium leaf spot (*Stemphylium botryosum*), and rust (*Uromyces striatus*) (Frate and Davis, 2008; Undersander et al., 2011). Various cultural techniques are usually utilized to manage the foliar diseases.

Root, stem, and crown diseases are less obvious but can cause just as much damage to alfalfa stands and yields. Anthracnose (*Colletotrichum trifoii*), Aphanomyces root rot (*Aphanomyces euteiches*), Phytophthora root rot (*Phytophthora megasperma*), Rhizoctonia root canker and crown rot (*Rhizoctonia solani*), Sclerotinia stem and crown rot (*Sclerotinia tifoliorum*, *S. sclerotiorum*), and Stagonospora crown and root rot (*Stagonospora meliloti*) are important diseases in this group with Phytophthora root rot being one of the most common diseases in alfalfa (Frate and Davis, 2008; Undersander et al., 2011). the exception of Rhizoctinia root canker and crown rot, resistant varieties are available and effective for the other diseases in this group.

Bacterial wilt (*Clavibacter michiganensis*), fusarium wilt (*Fusarium oxysporum*), and verticillium wilt (*Verticillium alfo-altrum*) are among the most important wilt diseases

(Frate and Davis, 2008). The pathogens for wilt diseases invade the vascular system of plants which leads to the plant wilting and usually dying. Resistant varieties are the first line of defense for the diseases in this group.

Parasitic nematodes are microscopic, unsegmented roundworms that reduce yields and cause considerable economic losses in alfalfa (Westerdahl and Frate, 2008). The stem nematode (*Ditylenchus dipsaci*), root-knot nematode (*Trichodorus spp*.), and the root-lesion nematode (*Pratylenchus penetrans*) are the most important nematodes consistently associated with damage to alfalfa. Nematodes feed on root hairs, feeder roots, and nitrogen-fixing nodules of alfalfa and do not typically kill plants (Undersander et al., 2011; Westerdahl and Frate, 2008). They stress the plants and act in conjunction with other stress factors in the field to reduce growth and yield. The key practices to manage nematodes include site selection, crop rotation, variety selection, certified seed, fallow, chemical nematicides, and other management techniques that prevent nematodes from spreading, such as using clean equipment and irrigation water (Westerdahl and Frate, 2008).

The phenotypic, agronomic, and environmental interaction assessments presented in Section VII have demonstrated that KK179 is not meaningfully different from conventional alfalfa. Therefore, if deregulated, KK179 would require the same management practices for diseases and other pests as for conventional alfalfa.

VIII.F. Weed Management

Annual and perennial weeds reduce alfalfa yield and quality and cause serious economic losses in alfalfa because they compete for the same resources required for alfalfa growth and development: water, nutrients, light, and space (Canevari et al., 2008). Weed competition in alfalfa occurs in two distinct time periods: seedling establishment and in established stands. Seedling alfalfa plants grow slowly and compete poorly with weeds. Forage yield losses due to the presence of weeds in new stands of alfalfa often exceed 1000 pounds per acre (Caddel et al., 2011). In many incidences, the overall tonnage of forage may be the same or actually higher when weeds are left uncontrolled (Canevari et al., 2008). However, the feeding value or nutritional value of the hay is drastically reduced. Weeds affect quality because most weeds are much lower in protein, higher in fiber, and are generally less palatable and less nutritious than alfalfa. In a California study, protein content was as low as 9% in hay that contained 80% weeds (Canevari et al., 2008). When weeds were controlled with herbicides, the protein content was over 20%. Reductions in forage quality also depend on the weed species present. Annual grasses have a significant impact on quality because they have high fiber content and decrease livestock intake (Undersander et al., 2011). Annual broadleaf weeds, such as curly dock, hoary alyssum, and yellow rocket are unpalatable and decrease animal intake. In addition, hay containing foxtail (*Setaria spp*.) and wild barley (*Hordeum spp*.) can cause livestock to develop serious mouth and throat ulcerations (Canevari et al., 2008). Some weeds can contribute “off” flavors in milk, and other weeds contain alkaloids that are toxic to livestock. Certain weed species can have a more direct impact on alfalfa production. For example, quackgrass and Canada thistle release toxins or growth-

inhibiting compounds upon decomposition, which can affect alfalfa growth and yield (Kommedahl et al., 1959; Wilson, 1981).

Annual, biennial, and perennial weeds are problematic during seedling establishment and in established stands. Common weeds found in alfalfa in the U.S. are presented in Table VIII-6. Tillage is an important part of a weed management program when establishing alfalfa. Tillage controls emerged annual weeds and sets back perennial weeds prior to planting. The absence of tillage during the production years naturally favors invasion by perennial weeds (Undersander et al., 2011). Controlling certain perennial weeds (*e.g.*, nutsedge) may be managed more effectively in other crops of the rotation sequence preceding alfalfa establishment. In the Midwest, alfalfa is often planted with a companion crop for erosion control, but this practice also helps suppress weeds while the alfalfa is becoming established (Undersander et al., 2011). Practices that encourage alfalfa germination and vigorous seedling growth are effective for weed management. A dense, vigorous alfalfa stand is an effective weed management strategy for establishing alfalfa and extending the life of the stand. Additional details on weed control when applying Roundup® agricultural herbicides to Roundup Ready alfalfa can be found in Section VIII.F.1. Details on weed control throughout this section are focused on conventional weed control programs.

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Table VIII-6. Common Weeds Present in Alfalfa in the U.S[1].

Annuals

Annual bluegrass	Creeping swinecress	Persian speedwell
Annual sowthistle	Dodder	Pigweed spp.
Barnyardgrass	Field pennycress	Prickly lettuce
Blessed milk thistle	Fillarees	Prostrate knotweed
Bristly oxtongue	Foxtail, yellow and	Roughseed buttercup
California burclover	green	Shepherds's purse
Cheeseweed (*Malva*	Henbit	Toad rush
spp.)	Hoary alyssum	Velvetleaf
Coast feddleneck	Italian ryegrass	Virginia pepperweed
Cocklebur	Kochia	Volunteer cereals
Common chickweed	London rocket	Wild celery
Common groundsel	Miner's lettuce	Wild oats
Common lambsquarters	Mustards	Yellow starthistle
Common purslane	Night-flowering catchfly	
Common ragweed	Nightshades	

Biennials

Buckhorn plantain	Mexican tea	Spotted knapweed

Perennials

Bermudagrass	Hemp dogbane	White cockle
Curly dock	Johnsongrass	Wirestem muhly
Dallisgrass	Nutsedge	Yellow rocket
Dandelion	Orange hawkweed	
Field bindweed	Quackgrass	

[1]Source:(Canevari et al., 2008; Undersander et al., 2011).

There are currently 16 herbicides registered for use in conventional alfalfa. According to an extensive survey conducted by the USDA from 1988 to 1992, herbicides are used on approximately 17% of all alfalfa acreage (seedling establishment and established stands) grown for forage (USDA-APHIS, 2010). Herbicides are used more extensively in the West (50%) compared to the Northeast (22%) and North Central (8%) areas of the U.S. (Hower et al., 1999). This may be due to the more arid environment in the West, in which weed competition has a greater economic impact. At the time of alfalfa establishment, total herbicide-treated acres were 1.1 million acres in spring-seeded alfalfa and 0.4 million acres in fall-seeded alfalfa (Hower et al., 1999). However, since more than one herbicide and more than one application were made on some acres, it is not possible to accurately calculate the percentage of spring- or fall-seeded alfalfa acres treated with herbicides. Mechanical and cultural weed control methods (*e.g.*, tillage and companion crops) are used for approximately 80% of the spring-seeded alfalfa and 18% of the fall-seeded alfalfa (USDA-APHIS, 2010). Companion crops were utilized on 49% and 3% of the spring- and fall -seeded alfalfa, respectively (Hower et al., 1999).

There are several herbicides available for effective weed control during seedling establishment, including 2,4-DB, benefin, bromoxinil, clethodim, hexazinone, imazamox, imazethapyr, sethoxydim, paraquat, and pronamide (USDA-APHIS, 2010). Table VIII-7 provides a summary of the crop tolerance and effectiveness ratings of herbicides on common weeds in direct-seeded alfalfa. The effectiveness and spectrum of weed species controlled with each of these herbicides varies; where rarely does one herbicide control all the weed problems in alfalfa in a particular area. In addition, the restrictions on alfalfa stage of growth for application and interval between application and harvest varies with each herbicide, making the selection of herbicide(s) more complex.

Alfalfa stands generally remain in production from three to six years or more. Eventually, weeds invade the alfalfa stand as the stand growth slows and stand density declines due to poor soil fertility, disease, insect problems, and winter injury (Undersander et al., 2011). The decision to apply an herbicide to remove undesirable weeds in established alfalfa will depend on the weed density, weed species, stand density, and intended use of the hay (Undersander et al., 2011). It is important to note that alfalfa does not spread into the open areas previously occupied by weeds. Approximately 5.1 million herbicide-treated acres of established alfalfa were treated annually with an herbicide during 1988 to 1992 in the U.S. (Hower et al., 1999). This low level of treated acres may be the result of the existence of alfalfa-grass mixtures, the low economic return from current herbicides, the ineffectiveness of past herbicide programs, the use of alternate weed control methods such as growing a companion crop, or cultural practices.

Several herbicides can be used in established stands of alfalfa including: 2,4-DB, clethodim, diuron, EPTC, hexazinone, metribuzin, norfluzaon, paraquat, pendimethalin, sethoxydim, terbacil, and trifluralin (USDA-APHIS, 2010). Table VIII-8 provides a summary of the crop tolerance and effectiveness ratings of herbicides on common weeds in established stands of alfalfa. The spectrum of weed species controlled varies significantly among these herbicides. In addition, some of the herbicides are applied for residual control, some control emerged weeds, and some have residual and

postemergence activity. The rotation restrictions with certain herbicides are an important consideration in the selection process.

The phenotypic, agronomic, and environmental interaction assessments presented in Section VII have demonstrated that KK179 is not meaningfully different from conventional alfalfa. Therefore, if deregulated, KK179 would require the same weed management practices as conventional alfalfa.

Table VIII-7. Alfalfa Tolerance and Herbicide Effectiveness Ratings on Direct Seedlings of Alfalfa[1]

	Preplant Incorporated Herbicides		Postemergence Herbicides							
	EPTC	**Trifluralin**	**Bromoxynil**	**2,4-DB**	**Glyphosate**	**Sethoxydim**	**Pendimethalin**	**Imazethapyr**	**Imazamox**	**Clethodim**
Alfalfa Tolerance	F/G	G	F/G	G	E[2]	E	E	G	G	E
Grasses Barnyardgrass	G/E	G/E	P	N	E	G/E	G/E	G	G	G/E
Foxtails	G/E	G/E	P	N	E	E	G/E	G	G/E	E
Quackgrass	P/F	P	N	N	G/E	F/G	P	P	P/F	G
Broadleaves C. chickweed	F	P	P	P	E	N	F/G	G	G	N
C. lambsquarters	F	F/G	G/E	G/E	G/E	N	G/E	F/G	G	N
C. ragweed	F	P	G/E	G/E	E	N	P	F	F/G	N
Eastern Black nightshade	F	P	G/E	F	E	N	P	E	E	N
Pennycress	P	P	G/E	F	E	N	P	G/E	G/E	N
Pigweed spp.	F/G	G/E	F	G/E	E	N	G/E	E	E	N
Shepherd's purse	P	P	E	F/G	E	N	G	G/E	G/E	N
Smartweed spp.	P	P	G	P	G/E	N	P	G	G	N
Velvetleaf	F/G	N	G	G/E	G/E	N	N	G/E	G/E	N
Wild mustard	P/F	P	G	F/G	E	N	N	G/E	G/E	N
Wild radish	P/F	N	G	F	E	N	N	G	G	N

Herbicide effectiveness ratings: E = excellent; G = good; F = fair; P = poor; N = no control.
[1]Source: (Cullen et al., 2012).
[2]Crop tolerance rating is for postemergence applications of glyphosate in Roundup Ready alfalfa varieties.

Table VIII-8. Alfalfa Tolerance and Postemergence Herbicide Effectiveness Ratings on Common Weeds in Established Alfalfa Stands[1].

	2,4-DB	Flumi-oxazin	Glyphosate[2]	Metribuzin	Sethox-ydim	Pendi-methalin	Imazeth-apyr	Imazamox	Clethodim	Hexazi-done
Alfalfa Tolerance	G	G/E	E[2]	F/G	E	E	G	G	E	G
Annuals										
Common chickweed	P	E	E	E	N	P	G	G	N	E
Field pennycress	F/G	E	E	G/E	N	P	E	E	N	G/E
Foxtails spp.	N	G/E	E	F	E	G/E	F/G	G/E	E	F
Shepherd's purse	F/G	E	E	E	N	F/G	G/E	G/E	N	E
Perennials										
Broadleaf plantain	F/G	-	G/E	P	N	P	F	F	N	F/G
Canada thistle	N	P	G	P	N	P	P	P/F	N	N
C. dandelion	P	P	G	G/E	N	P	P	P/F	N	G/E
Curly dock	P/F	P	-	F	N	P	P/F	P/F	N	F
Hemp dogbane	N	-	E	P	N	P	P	-	N	N
Hoary alyssum	F	P[3]	-	F/G	N	P	F	F	N	G
Perennial sowthistle	N	-	E	P	N	-	G	G	N	P
Quackgrass	N	P	G/E	F/G	F/G	P	P	P/F	G	F/G
White cockle	P	P[3]	F/G	G	N	P	P	P	N	F
Wirestem muhly	N	-	E	P	F/G	-	P	P	F/G	F
Yellow nutsedge	N	P	F	F	N	P	P/F	F	N	F
Yellow rocket	P	P[3]	G/E	G	N	P	F/G	F/G	N	G/E

Herbicide effectiveness ratings: E = excellent; G = good; F = fair; P = poor; N = no control; - = no data.

[1]Source: (Cullen et al., 2012).

[2]Crop tolerance rating is for postemergence applications of glyphosate in Roundup Ready alfalfa varieties.

[3]Provides excellent control of seedlings.

VIII.F.1 Weed Management in Roundup Ready Alfalfa

Roundup Ready alfalfa treated with postemergence applications of Roundup agricultural herbicide products provides alfalfa growers broad spectrum weed control and excellent crop safety for seedling establishment and established stands. Roundup Ready alfalfa growers can experience more effective control of problem weeds in alfalfa with greater application flexibility and simplicity compared to conventional weed management options. Tables VIII-7 and VIII-8 provide a summary of the crop tolerance and effectiveness ratings of several herbicides, including glyphosate, on common weeds in direct-seeded and established alfalfa stands. Glyphosate is rated excellent for Roundup Ready alfalfa tolerance, while several other herbicides are only rated good or fair to good for alfalfa tolerance. Glyphosate is rated good to excellent on almost all the common weeds listed in direct seedings and established stands of alfalfa. Postemergence application of glyphosate provides improved control of some of the most difficult to control perennial weeds (bermudagrass, nutsedge, johnsongrass, and dandelion) (Canevari et al., 2008). It is expected that adequate control of these tough perennials could add years to alfalfa stand life (Canevari et al., 2008).

Roundup agricultural herbicide products can be applied pre-plant, at planting, and preemergence during alfalfa seedling establishment and in-crop postemergence to established stands. In-crop postemergence applications can be made to Roundup Ready alfalfa from emergence of alfalfa until five days prior to cutting, which will not limit weed management decisions. Single in-crop applications must not exceed 1.54 lbs a.e. (acid equivalent) of glyphosate per acre and the combined total per year for in-crop applications on newly established and established stands must not exceed 4.64 lbs a.e. per acre. The combined total per year for all applications, including preplant during the year of establishment, must not exceed 6.0 lbs a.e. of glyphosate (Monsanto Company, 2012).

Since glyphosate controls only emerged weeds with no residual control, it is important to apply Roundup agricultural herbicide products after most of the weeds have emerged or when there is enough crop canopy to outcompete late-emerging weeds. Generally, the best time for making applications in seedling alfalfa is when the alfalfa is between the 3- and 6-trifoliate leaf stages (Canevari et al., 2008). Earlier applications may allow subsequent weed emergence. With later applications, weeds may be too large for adequate control and can result in heavy weed infestations, such that alfalfa stand density or vigor may be affected before herbicide application. A diverse weed control system that incorporates cultural methods as well as rotations or tank mixes of glyphosate with other mode-of-action herbicides can minimize the risk of weed species shifts or weeds developing resistance to glyphosate (Canevari et al., 2008).

VIII.G. Alfalfa Stand Termination and Volunteer Management

The productivity of alfalfa fields declines over time due to loss of plants and weakening of crowns from diseases, insect pests, weed competition, equipment traffic, soil compaction, and other factors (Canevari and Putnam, 2008). Eventually, the forage yield and quality decline to the point that the stand must be removed and the field rotated to another crop. In some cases, it may be economically feasible to extend the life of the

stand by overseeding with grasses and other legumes. However, there must be a potential market for the alfalfa-grass mixture. The plant density as well as the health, size, and regrowth potential of the alfalfa plants are factors determining stand viability (Canevari and Putnam, 2008). When the plant density falls below four to six plants per square foot, yields begin to decline. However, stem densities may be more important than plant density. Growers should consider stand removal when stem densities fall below approximately 39 stems per square foot, or when yields fall 25% below normal and weeds become a factor (Canevari and Putnam, 2008).

Alfalfa stand removal or termination is achieved in the fall through the use of deep tillage, herbicides, or both. Since normal tillage operations alone often do not provide complete termination, herbicides are commonly used to effectively control alfalfa. For most effective control with herbicides, termination should be done in the fall when the alfalfa plants have sufficient vegetative growth and the herbicides are more effective. Fall applications should be made after the last cutting to at least 6 to 8 inches alfalfa regrowth and before the first killing frost (Coulter, 2010). To terminate seed production fields, multiple tillage operations are used, followed by irrigation to induce germination of dropped seed and the decomposition of the killed plants. Herbicides in combination with shallow tillage may be used to kill the seed parent plants and recently germinated seedlings.

Volunteer alfalfa plants can arise from viable alfalfa crowns in terminated alfalfa forage production fields. In former seed production fields, volunteers may be either from surviving or newly germinated plants. Growers currently use a combination of tillage and herbicides for volunteer control.

Several herbicides are labeled for control of alfalfa, including: 2,4-D, chlorpyralid, dicamba, carfentrazone, flumioxazin, and glufosinate. Glphosate only provides partial control of conventional alfalfa and is not recommended for volunteer management. Research has demonstrated that 2,4-D, chlorpyralid, dicamba, or a combination of the 2,4-D and dicamba provide excellent control of alfalfa (Coulter, 2010; Dillehay and Curran, 2006; Mayerle, 2002). Table VIII-9 lists the herbicides labeled for preplant and in-crop applications that can be used for stand termination and to control volunteer alfalfa in crops that immediately follow alfalfa in the rotation. In certain rotational crops (*i.e.*, potato, tomato), herbicides are available for stand termination or volunteer control preplant to the crop, but no herbicides are labeled for in-crop control of volunteers. In these rotational crops, tillage and hand weeding are used for in-crop control of volunteer alfalfa.

The phenotypic, agronomic, and environmental interaction assessments presented in Section VII have demonstrated that KK179 is not meaningfully different from conventional alfalfa. Therefore, if deregulated, stand termination and volunteer control of KK179 would use the same management practices as utilized for conventional alfalfa.

Table VIII-9. Herbicides for Alfalfa Stand Termination and Control of Volunteer Alfalfa[1].

Crop/Product	Rate/Acre (lbs a.e or a.i.)	Preplant/Pre	In-Crop
Corn			
2,4-D	1.0	√	√
Dicamba	0.5	√	√
2,4-D/Dicamba	0.5/0.5	√	√
Chlorpyralid	0.094-0.188	√	√
Cotton			
Dicamba	0.5 fl oz	√	-
Carfentrazone	0.004-0.008	√	√
Flumioxazin	0.06	√	-
Trifloxysulfuron	0.01	-	√
Small Grains			
2,4-D	1.0	√	√
Dicamba	0.5	√	√
2,4-D/Chlorpyralid	0.75 + 0.07	√	√
Potato			
2,4-D	1.0	√	-
Dicamba	0.5	√	-
2,4-D/Dicamba	0.5/0.5	√	-
Sugarbeets			
Chlorpyralid	0.094-0.188	√	√
Glufosinate	0.42	√	-
Tomato			
2,4-D	1.0	√	-
Dicamba	0.5	√	-
2,4-D/Dicamba	0.5/0.5	√	-

[1] Sources: (CDMS, 2011; Coulter, 2010; Dillehay and Curran, 2006; Rogan and Fitzpatrick, 2004; USDA-APHIS, 2010; Van Deynze et al., 2004).

√ Indicates that herbicide provides control of volunteer alfalfa.

- Indicates herbicide does not provide control or is not labeled for the identified application.

VIII.H. Management of Feral Alfalfa

Alfalfa has occasionally become feral, or naturalized, by establishment outside of agricultural fields or intentionally planted in non-agricultural locations (USDA-APHIS, 2010). The *Medicago sativa* subspecies *sativa* (purple-flowered alfalfa used in cultivation) has naturalized populations in all 50 states (USDA-APHIS, 2010). *M. sativa* subspecies *falcata* (yellow-flowered or Siberian alfalfa) is naturalized in the northern and western states and is being promoted as a rangeland enhancer for grazing. Like alfalfa under cultivation, feral alfalfa originated from introduced varieties. Feral alfalfa can be found in air fields, canals, cemeteries, ditch banks, fence rows, highways, irrigation ditches, pipelines, railroads, rangeland, rights-of-way, roadsides, wasteland, preserves, parks, and recovery areas. Feral populations exist near locations used for alfalfa seed and forage production (USDA-APHIS, 2010).

In the event of the trait for reduced G lignin and total lignin being transferred from KK179 to feral populations by cross-pollination, no environmental consequences are expected to occur. Furthermore, evaluations have shown that the introduced trait for reduced G lignin and total lignin in KK179 does not enhance weediness or plant pest potential relative to conventional alfalfa. Also, Monsanto and FGI are not aware of a conceivable mechanism by which the trait could confer a selective advantage or to be selected for in an unmanaged setting.

In general, alfalfa is not a species specifically targeted for weed control in unmanaged areas or in roadsides. In fact, alfalfa may be considered a desirable species along some roadsides in South Dakota and Wisconsin and is encouraged to grow. In situations where control is desired, feral alfalfa can be controlled or discouraged using physical or chemical methods. Herbicides, such as 2,4-D, dicamba, or a combination of these herbicides, effectively control feral alfalfa, including Roundup Ready and KK179 alfalfa, in most sites. Glyphosate is not generally the herbicide of choice to control feral alfalfa, as discussed in Section VIII.G, because it is not highly effective on conventional alfalfa.

The phenotypic, agronomic, and environmental interaction assessments presented in Section VII have demonstrated that KK179 is not meaningfully different from conventional alfalfa. Therefore, if deregulated, control of KK179 would use the same physical and herbicide management practices as utilized for conventional alfalfa.

VIII.I. Crop Rotation Practices in Alfalfa

The rotation of alfalfa with other crops is an integral part of a farm management program to maintain soil productivity, reduce soil erosion, avoid pathogen and pest buildup, and adjust to market conditions (USDA-APHIS, 2010). Alfalfa provides greater water infiltration, improves soil tilth, and provides nitrogen for subsequent crops in rotation (Canevari and Putnam, 2008; Orloff and Putnam, 1997). The extensive root system of alfalfa improves soil tilth and soil structure by creating channels that encourage water penetration and biological activity in the root zone. Considerable organic matter is added to the soil over the life of the stand which greatly benefits the growth and yield of subsequent crops, such as corn, tomato, wheat, or specialty crops. Alfalfa fixes atmospheric nitrogen through the symbiotic relationship with *Rhizobium* bacteria which can provide from 40 to 60 pounds of nitrogen per acre to crops that follow alfalfa (Orloff, 2008). In turn, rotations with other crops benefit alfalfa by breaking disease and insect cycles and improving weed control and soil fertility (Orloff, 2008; Orloff and Putnam, 1997).

Alfalfa is rotated most often with corn, cotton, wheat, oats, barley, sugar beets, and tomatoes (Putnam et al., 2008c; USDA-APHIS, 2010). Alfalfa-to-alfalfa rotations are uncommon because of the potential for autotoxicity and the inefficient use of residual soil nitrogen credits. Approximately 18% of existing alfalfa stands are terminated and rotated to a different crop each year (Hower et al., 1999). Current crop rotation options or patterns in the U.S. where alfalfa is grown are expected to remain the same upon the introduction of KK179.

VIII.J. Impact of the Introduction of KK179 on Agricultural Practices

Lignin plays an important role in terms of providing strength to plants (Undersander et al., 2009). and allowing water to move up the plant stem without leakage. As a component of plant cell walls, lignin molecules bind with hemicellulose and fill the spaces between cellulose, hemicellulose, and pectins. Lignin increases as plants mature and makes up the major portion of fiber in alfalfa forage (Undersander et al., 2009). However, lignin is indigestible and as a result, has a significant impact on the overall quality of alfalfa. It reduces digestion of the cellulose in the rumen of livestock. Therefore, forage and livestock producers desire alfalfa forage with lower lignin values as well as high protein content.

KK179 alfalfa is a biotechnology-derived alfalfa with an intended reduction in G lignin and total lignin compared to conventional alfalfa at the same stage of growth. Because of its lower lignin content, KK179 will provide growers with production benefits, including: 1) greater flexibility in harvesting schedules and 2) potentially lower production costs.

Growers will have the flexibility at each forage harvest to choose one of two production strategies to improve the value of alfalfa production on their farm. They can maximize forage quality while maintaining yield, or maximize yield while maintaining forage quality, depending on plant growth stage at harvest. When implementing the first production strategy (maximizing forage quality while maintaining yield), the cutting

schedules and the timing of harvest remains the same as is used with conventional varieties. For example, if the grower was producing high quality alfalfa hay for dairy cows, the grower would typically harvest alfalfa at the early to late bud stage. KK179 harvested at this crop stage will provide alfalfa forage with lower levels of lignin compared to conventional alfalfa. As a result, the quality of the forage is more likely to meet or exceed the intended quality standard targeted by the grower.

The second production strategy (maximizing yield while maintaining forage quality) involves delaying harvest to maximize yield without forfeiting forage quality compared to conventional alfalfa. KK179 can be harvested later and still produce high quality alfalfa forage that is comparable to earlier harvest timings with conventional alfalfa. However, the forage tonnage or yield from the cutting will likely be higher with KK179. Alfalfa forage yields during this plant growth stage can increase at the rate of 200 pounds per acre per day (Undersander et al., 2009). Therefore, delaying harvest can result in significant increases in forage yield. Additionally, when rain or adverse weather conditions coincide with a planned harvest interval, growers now will have the flexibility to delay harvest. This will allow growers to postpone harvest to when more favorable weather conditions occur, while still maintaining acceptable forage quality at the later harvest timing.

A delayed harvest schedule, with its longer harvest intervals, will potentially lower production costs over the life of the KK179 stand. A delayed harvest schedule likely will lead to one less forage harvest per year in the North Central region (*i.e.*, three compared to four cuttings). The elimination of one cutting will result in a reduction in harvesting costs. The overall forage yield for the year or season is expected to be the same or even higher with fewer cuttings because of the longer harvest intervals and resulting higher forage yields with each cutting. Fewer harvests also means fewer trips across the field, resulting in less labor, fuel consumption and soil compaction, plus potentially less crown damage to alfalfa plants in established stands. In addition, longer harvest intervals have been shown to extend the life of the alfalfa stand because the plants have a longer period of time to replenish carbohydrate root reserves (Orloff and Putnam, 2008).

The phenotypic, agronomic, and environmental interaction assessments presented in Section VII have demonstrated that KK179 is not meaningfully different from conventional alfalfa. Therefore, KK179 will utilize the same agronomic practices as conventional alfalfa production, including: tillage operations, seedbed preparation, pest management, and harvesting procedures. In addition, KK179 alfalfa growers will have increased flexibility in their forage harvest schedules, but the resulting hay quality will continue to meet the established USDA Agricultural Marketing Services' quality classifications of supreme, premium, good, fair, and utility. These classifications are based on several factors, including: the presence of weeds, fiber content, protein content, general color, and presence of mold (USDA-APHIS, 2010).

KK179 will be combined with Roundup Ready alfalfa utilizing traditional breeding techniques. The combined traits will allow growers planting Roundup Ready × KK179 alfalfa 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 maximizes the profitability of alfalfa 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 alfalfa forage production.

VIII.K. Stewardship of KK179

Monsanto and FGI develop effective alfalfa products and technologies and are committed to assuring that their products and technologies are safe and environmentally responsible. Monsanto and FGI demonstrate this commitment by implementing product stewardship processes throughout the lifecycle of a product and by participation in the Excellence Through Stewardship® (ETS) Program[1] and the USDA-APHIS Biotechnology Quality Management System (BQMS)[2]. These policies and practices include rigorous field compliance and quality management systems and verification through auditing. Stewardship Principles are also articulated in Technology Use Guides (TUG) distributed annually to growers who utilize Monsanto branded traits (Monsanto Company, 2012).

As an integral action of fulfilling this commitment, Monsanto and FGI will seek biotechnology regulatory approvals for KK179 in all key alfalfa import countries with a functioning regulatory system to assure global compliance and support the flow of international trade. These actions will be consistent with the Biotechnology Industry Organization (BIO) Policy on Product Launch Stewardship[3]. Monsanto and FGI will continue to monitor other countries that are key importers of alfalfa from the U.S. for the development of formal biotechnology approval processes. If new functioning regulatory processes are developed, Monsanto and FGI will make appropriate and timely regulatory submissions.

Monsanto and FGI also commit to industry best practices on seed quality assurance and control to ensure the purity and integrity of KK179 seed. As with Monsanto's and FGI's other alfalfa products, before commercializing KK179 in any country, a detection method for KK179 will be made available to alfalfa producers, processors, and buyers.

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[1] http://www.excellencethroughstewardship.org/.

[2] http://www.aphis.usda.gov/biotechnology/bqms_main.shtml

[3] http://www.excellencethroughstewardship.org/facts/documents/Guide%20for%20Product%20Launch%20Stewardship.pdf.

IX. ENVIRONMENTAL ANALYSIS

IX.A. Introduction

This section provides a brief review and assessment of the plant pest potential of KK179 and its impact on agronomic practices and on the environment. USDA-APHIS has responsibility, under the Plant Protection Act (PPA) (7 U.S.C. § 7701-7772), to prevent the introduction and dissemination of plant pests into the U.S. Regulation 7 CFR § 340.6 provides that an applicant may petition USDA-APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and should no longer be regulated. If USDA-APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article. According to the PPA, the definition of "plant pest" includes the living stage of any of the following, or a similar article that can directly or indirectly injure, damage, or cause disease in any plant or plant product: (A) a protozoan; (B) a nonhuman animal; (C) a parasitic plant; (D) a bacterium; (E) a fungus; (F) a virus or viroid; or (G) an infectious agent or other pathogens. (7 U.S.C. § 7702[14]).

The regulatory endpoint under the PPA for biotechnology-derived crop products does not involve zero risk, but rather a determination that the regulated article is not expected to pose a plant pest risk. Information in this petition related to plant pest risk characteristics of KK179 include: 1) mode-of-action and changes to plant metabolism; 2) composition; 3) characteristics of the expressed product; 4) potential for weediness of the regulated article; 5) impacts to non-target organisms (NTOs); 6) disease and pest susceptibilities; 7) impacts on agronomic practices; and 8) impacts on the weediness of any other plant with which it can interbreed, as well as the potential for gene flow.

The following lines of evidence form the basis for the plant pest risk assessment in this petition: 1) insertion of a single functional copy of the *CCOMT* suppression cassette; 2) safety and mode-of-action of the *CCOMT* suppression cassette; 3) compositional equivalence of KK179 forage to conventional alfalfa with the exception of the intended reduction in G lignin and total lignin; 4) phenotypic and agronomic characteristics demonstrating no increased plant pest potential including disease and pest susceptibilities; 5) negligible risk to NTOs including organisms beneficial to agriculture; 6) familiarity with alfalfa as a cultivated crop; and 7) no greater likelihood to impact agronomic practices, cultivation practices, or the management of weeds, diseases, and insects compared to commercially grown alfalfa.

Using the assessment above, the data and analysis presented in this petition lead to a conclusion that KK179 is not expected to be a plant pest, and therefore should no longer be subject to regulation under 7 CFR § 340.

IX.B. Plant Pest Assessment of KK179

This section summarizes the details of the genetic insert, characteristics of the genetic modification, safety of expressed product, composition, and phenotypic, agronomic,

environmental interaction characteristics of KK179 used to evaluate the feed, food, and environmental safety of KK179.

IX.B.1. Characteristics of the Genetic Insert

IX.B.1.1. Genetic Insert

As described in Section IV, KK179 was developed by *Agrobacterium tumefaciens*-mediated transformation of conventional alfalfa R2336 leaf tissue using the plasmid vector PV-MSPQ12633. PV-MSPQ12633 contains two T-DNAs, each delineated by Left and Right Border regions to facilitate transformation. The first T-DNA is designated as T-DNA I and contains the *CCOMT* suppression cassette, which is regulated by the *Pal2* promoter and the *nos* 3′ UTR. The second T-DNA, designated as T-DNA II, contains the *nptII* expression cassette, which is regulated by the *35S* promoter and the *nos* 3′ UTR. During transformation, both T-DNAs were inserted into the alfalfa genome where T-DNA II, containing the *nptII* expression cassette, functioned as a marker gene for the *in vitro* selection of transformed plantlets. Subsequently, conventional breeding methods and heritable segregation, along with a combination of analytical techniques, were used to isolate those progeny plants that contained the *CCOMT* suppression cassette (T-DNA I) but did not contain the *nptII* expression cassette (T-DNA II).

Molecular analyses demonstrated that KK179 contains a single copy of the *CCOMT* suppression cassette integrated into the alfalfa genome at a single locus. No T-DNA II or backbone DNA sequences from PV-MSPQ12633 were detected in KK179. Recorded data confirmed the organization and sequence of the insert, in addition to the stability of the insert over several generations.

IX.B.1.2. Mode-of-Action

KK179 reduces G lignin and total 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 alfalfa, assembled to form an inverted repeat DNA sequence. The inverted repeat sequence produces double-stranded RNA (dsRNA) which 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 subunit compared to conventional alfalfa at the same stage of growth. The reduction in G lignin subunit synthesis leads to reduced accumulation of total lignin, measured as acid detergent lignin (ADL) described in Section I.B.3.

Analyses of KK179 RNA by Northern blot confirm the suppression of endogenous *CCOMT* RNA in alfalfa forage. Additionally, analysis of monomeric lignin subunits, which constitute the building blocks of 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 by a commercial forage testing lab, 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.

IX.B.1.3. Safety of KK179 Expressed Products

As described in Section V, the *CCOMT* suppression cassette encodes for dsRNA and is extremely unlikely to encode for a protein. Double stranded RNAs are composed of nucleic acids and are commonly found in eukaryotes, including plants, for endogenous gene suppression. Nucleic acids have a long history of safe consumption and are considered GRAS by the U.S. FDA as there is no evidence to suggest dietary consumption of RNA is associated with mammalian toxicity or allergenicity. 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 toxicity or allergenicity of dietary RNA, the RNA-based suppression of endogenous CCOMT gene expression in KK179 poses no novel risks as a result of exposure to expressed products of the DNA insert.

IX.B.2. Compositional Characteristics of KK179

Compositional comparisons based on OECD guidance were presented in Section VI.B. to assess whether levels of nutrients, anti-nutrients, and secondary metabolites in forage derived from KK179 are comparable to levels in the conventional control and several conventional commercial reference varieties for which there is an established history of safe consumption. Nutrients assessed in this analysis included 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 assessed 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 assessed included *p*-coumaric acid, ferulic acid, sinapic acid, total polyphenols, free phenylalanine, and canavanine.

Compositional and nutritional comparisons were conducted to determine statistically significant differences at the 5% level of significance between KK179 and the conventional control. The relevance of each statistically significant difference observed was assessed through evaluation of: 1) the magnitude of the difference in the mean values of components; 2) whether the mean value is within the range of natural variability of that component as established by the 99% tolerance interval of the conventional commercial reference varieties; and 3) an assessment of the differences within the context of natural variability of available commercial alfalfa composition published in the scientific literature.

Assessment of the compositional analyses confirmed that the differences observed were not meaningful to feed and food safety or the nutritional quality of KK179 compared to the conventional control. With the exception of the intended changes in G lignin and total lignin (ADL) described in Section I.B.3., the levels of assessed components in KK179 were determined to be within the 99% tolerance interval established from the conventional commercial reference varieties grown concurrently. While compositional analysis confirmed that the intended reduction in G lignin and total lignin, this compositional change does not pose meaningful or novel feed/food safety or environment safety concerns, as similar levels of G lignin and total lignin are also found in conventional alfalfa forage harvested at earlier stages of growth. With the exception of the intended reduction in G lignin and total lignin, these results support the overall conclusion that KK179 forage is compositionally equivalent to conventional alfalfa in accordance with OECD guidelines.

IX.B.3. Phenotypic and Agronomic and Environmental Interaction Characteristics

Extensive information and data were used to assess whether the introduction of the trait for reduced G lignin and total lignin altered the plant pest potential of KK179 compared to the conventional control. Phenotypic, agronomic, and environmental interaction characteristics of KK179 were evaluated and compared to those of the conventional control and considered within the variation among conventional commercial reference varieties. These assessments included seven general data categories: 1) seed germination, dormancy, and emergence; 2) vegetative growth; 3) winter survival; 4) reproductive growth including pollen, flower, and seed characteristics; 5) lodging and seed retention on the plant; 6) plant response to abiotic stress and interactions with diseases and arthropods; and 7) plant-symbiont interactions. Results from the phenotypic, agronomic, and environmental interaction assessment demonstrate that KK179 does not possess: 1) increased weedy characteristics; 2) increased susceptibility or tolerance to specific abiotic stresses, diseases or arthropods; or 3) characteristics that would confer a plant pest risk compared to the conventional control. Taken together, the results of the analysis support a determination that KK179 is not expected to pose a plant pest risk compared to conventional alfalfa.

IX.B.3.1. Seed Dormancy and Germination

Seed germination and dormancy mechanisms vary with species and often have complex genetic origins. Seed dormancy is a characteristic that is often associated with plants considered weeds (Anderson, 1996; Lingenfelter and Hartwig, 2007). Apart from the impervious (hard) seed coat, alfalfa has no physiological seed dormancy mechanism (Copeland and McDonald, 2001; Rolston, 1978). A decrease in the percent of hard seed was observed in KK179 relative to the conventional control, however, this difference is not considered a characteristic associated with increased weediness or plant pest potential. No other changes in the seed dormancy or germination characteristics were observed in either scarified or non-scarified seed that would be indicative of increased plant weediness or plant pest potential of KK179 compared to the conventional control as described in Section VII.C.1.

IX.B.3.2. Plant Growth and Development

Evaluations of plant growth and development in the field described in Section VII.C.2. were based on assessment of phenotypic characteristics including emergence, early season vigor, lodging, crop growth stage, regrowth after forage harvest, fall plant height, total forage yield, spring vigor, spring stand recovery, and spring stand count. Also assessed were phenotypic characteristics during seed production such as seedling vigor, seed maturity, lodging at seed maturity, and split pods. Of the growth and development characteristics assessed between KK179 and the conventional control, no statistically significant differences were detected at a 5% level of significance ($\alpha = 0.05$). The data support the conclusion that there is no biologically meaningful change in terms of weediness or plant pest potential of KK179 compared to conventional alfalfa.

IX.B.3.3. Response to Abiotic Stressors

No biologically meaningful differences were observed during comparative field observations between KK179 and the conventional control in their response to abiotic stressors, such as cold, heat, drought, wind, as described in Section VII.C.2. The lack of significant biological differences in plant responses to abiotic stress supports the conclusion that the introduction of the trait for reduced G lignin and total lignin is not expected to result in increased plant pest potential for KK179 compared to conventional alfalfa.

IX.B.3.4. Reproductive Characteristics

Evaluations of pollen morphology and viability and flower morphology in KK179 and the conventional control as described in Section VII.C.3 provide information useful in a plant pest risk assessment as it relates to the potential for gene flow and introgression of the biotechnology-derived trait into other alfalfa varieties and sexually compatible related species. Pollen morphology and viability and flower morphology evaluations demonstrated no statistically significant differences ($\alpha = 0.05$) between KK179 and the conventional control for any of the assessed characteristics. Taken together, these comparative assessments indicate that KK179 is not different than conventional alfalfa and is not expected to have increased weediness or plant pest potential compared to conventional alfalfa.

IX.B.3.5. Interactions with Non-target Organisms Including Those Beneficial to Agriculture

Evaluation of KK179 for potential effects on NTOs is a component of the plant pest risk assessment and was evaluated using a combination of biochemical information and experimental data. The information and data included molecular characterization, safety of expressed products, the history of environmental exposure to reduced lignin, results from the environmental interactions assessment described earlier, and the demonstration of compositional, agronomic, and phenotypic equivalence to conventional alfalfa. The nature of KK179 as a product with no pesticidal activity means all exposed organisms can be considered NTOs. Observational data on environmental interactions for KK179

and a conventional alfalfa control were collected at select U.S. sites in 2010 and 2011. Multiple conventional commercial conventional reference varieties were included in the analysis to establish a range of natural variability for each characteristic. The environmental interactions evaluation, described in Section VII.C., included qualitative assessment of plant response to insects and disease and quantitative assessment of arthropod pests and abundance. The results of these assessments indicated that the presence of the trait for reduced G lignin and total lignin does not alter plant-insect interactions, including beneficial arthropods and pests, nor does it alter disease susceptibility of KK179 compared to conventional alfalfa. The results also indicated that reduction of lignin to levels already present in the environment does not alter plant-insect interactions or disease susceptibility.

In the field, alfalfa forms a complex symbiotic relationship with members of the bacterial family *Rhizobiaceae* and *Bradyrhizobiaceae*. No significant differences in parameters related to symbiont interactions were detected between KK179 and the control for, indicating no impact on either the symbiotic relationship or the symbiotic nitrogen-fixing bacteria. This assessment demonstrated that the symbiosis between nitrogen fixing bacteria and alfalfa was not altered as a result of the introduction of the trait for reduced G lignin and total lignin (Section VII).

Taken together, these data combined with biochemical information support the conclusion that KK179 is not expected to have an effect on NTOs including those beneficial to agriculture.

IX.C. Weediness Potential of KK179

Alfalfa is a widely adapted crop that can be found in all parts of the continental United States, Alaska, and Hawaii, preferring fertile, well-drained soils. Because of its adaptability, it also survives outside of cultivation. Little evidence exists to suggest that alfalfa behaves as a weed, other than as a volunteer in agricultural settings (USDA-APHIS, 2010). Weed control experts from states where alfalfa is cultivated extensively, including Arizona, California, Idaho, Oregon, Pennsylvania, South Dakota, Washington, and Wisconsin, have communicated that they do not consider alfalfa a weed (Rogan and Fitzpatrick, 2004; USDA-APHIS, 2010). Out of 12 weed lists available in the USDA PLANTS Database (USDA-NRCS, 2012), *Medicago sativa* is found in only one weed identification guide by the Southern Weed Science Society (SWSS). The author of the SWSS entry for alfalfa has clarified that alfalfa is not an invasive weed and does not displace native species (USDA-APHIS, 2010).

Though not considered a weed, alfalfa does exist in a feral state outside of agricultural settings. These plants originated from introduced varieties and can be found in sparse populations throughout the U.S. (USDA-APHIS, 2010; USDA-NRCS, 2012). As described in Section VIII.H., alfalfa can be found in many non-agricultural areas. It has been intentionally used for numerous non-agricultural purposes including: rehabilitation of overgrazed rangelands to improve wildlife habitat and for livestock; erosion-control projects in forest interiors; improvement of compacted soils; use in seed mixes for USDA's Conservation Reserve Program (CRP); revegetation of areas damaged by

wildfire; and erosion reduction in mined soils (USDA-APHIS, 2010). These uses have in some cases led to establishment of feral alfalfa populations. Surveys have confirmed that minor feral populations exist in six major alfalfa-producing states, in areas where alfalfa seed or forage is produced (Rogan and Fitzpatrick, 2004). The *falcata* subspecies of alfalfa (yellow-flowered) is naturalized on rangelands in the northern and western States, where it has been promoted as a rangeland enhancer for grazing (USDA-NRCS, 2012). In situations where control of feral alfalfa is desired, it can be controlled like cultivated alfalfa using cultural or chemical methods as described in Sections VIII.G and VIII.H.

Feral alfalfa can be found in various soils and environments in coexistence with many other plant species. As expected, feral alfalfa populations are not managed and lack regular external inputs like irrigation, herbicides, insecticides, and fertilizers. Thus, they are exposed to numerous environmental factors limiting establishment of seedlings including low or excessive water conditions, low light intensity, extreme soil temperatures, and low pH soils (Bagavathiannan and van Acker, 2009). Because active pest control and genetic resistance to pests are largely absent, feral alfalfa is more negatively impacted by endemic pests than alfalfa under cultivation (Bagavathiannan et al., 2011b). As a result, the competitiveness and numbers of viable, vigorous seeds produced by feral alfalfa are likely constrained compared to cultivated alfalfa with improved genetics under management (Van Deynze et al., 2008).

Seed dormancy is an important characteristic often associated with plants that are weeds (Anderson, 1996). Dormancy mechanisms, including hard seed, vary with species and are usually based on complex processes. For most crops, the number of hard seed is negligible or nonexistent. However, when alfalfa seed is produced, a portion of the seed is "hard," that is, the seeds do not absorb water after a prescribed period of time because of an impermeable seed coat (AOSA, 2007b). The percentage of hard seed in alfalfa varies widely, and depends largely on environmental conditions during and after seed maturation, harvesting and seed conditioning methods and on genetic factors (Bass et al., 1988). Seed aging, weathering or mechanical scarification make the seed coat permeable to water and allows rapid germination under favorable conditions. Apart from an impervious seed coat, alfalfa has no physiological seed dormancy mechanism to delay germination.

The viability of most alfalfa seed in soil declines over time (Bass et al., 1988). A portion of the residual alfalfa seed can persist in the soil for several years, and if it remains viable may germinate as volunteers (Bass et al., 1988; Mueller, 2008). Alfalfa that has germinated and emerged unintentionally in a subsequent crop, also known as volunteer alfalfa, may compete with the succeeding rotational crop. However, problems controlling volunteer alfalfa are not common (Van Deynze et al., 2008). Volunteers, including ones with herbicide-tolerant traits, can be managed with pre-plant or selective post-emergent herbicide applications or by mechanical means as described in Section VIII.G.

In comparative studies described in Section VII.C. between KK179 and the conventional control, phenotypic, agronomic, and environmental interaction data were evaluated for changes that would impact the plant pest potential, in particular, weediness potential. Results of these evaluations show that there are no biologically significant differences

between KK179 and the conventional control for traits potentially associated with weediness. Furthermore, comparative field observations between KK179 and the conventional control including their responses to abiotic stressors, such as cold, heat, drought, and wind, indicated no differences and, therefore, no increased weediness potential. Data on environmental interactions also indicate that KK179 does not confer any biologically meaningful increased susceptibility or tolerance to specific diseases or insect pests. Collectively, these findings support the conclusion that KK179 has no increased weediness potential compared to alfalfa and is not expected to become a weed.

IX.D. Potential for Gene Flow and Introgression

Gene flow refers to all mechanisms that may result in the movement of genes between populations. Pollen-mediated gene flow, also referred to as cross-pollination or hybridization, occurs when pollen of one plant successfully fertilizes ovules of a second sexually compatible plant. Pollen-mediated gene flow is affected by both biotic and abiotic factors such as the degree of sexual compatibility of the pollen donor and recipient plants, proximity and frequency of pollen sources and sinks, pollen dispersal, flowering phenology, synchrony of flowering times, environmental conditions such as temperature, humidity and field architecture. Because pollen-mediated gene flow is a natural biological process, it does not constitute an environmental risk in and of itself. Seed-mediated gene flow is an additional mechanism whereby genes may be dispersed between locales and populations.

Gene introgression is a process whereby one or more genes successfully incorporate into the genome of a recipient plant. Gene introgression must be considered in the context of the transgenes inserted into the biotechnology-derived plant and the likelihood that the presence of the transgenes and their subsequent transfer to recipient plants and plant populations will result in increased plant pest potential.

The baseline potential for gene flow and introgression in conventional alfalfa in agricultural and feral settings has been comprehensively reviewed by USDA-APHIS (USDA-APHIS, 2010) and is relevant to the environmental analysis of KK179. The lack of differences between the KK179 and conventional control with respect to plant pest potential, described in Section VII.C., includes aspects related to gene flow and introgression such as pollen, flower, and seed characteristics. The following section summarizes the previous USDA-APHIS review and more recently published information that can be used to address the potential for gene flow and introgression from the introduction of KK179.

IX.D.1. Gene Flow Among Alfalfa (*Medicago sativa)* Populations

Alfalfa is dependent on cross-pollination by insects; therefore, pollen-mediated gene flow between different alfalfa populations is possible. Many factors influence the probability of successful gene flow between alfalfa populations, including: timing and degree of flowering; relative abundance of pollen sources; the presence and activity of pollinators; proximity of alfalfa populations; physical barriers between populations; the relative scale of the alfalfa populations; probability of seed maturation and germination; probability of seedling survival; starting gene frequency within the source and sink alfalfa populations; and cultural practices employed (Putnam, 2006; Van Deynze et al., 2008). How these factors interact to impact the gene flow potential in alfalfa has been extensively studied and reviewed by academic researchers and the alfalfa industry with respect to potential impacts on commercial alfalfa cultivation and the environment (Bagavathiannan et al., 2011b; a; Fitzpatrick et al., 2003; Hammon et al., 2006; NAFA, 2008; Putnam, 2006; St. Amand et al., 2000; Teuber et al., 2011; Teuber et al., 2005; Teuber et al., 2007; Van Deynze et al., 2008; Van Deynze et al., 2005). The gene flow literature has also been reviewed by USDA-APHIS (USDA-APHIS, 2010).

The development and introduction of Roundup Ready alfalfa containing the glyphosate tolerance trait has provided a marker system to examine in detail the dynamics of gene flow for alfalfa, both conventional and biotechnology-derived, under experimental and commercial conditions. Studies conducted under commercial seed production conditions in areas of intended use for KK179 have provided information on the realistic scenarios for gene flow (Fitzpatrick et al., 2003; Hagler et al., 2011; Teuber et al., 2011; Teuber et al., 2007). These studies along with previous studies and reviews have provided a comprehensive assessment of gene flow as a commercial and environmental risk factor (USDA-APHIS, 2010; Van Deynze et al., 2008). More recent studies have confirmed previous findings (Hagler et al., 2011; Teuber et al., 2011).

For purposes of analyzing gene flow, alfalfa populations can be categorized into three major types as sources and recipients of pollen: 1) alfalfa fields intended for hay production; 2) alfalfa fields intended for seed production; and 3) naturalized or feral alfalfa populations existing outside of managed conditions. These categories provide a comprehensive range of scenarios shown in Table IX-1 under which pollen flow can occur and provide a framework to evaluate pollen-mediated gene flow (Van Deynze et al., 2008).

Table IX-1. Potential Scenarios for Pollen-mediated Gene Flow in Alfalfa (adapted from Van Deynze et al., 2008)

↓ From To→	**Hay**	**Seed**	**Feral**
Hay	Hay-to-Hay	Hay-to-Seed	Hay-to-Feral
Seed	Seed-to-Hay	Seed-to-Seed	Seed-to-Feral
Feral	Feral-to-Hay	Feral-to-Seed	Feral-to-Feral

Commercial alfalfa seed production typically requires the intentional introduction of large numbers of bee colonies in or near fields during the peak of flower production in order to achieve high rates of pollination and uniform seed ripening. Forage production does not entail the use of bees by growers at any stage. The primary pollinators used are leafcutter bees, honey bees, and to a lesser extent alkali bees. Leafcutter bees and alkali bees pollinate at rates of over 80% while honey bees pollinate at 22% (Beekman and Ratnieks, 2000; Cane, 2002; Pitts-Singer and Cane, 2011). Leafcutter bees have a foraging range under 1 mile while honey bees range up to 3 miles and alkali bees even further (Beekman and Ratnieks, 2000; Hagler et al., 2011; Pitts-Singer and Cane, 2011). The studies show that cross-pollination rates decrease with increasing distance from the source of pollen and vary with the predominant pollinator species (USDA-APHIS, 2010; Van Deynze et al., 2008).

Gene Flow into Hay Production Fields

Greater than 99.5 percent of alfalfa planted in the U.S. is cultivated exclusively for alfalfa hay (forage) production (USDA-NASS, 2011a). The most commonly occurring alfalfa field interface is hay field-to-hay field, however, pollen-mediated gene flow is highly improbable between adjacent hay fields (Putnam, 2006; Van Deynze et al., 2008). Several factors in forage production limit potential gene flow from and into hay production fields: 1) harvest takes place at vegetative and early flower stages when little

to no pollen is produced and few flowers are present; 2) few natural pollinators of the optimal type are present; 3) biomass with flowers is removed on a regular basis which prevents seed setting; and 4) the competition and natural autotoxicity of the alfalfa prevents new seedlings resulting from rare outcrossing events to successfully grow within established stands (Canevari and Putnam, 2008) Thus, normal forage production practices significantly lower the risk of pollen-mediated gene flow between hay production fields and outside populations (Van Deynze et al., 2008).

The most important factor for avoiding seed-mediated gene flow in a hay production setting is the use of certified seed (Putnam, 2006; Teuber et al., 2007). Forage growers routinely use certified seed of registered varieties for sowing alfalfa stands. High quality seed decreases the risk of seed-mediated gene flow during stand establishment and the introduction of varietal mixtures and off-types.

It is also improbable that pollen from an adjacent seed field would result in gene flow into a hay field. Normal forage production practices, which include multiple harvests per year of the hay field, coupled with physical isolation distance requirements of certified alfalfa seed production fields keep the potential for gene flow from seed production fields to hay production fields very low (USDA-APHIS, 2010; Van Deynze et al., 2008). Under the remote possibility that an outcrossed seed in a hay field were to mature, competition from the stand and alfalfa's natural autotoxicity would reduce the probability of successful germination and survival within an existing stand.

Gene Flow into Seed Production Fields

Pollen-mediated gene flow between adjacent seed production fields (seed-to-seed), while a higher risk scenario, is considered a common, managable, and measurable occurrence for conventional cultivars of most outcrossing crops including alfalfa. This scenario applies to a much smaller area of approximately 121,000 acres, or 0.5 percent of the total U.S. alfalfa acres, concentrated in areas optimal for alfalfa seed production (USDA-APHIS, 2010). Conventional alfalfa seed production occurs in approximately 20 U.S. states. In the case of Roundup Ready alfalfa, the alfalfa industry has, for purposes of coexistence and trait stewardship, opted to limit seed production contracts to only ten states: Arizona, Colorado, Idaho, Montana, Nevada, Oregon, Texas, Utah, Washingon, and Wyoming. Professional seed growers in these states routinely utilize state seed certification services and, together, produce the majority of the U.S. alfalfa seed crop.

Certified seed growers have relied for many decades on physical isolation to minimize and mitigate pollen-mediated gene flow in order to manage genetic purity of commercial varieties (Brown et al., 1986; Dunkle, 2011; Kalaitzandonakes, 2011; Van Deynze, 2011). Gene flow studies of alfalfa seed production fields have confirmed that current AOSCA and OECD seed isolation and production standards can be used to meet Federal Seed Act standards (7 CFR § 201.76) (AOSCA, 2011; Fitzpatrick et al., 2003; Hammon et al., 2006; OECD, 2012; Teuber et al., 2011; Teuber et al., 2005; Teuber and Fitzpatrick, 2007; Teuber et al., 2007; Teuber et al., 2004). For special situations such as identity preserved crops where even greater purity is desired, seed producers use additional isolation or sanitation to ensure high seed purity.

Current AOSCA isolation requirements for certified alfalfa seed production have also been shown to effectively mitigate gene flow from hay production fields into seed fields (Teuber et al., 2007). The normal forage production practice of harvesting a hay field at or before 10 percent bloom during the seed production pollination period is sufficient to ensure that pollen-mediated gene flow from hay production fields into seed production fields is low. Furthermore, this practice is a stewardship requirement for Roundup Ready alfalfa hay growers, under terms of the technology use agreement (Monsanto Company, 2012). Research has shown that harvesting hay at stages of 20 to 50% bloom does not significantly raise the potential gene flow to neighboring seed production fields and risk remains very low (Teuber and Fitzpatrick, 2007; Van Deynze et al., 2008).

Further studies under actual commercial alfalfa seed grower conditions have shown that the use of minimum isolation distances specific to pollinator species and identity-preserved production protocols successfully mitigate gene flow to levels observed for other biotechnology-derived crop species to produce seeds of high genetic purity (>99 %). Adventitious presence levels can be managed to less than 0.5 percent (Fitzpatrick, 2007; Fitzpatrick et al., 2007; Fitzpatrick and Lowry, 2010). Levels of actual gene flow under these conditions have also been shown to be several times less than those predicted by research models developed using smaller research plots (Fitzpatrick, 2007; Fitzpatrick et al., 2007).

The information from these studies has allowed the alfalfa industry in conjunction with AOSCA to develop coexistence strategies to provide additional measures for seed producers interested in further reducing the risk of pollen-mediated gene flow to their commercially-sensitive seed production crops from hay production fields. The strategies include FGI Best Practices for Stewardship in Roundup Ready Seed Production, NAFA Best Management Practices for Roundup Ready Seed Production, and NAFA Best Management Practices for Adventitious Presence-Sensitive Alfalfa Seed Production (Fitzpatrick, 2007; NAFA, 2011b; 2012b; c; a) with or without identity-preserved protocols such as the AOSCA Alfalfa Seed Stewardship Program (ASSP) (AOSCA, 2012). The protocols in these strategies require pollinator-specific isolation distances to proactively mitigate adventitious presence in conventional seed and are considered applicable to other traits in alfalfa (Van Deynze et al., 2008). Other measures may include planting larger size seed production fields (>5 acres) and harvesting the seed field borders as a separate lot. The AOSCA ASSP was launched in 2010 by participating state seed certification agencies to offer a voluntary identity-preserved, process-based certificate to seed producers concerned about low level presence of biotechnology-derived traits (AOSCA, 2012). The establishment of formal, voluntary grower opportunity zones in many seed production areas since 2011 has facilitated local coordination of coexistence efforts of alfalfa seed growers for both conventional and biotechnology-derived alfalfa (NAFA, 2011a).

Gene Flow via Feral Alfalfa

As described in Section IX.C, populations of feral alfalfa have existed in the U.S. since its introduction due to natural dispersal from cultivated alfalfa and from intentional introductions for rangeland development and other purposes (Kendrick et al., 2005; USDA-APHIS, 2010). Gene flow to feral alfalfa plants from large-scale seed or hay

production fields of conventional alfalfa and biotechnology-derived alfalfa has been shown to occur (Hammon et al., 2006; St. Amand et al., 2000). However, typical conditions and practices for hay and seed production all but preclude the chance of gene flow into hay or seed production fields as previously described (USDA-APHIS, 2010; Van Deynze et al., 2008).

Certified alfalfa seed production requires minimum isolation from all sources of alfalfa including feral populations (AOSCA, 2011). Removing or mowing feral alfalfa plants near cultivated fields prevents synchronous bloom and reduces the risk of gene flow to near zero (USDA-APHIS, 2010). Seed producers are known to control these populations as there are fewer feral populations in areas of intensive alfalfa seed production compared to hay production areas (Kendrick et al., 2005). This practice also lowers the potential for gene flow into as well as from feral populations (Van Deynze et al., 2008). As explained previously, normal forage production practices also restrict gene flow from feral populations to hay production fields to extremely low levels.

A number of factors relative to feral alfalfa itself further reduce the risk of pollen-mediated gene flow from feral alfalfa populations to extremely low levels (USDA-APHIS, 2010; Van Deynze et al., 2008). First, most feral populations are found in relatively small populations at low densities. Second, the asynchronous timing of flowering and the low density of pollinators in feral areas limit the effectiveness of feral alfalfa populations as sources, sinks or bridges for gene flow. Third, feral populations, as described in Section IX.C., are not managed, and therefore are more susceptible to environmental stresses. Under these conditions, pollen and seed production on feral plants are expected to be considerably less prolific compared to alfalfa plants in managed seed production fields. These same factors are also determinant in the relatively low potential for gene flow between feral alfalfa populations (USDA-APHIS, 2010; Van Deynze et al., 2008).

A final consideration is that any gene, were it to be introgressed into a feral population, including a biotechnology-derived gene, would be present at a limited frequency unless there were pressure from a selection mechanism to raise the frequency (Ellstrand et al., 1999). Monsanto and FGI are not aware of a conceivable mechanism by which the introduced trait for reduced G lignin and total lignin could confer a selective advantage to feral alfalfa plants or be selected for in an unmanaged setting. The data presented in this petition strongly suggest that the trait in KK179 would not confer a selective advantage to a feral alfalfa plant. These factors taken together lead to the conclusion that gene flow from KK179 under scenarios involving feral populations is not expected to be greater than it is for conventional alfalfa and is unlikely to affect seed production, hay production, or existing feral alfalfa populations.

As summarized in Section IX.C., comparative studies between KK179 and the conventional control to evaluate possible changes in plant pest potential, and weediness potential in particular, did not reveal biologically significant differences in characteristics associated with weediness. Collectively, these findings also support the conclusion that KK179 has no increased potential compared to conventional alfalfa to outcross or hybridize with cultivated alfalfa under hay production or seed production conditions.

IX.D.2. Hybridization with Annual Species of Subgenus *Medicago*

Medicago sativa is very distantly related to the annual members of *Medicago* (Lesins and Lesins, 1979). No annual species of this genus is native to North America. One annual species, *M. lupulina* (black medic), however, is found in the U.S. and is considered a weed in lawns and unmanaged areas, as well as in forage seed crops due to its seeds contaminating other small-seeded forage legume seed crops. Crosses between the annual and perennial species do not occur naturally, and even artificial cross-fertilization is unsuccesful (Fridriksson and Bolton, 1963; Sangduen et al., 1983). Significant biological barriers exist between annual and perennial species, which prevent successful unassisted hybridization. Annual species are self-pollinating, while perennial species outcross and require bees to facilitate pollination. Ploidy and karyotype differences between alfalfa and the annual *Medicago* species also prevent successful hybridization. Additional reproductive barriers include both pre- and post-fertilization abnormalities, such as abnormal pollen tube growth (Sangduen et al., 1983) and post-fertilization abortion of ovules (Fridriksson and Bolton, 1963). Successful hybridizations between *M. sativa* and *M. lupulina* were reported once several decades ago, but numerous attempts to repeat the crosses have failed and the ability of the two species to hybridize is disputed (USDA-APHIS, 2010). Expert opinion concludes that no annual species is known to naturally hybridize with *M. sativa* (McCoy and Bingham, 1988; Quiros and Bauchan, 1988). USDA-APHIS has also concluded, due to lack of confirmatory evidence, hybridization between *M. lupulina* and *M. sativa* is very unlikely to occur (USDA-APHIS, 2010). Recent research further supports that *M. lupulina* and *M. sativa* hybridization is unlikely (Chandra et al., 2011; Steele et al., 2010).

IX.D.3. Hybridization with the Perennial Species of Subgenus *Medicago*

The *M. sativa* complex has been sexually hybridized with 12 other perennial *Medicago* species (McCoy and Bingham, 1988). Many of these interspecific hybrids have only been accomplished by using *in vitro* embryo culture of the hybrid in the laboratory (McCoy and Smith, 1986), making them highly unlikely to occur in nature. Three of these perennial species, *M. prostrata, M. cancellata,* and *M. saxatilis* are considered the most capable of successfully crossing with *M. sativa* (Lesins, 1961; 1962; 1970; Quiros and Bauchan, 1988). No perennial *Medicago* species are present naturally in the Americas, Australia, New Zealand, or South Africa (Quiros and Bauchan, 1988; USDA-APHIS, 2010). therefore, no risk of interspecific hybridization exists in the United States. This was confirmed by a search of survey databases for *Medicago* populations in the U.S. which only produced matches for *Medicago sativa* itself and for species that are sexually incompatible with *M. sativa* complex members (USDA-APHIS, 2010).

IX.D.4. Transfer of Genetic Information to Species with which Alfalfa Cannot Interbreed (Horizontal Gene Flow)

Monsanto and FGI are not aware of any reports confirming the transfer of genetic material from alfalfa to other sexually incompatible plant species. The probability for horizontal gene flow to occur is judged to be exceedingly small. The ecological risk associated with gene flow and introgression from KK179 derives from the presence of

the *CCOMT* gene segments. The consequences of the introgression of the *CCOMT* gene segments from KK179 into a sexually compatible species are neglible since, as data presented in this petition confirm, it confers no increased plant pest potential to alfalfa. If the extremely remote possibility of horizontal gene transfer were to occur, it is highly unlikely the consequences would be greater than gene flow to sexually compatible species.

IX.E. Potential Impact of KK179 on Alfalfa Agronomic Practices

Current agronomic practices for alfalfa were assessed to determine whether cultivation of KK179 has the potential to impact current alfalfa cultivation practices and management as described in Section VIII. Typically, alfalfa fields are managed agricultural areas dedicated to forage production. KK179 is likely to be used in common rotations on land previously used for agricultural purposes. Cultivation of KK179 is not expected to differ from current cultivation of conventional or Roundup Ready alfalfa. Certified seed production will continue to use well-established industry practices to deliver high-quality seed containing KK179 to growers.

KK179 is similar to conventional alfalfa in its agronomic, phenotypic, ecological, and compositional characteristics, and has levels of resistance to insects and diseases comparable to conventional alfalfa. KK179 will utilize the same agronomic practices as conventional alfalfa production, including tillage operations, seedbed preparation, pest management, and harvesting procedures. Therefore, no impacts on current cultivation and management practices for alfalfa are expected following the introduction of KK179. KK179 will allow growers the flexibility to alter timing of harvest to a limited extent. The resulting hay quality will continue to meet the established USDA Agricultural Marketing Services' quality classifications of supreme, premium, good, fair, and utility. Growers will have the flexibility at each harvest to choose one of two production strategies to improve the value of alfalfa production on their farm. Either they can 1) maximize forage quality while maintaining yield or 2) maximize yield while maintaining forage quality, depending on plant growth stage at harvest. When implementing the first production strategy, the cutting schedules and the timing of harvest remains the same as those used with conventional alfalfa. KK179 harvested at this crop stage will produce forage with lower levels of lignin compared to conventional alfalfa. As a result, the quality of the forage is more likely to meet or exceed the intended quality standard targeted by the grower. The second production strategy involves delaying harvest to maximize yield without forfeiting forage quality compared to conventional alfalfa. KK179 can be harvested later and still produce high quality forage that is comparable to earlier harvest timings with conventional alfalfa. This strategy is expected to result in increased forage tonnage or yield from the harvest. Growers will also have flexibility to delay harvest when rain or adverse weather conditions coincide with a planned harvest interval. This will allow them to postpone harvest to when more favorable weather conditions occur, while maintaining acceptable forage quality at a later harvest timing.

A delayed harvest schedule with its longer cutting intervals could potentially lower production costs over the life of the KK179 alfalfa stand. A delayed harvest schedule could lead to one less forage harvest per year in the North Central region (*i.e.*, three

compared to four cuttings). The elimination of one cutting could result in a substantial reduction in harvesting costs. At the same time, the overall forage yield for the year or season is expected to be similar or even higher because of the longer cutting intervals and resulting higher forage yields with each cutting. Fewer cuttings also means fewer trips across the field, which results in less labor, fuel consumption, and soil compaction, plus potentially less crown damage to alfalfa plants in established stands.

Based on this assessment, the introduction of KK179 is not expected to have an effect on current U.S. alfalfa cultivation practices and will provide growers increased flexibility to better manage the yield-quality relationship and harvesting schedules to meet market needs and intended on-farm uses for their alfalfa forage production.

IX.F. Summary of Plant Pest Assessments

Plant pests, as defined in the Plant Protection Act, are the living stage of any of the following, or a similar article, that can directly or indirectly injure, damage, or cause disease in any plant or plant product: (A) a protozoan; (B) a nonhuman animal; (C) a parasitic plant; (D) a bacterium; (E) a fungus; (F) a virus or viroid; or (G) an infectious agent or other pathogens (7 U.S.C. § 7702[14]). Data presented in Sections V through VII of this petition confirm that KK179, with the exception of reduced G lignin and total lignin in forage compared to conventional alfalfa cut at the same stage of growth, is not significantly different from conventional alfalfa, in terms of plant pest potential. Monsanto and FGI are not aware of any study results or observations associated with KK179 that would suggest that an increased plant pest risk would result from its introduction.

The plant pest assessment was based on multiple lines of evidence developed from a detailed characterization of KK179 compared to conventional alfalfa, followed by a risk assessment on detected differences. The plant pest risk assessment in this petition was based on the following lines of evidence: 1) insertion of a single functional copy of the *CCOMT* supppression cassette; 2) safety of the expressed product, dsRNA; 3) compositional equivalence of KK179 forage compared to a conventional control; 4) phenotypic and agronomic characteristics demonstrating no increased plant pest potential; 5) negligible risk to NTOs including organisms beneficial to agriculture; 6) familiarity with alfalfa as a cultivated crop and 7) no greater likelihood to impact agronomic practices, including land use, cultivation practices, or the management of weeds, diseases and insects, than conventional alfalfa.

Based on the data and information presented in this petition, Monsanto and FGI have concluded that, like conventional alfalfa and previously deregulated Roundup Ready alfalfa products, KK179 is not expected to be a plant pest. Results also support a conclusion of no increased weediness potential of KK179 compared to conventional alfalfa. Therefore, Monsanto and FGI request a determination from USDA-APHIS that KK179 and any progeny derived from crosses between KK179 and other commercial alfalfa be granted non-regulated status under 7 CFR § 340.

X. ADVERSE CONSEQUENCES OF INTRODUCTION

Monsanto and FGI are not aware of any study results or observations associated with KK179 indicating that there would be unexpected environmental consequences from the introduction of KK179. KK179 contains an DNA insert with gene segments from the endogenous CCOMT enzyme gene under the control of a promoter to drive a pattern of expression similar to that of lignin (Guo et al., 2001; Leyva et al., 1992). The assembled gene segments produce a transcript with an inverted repeat sequence that forms double stranded RNA (dsRNA), which specifically results in the degradation of *CCOMT* transcripts via an RNA interference mechanism and leads to the suppression of the endogenous *CCOMT* gene (Siomi and Siomi, 2009). The DNA insert is not capable of expressing protein products. The suppression of the CCOMT gene in the lignin biosynthetic pathway significantly lowers G lignin synthesis, which leads to reduced accumulation of total lignin in forage tissue. As demonstrated by field results and laboratory tests, the only phenotypic difference between KK179 and conventional alfalfa is reduction in G lignin and total lignin.

The data and information presented in this petition demonstrate that KK179 is not expected to pose an increased plant pest risk compared to conventional alfalfa. This conclusion is reached based on multiple lines of evidence developed from a detailed characterization of the product compared to conventional alfalfa, followed by risk assessment on detected differences. The characterization evaluation included molecular analyses, which confirmed the insertion of a single functional copy of the CCOMT suppression cassette at a single locus within the alfalfa genome. Additionally, there is a history of safe use of RNA as an expressed product and no evidence of protein production from the CCOMT suppression cassette. Analyses of key nutrients, anti-nutrients, and toxicants of KK179 seed demonstrate that KK179 is compositionally equivalent to conventional alfalfa, with the exception of the intended reduction in G lignin and total lignin. The phenotypic evaluations of KK179, including an assessment of seed germination and dormancy characteristics, plant growth and development characteristics, reproductive characteristics, and environmental interactions also indicated KK179 is unchanged compared to conventional alfalfa. There is no indication that KK179 would have an effect on non-target organisms including ones beneficial to agriculture. Therefore, based on the lack of increased pest potential compared to conventional alfalfa, the risks for humans, animals, and other NTOs from KK179 are negligible under the anticipated conditions of use.

The introduction of KK179 will not result in changes to cultivation practices or the management of weeds, diseases and insects in alfalfa production systems. Growers familiar with cultivation of conventional or Roundup Ready alfalfa will continue to employ the same crop rotational practices, weed control practices and/or volunteer control measures currently in place for conventional or Roundup Ready alfalfa products.

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APPENDICES

Appendix A: USDA Permits

Field trials of KK179 have been conducted in the U.S. since 2007. The protocols for these trials include field performance, breeding and observation, agronomics, and generation of field materials and data necessary for this petition. In addition to the KK179 phenotypic assessment data, observational data on pest and disease stressors were collected from these product development trials. The majority of the final reports have been submitted to the USDA. However, some final reports, mainly from the 2010-12 seasons, are still in preparation. A list of trials conducted under USDA permits and the status of the final reports for these trials are provided in Table A-1.

Table A-1. USDA Notifications and Permits Approved for KK179 and Status of Trials Conducted under These Permits

USDA No.	Effective Date	Release State (Sites)	Trial #	Trial Status
Year Field Trials – 2007				
07-035-101 n 07-317-101 rm 10-328-101 rm	4/1/2007 1/9/2008 1/9/2011	WI	7.001	Submitted to USDA
USDA No.	**Effective Date**	**Release State (Sites)**		**Trial Status**
Year Field Trials – 2008				
08-034-101 rm	04/14/2008	CA	8.010	Submitted to USDA
08-011-102 rm	05/09/2008	WI (2)	8.032	Submitted to USDA
			8.033	Submitted to USDA
07-317-101 rm	01/09/2008	WI (2), ID (2), OK	8.054	Submitted to USDA
			8.056	Submitted to USDA
			8.103	Submitted to USDA
			8.105	Submitted to USDA
			8.151	Submitted to USDA
USDA No.	**Effective Date**	**Release State (Sites)**		**Trial Status**
Year Field Trials – 2009				
08-359-101 rm 11-353-103 rm	03/18/2009 03/15/2012	ID	9.018	Submitted to USDA
08-345-111 rm 11-353-102 rm	03/18/2009 03/15/2012	ID (4)	9.019	Submitted to USDA
			9.024	Submitted to USDA
			9.025	Submitted to USDA
			9.028	Submitted to USDA
08-359-101 rm 11-353-103 rm	03/18/2009 03/15/2012	ID	9.0221-4	Submitted to USDA
			9.0225-22	Submitted to USDA
09-037-101 rm 11-353-104 rm	04/15/2009 04/15/2012	PA	9.035	Submitted to USDA
08-345-108 rm 11-350-104 rm	03/18/2009 03/15/2012	WI (6)	9.049	Submitted to USDA
			9.050	Submitted to USDA
			9.051	Submitted to USDA
			9.055	Submitted to USDA

			9.056	Submitted to USDA
			9.062	Submitted to USDA
08-345-109 rm 11-353-101 rm	03/18/2009 03/15/2012	IA (2)	9.076	Submitted to USDA
			9.078	Submitted to USDA
08-345-107 rm 11-350-103 rm	03/18/2009 03/15/2012	WA	9.092	Submitted to USDA
08-345-110 rm	03/18/2009	OK	9.162	Submitted to USDA
09-096-103 rm	8/1/2009			No plantings
USDA No.	**Effective Date**	**Release State (Sites)**		**Trial Status**
Year Field Trials – 2010				
09-149-103 rm 12-073-103 rm	08/01/2009 08/01/2012	CA (2)	10.001	Submitted to USDA
			10.004	In Progress
08-034-101 rm 11-066-102 rm	04/14/2008 04/15/2011	CA	10.027	In Progress
09-037-101 rm	04/15/2009	PA	10.033	In Progress
08-345-107 rm 11-350-103 rm	03/18/2009 03/15/2012	WA (2)	10.041	Submitted to USDA
			10.043	Submitted to USDA
08-345-108 rm 11-350-104 rm	03/18/2009 03/15/2012	WI (6)	10.101	Submitted to USDA
			10.103	In Progress
			10.106	In Progress
			10.109	In Progress
			10.110	In Progress
			10.111	In Progress
09-345-102 rm	04/15/2010	WI (2)	10.107	In Progress
			10.114	In Progress
08-011-102 rm 11-070-103 rm	05/09/2008 05/09/2011	WI (2)	10.151	Submitted to USDA
			10.152	In Progress
09-308-108 rm	03/24/2010	ID	10.201	Submitted to USDA
08-345-111 rm 11-353-102 rm	03/18/2009 03/15/2012	ID (3)	10.204	Submitted to USDA
			10.211	Submitted to USDA
			10.212	Submitted to USDA
08-359-101 rm	03/18/2009	ID	10.213	Submitted to USDA
10-004-102 rm	04/15/2010	ID	10.215	In Progress
09-357-107 rm	04/01/2010	KS (2)	10.241	In Progress

			10.242	Submitted to USDA
09-308-102 rm	04/01/2010	IL	10.251	Submitted to USDA
09-357-106 rm	04/01/2010	IL	10.252	Submitted to USDA
08-345-109 rm 11-353-101 rm	03/18/2009 03/15/2012	IA (5)	10.262	Submitted to USDA
			10.267	Submitted to USDA
			10.268	Submitted to USDA
			10.269	Submitted to USDA
			10.270	Submitted to USDA
09-308-107 rm	04/01/2010	CA	10.301	In Progress
09-308-104 rm	04/01/2010	IA	10.311	In Progress
09-357-103 rm	04/01/2010	TX	10.321	In Progress
09-308-103 rm	04/01/2010	WI	10.331	In Progress
09-357-104 rm	04/06/2010	NY	10.341	Submitted to USDA
09-345-103 rm	04/15/2010			No plantings
10-225-104 rm				No plantings
USDA No.	**Effective Date**	**Release State (Sites)**		**Trial Status**
Year Field Trials - 2011				
10-364-115 rm	04/01/2011	ID (2)	11.001	In Progress
			11.002	In Progress
11-066-102 rm	04/15/2011	CA	11.052	In Progress
10-341-101 rm	03/15/2011	CA (2)	11.053	In Progress
			11.054	In Progress
10-341-103 rm	04/15/2011	WI (9)	11.101	In Progress
			11.102	In Progress
			11.103	In Progress
			11.104	In Progress
			11.105	In Progress
			11.107	Submitted to USDA
			11.108	Submitted to USDA
			11.110	In Progress
			11.111	In Progress
10-363-114 rm	04/15/2011	WI	11.151	Submitted to USDA
10-341-102 rm	04/15/2011	PA (5), WA (6), ID (7), OK (2)	11.004	In Progress
			11.005	In Progress
			11.006	In Progress
			11.008	Submitted to USDA
			11.009	In Progress

			11.010	Submitted to USDA
			11.011	Submitted to USDA
			11.201	Submitted to USDA
			11.202	Submitted to USDA
			11.203	Submitted to USDA
			11.204	In Progress
			11.205	In Progress
			11.261	Submitted to USDA
			11.262	Submitted to USDA
			11.263	In Progress
			11.264	In Progress
			11.267	In Progress
			11.268	In Progress
			11.351	In Progress
			11.352	In Progress
10-341-104 rm	04/15/2011	IA (8)	11.241	In Progress
			11.242	In Progress
			11.243	In Progress
			11.245	In Progress
			11.246	In Progress
			11.247	In Progress
			11.248	In Progress
			11.249	In Progress
11-070-103 rm	05/09/2011	WI (6)	11.281	In Progress
			11.282	In Progress
			11.283	Submitted to USDA
			11.284	Submitted to USDA
			11.285	In Progress
			11.286	In Progress
USDA No.	**Effective Date**	**Release State (Sites)**		**Trial Status**
Year Field Trials - 2012				
10-341-101 rm	03/15/2011	CA	12.001	In Progress
11-342-102 rm-a1	03/15/2012	CA (2)	12.002	In Progress
			12.003	In Progress
11-349-101 rm	04/15/2012	WI (4)	12.101	In Progress
			12.104	In Progress
			12.105	In Progress
			12.109	In Progress
11-353-103 rm	03/18/2012	ID	12.201	In Progress

10-364-115 rm	04/01/2011	ID (2)	12.202	In Progress
			12.203	In Progress
11-349-103 rm	04/15/2012	ID (3)	12.205	In Progress
			12.206	In Progress
			12.207	In Progress
11-349-104 rm	04/15/2012	IA (2)	12.401	In Progress
			12.402	In Progress
10-364-116 rm	04/15/2011	WI	12.501	In Progress
11-203-101 rm	10/1/2011			No plantings
11-349-103 rm	04/15/2012	PA	12.302	In Progress
11-349-103 rm	04/15/2012	WA	12.703	In Progress
11-066-102 rm	04/15/2011	CA (2)	12.711	In Progress
			12.715	In Progress
09-357-107 rm	04/01/2010	KS (2)	12.721	In Progress
			12.722	In Progress
09-308-103 rm	04/01/2010	WI	12.731	In Progress
09-308-104 rm	04/01/2010	IA	12.741	In Progress

Appendix B: Materials and Methods Used for Molecular Analyses of KK179

B.1. Materials

The genomic DNA used in molecular analyses was isolated from leaf tissues of the P_0 generation of KK179, the conventional control C_0, and conventional parental controls R2336 and Ms208. For generational stability analysis, genomic DNA was extracted from leaf tissue of the P_0, MBC1, MBC2, and Syn1 generations of KK179. PV-MSPQ12633 (Figure III-1) was used as a positive hybridization control in Southern blot analyses. Probe templates generated from PV-MSPQ12633 were used as additional positive hybridization controls. Additional reference standards, the 1 kb DNA Extension Ladder, and λ DNA/Hind III Fragments from Invitrogen (Carlsbad, CA) were used for size estimations on Southern blots and agarose gels.

B.2. Characterization of the Materials

The identity of the source materials was verified by methods used in molecular characterization to confirm the presence or absence of KK179. The stability of the genomic DNA was confirmed by observation of interpretable signals from digested DNA samples on ethidium bromide-stained agarose gels and /or specific PCR products, and the samples did not appear visibly degraded on the ethidium bromide-stained gels.

B.3. DNA Isolation for Southern Blot and PCR Analyses

KK179, conventional control C_0, and conventional parental controls R2336 and Ms208 genomic DNA samples were isolated from alfalfa leaf tissue. Prior to extraction, leaf tissue was processed to a fine powder by mortar and pestle using liquid nitrogen. Genomic DNA was extracted using a hexadecyltrimethylammonium bromide (CTAB) based method. Briefly, 5 ml of CTAB buffer (1.5% w/v CTAB, 75 mM Tris HCl, 100 mM EDTA, 1.05 M NaCl, and 0.75% w/v PVP) and 50 µg RNase A were added to approximately 1 ml of ground leaf tissue and incubated at 60-70°C for 40-50 minutes with intermittent mixing. The samples were cooled to room temperature. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the samples, mixed by hand for two to three minutes, and then centrifuged at ~10,300 × g for eight minutes at room temperature. The upper aqueous phase was transferred to a clean tube and the above purification step was repeated once. Approximately 5 ml of chloroform was added to the samples and mixed by hand for two to three minutes, then centrifuged at 10,300 × g for eight to 10 minutes. The upper aqueous phase was put into a clean tube and the chloroform step was repeated twice. After the last chloroform step, the aqueous phase was put into a clean tube and the DNA was precipitated with approximately 4 ml of 100% ethanol. The sample was centrifuged at 5,100 × g for five to seven minutes to pelletize the precipitated DNA. The DNA pellets were washed with 10-12 ml of 70% ethanol by centrifuging the samples at 5,100 × g for five to seven minutes. The DNA pellets were air dried, then resuspended in 500 µl of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0). All extracted DNA was stored in a 4°C refrigerator or a 20°C freezer.

B.4. Quantification of Genomic DNA

PV-MSPQ12633 DNA, probe templates, and extracted genomic DNA were quantified using a Qubit™ Fluorometer (Invitrogen, Carlsbad, CA) according to manufacturer's instruction.

B.5. Restriction Enzyme Digestion of Genomic DNA

Approximately ten micrograms (µg) of genomic DNA extracted from the test and control substances were digested with the appropriate restriction enzymes. When digesting genomic DNA with the restriction enzyme combination *Xmn* I (New England Biolabs (NEB), Ipswich, MA) and *Dra* III (NEB), 10X Buffer 1 (NEB) was used. The digests were performed at 37°C in a total volume of ~500 µl using ~50 units of each restriction enzyme. When digesting genomic DNA with the restriction enzyme combination *Xba* I (NEB) and *Swa* I (NEB), 10X Buffer 2 (NEB) was used. In these digests, the DNA was first incubated overnight with ~50 units of *Xba* I at 37°C in a volume of ~500 µl. After the *Xba* I digest, 23 µl of 1 M Tris HCl (Fisher Scientific, Pittsburgh, PA) and 5 µl of 5 M NaCl (Sigma-Aldrich Corp., Saint Louis, MO) were added to the digests the next day to optimize the buffer condition for *Swa* I. Approximately 50 units of *Swa* I was added to the digests and incubated for five hours at room temperature. For the purpose of running positive hybridization controls, ~10 µg of genomic DNA extracted from the conventional control was digested, and the appropriate positive hybridization control(s) were added to these digests prior to loading the gel.

B.6. Agarose Gel Electrophoresis

Digested DNA was resolved on 0.8% (w/v) agarose gels. For all Southern blot analyses, individual digests containing ~10 µg each of KK179 and conventional control genomic DNA were loaded on the same gel in a long run/short run format. The long run allows for greater resolution of large molecular weight DNA, whereas the short run allowed the detection of small molecular weight DNA. The positive hybridization controls were only run in the short run format. For the insert stability analysis, individual digests of ~10 µg of genomic DNA extracted from leaf tissue across multiple generations of KK179 were loaded on the agarose gel in a single run format.

B.7. DNA Probe Preparation for Southern Blot Analyses

Probe templates were prepared by PCR amplification using PV-MSPQ12633 as the template. The PCR products were separated on an agarose gel by electrophoresis and purified from the gel using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. The probe templates were designed based on the nucleotide composition (% GC) of the sequence, in order to optimize the detection of DNA sequences during hybridization. When possible, probes possessing a similar melting temperature (Tm) were combined in the same Southern blot hybridization. Approximately 25 ng of each probe template were radiolabeled with

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[α-^{32}P]-deoxycytidine triphosphate (dCTP) (6000 Ci/mmol) or [α-^{32}P]-deoxyadenosine triphosphate (dATP) (6000 Ci/mmol) using the random priming method (Invitrogen). Probe locations relative to the genetic elements in PV-MSPQ12633 are depicted in Figure IV-1.

B.8. Southern Blot Analyses of Genomic DNA

Genomic DNA isolated from the test and control substances were digested and evaluated using Southern blot analyses (Southern, 1975). In Southern blots hybridized with a single probe, ~0.1 and ~1.0 genome equivalent of the PV-MSPQ12633 DNA previously digested with *Xba* I or *Eco* RI (NEB) was added to digested conventional control genomic DNA to serve as a positive hybridization control. In Southern blots hybridized with multiple probes, ~1.0 genome equivalent of the PV-MSPQ12633 DNA previously digested with *Xba* I or *Eco* RI (NEB), as well as ~0.1 and ~1.0 genome equivalent of the appropriate probe templates were added to digested conventional control genomic DNA to serve as positive hybridization controls. The DNA was then separated by agarose gel electrophoresis and transferred onto a nylon membrane. Southern blots were hybridized and washed at 55°C or 60°C, depending on the calculated Tm of the probe(s) used. The table below lists the hybridization and radiolabeling conditions of the probes used in this study. Multiple exposures of each blot were then generated using Kodak Biomax MS film (Eastman Kodak, Rochester, NY) in conjunction with at least one Kodak Biomax MS intensifying screen in a -80°C freezer.

Table B-1. Hybridization Conditions of Utilized Probes

Probe	Probe Type	Labeling Method	Probe labeled with dNTP (^{32}P)	Hybridization/Wash Temperature (°C)
1	T-DNA I	RadPrime	dATP	55
2	T-DNA I	RadPrime	dATP	55
3	T-DNA I	RadPrime	dATP	55
4	T-DNA I	RadPrime	dATP	55
5	T-DNA II	RadPrime	dCTP	60
6	Backbone	RadPrime	dCTP	60
7	Backbone	RadPrime	dCTP	60
8	Backbone	RadPrime	dCTP	60
9	Backbone	RadPrime	dCTP	60

B.9. DNA Sequence Analysis of the Insert

Overlapping PCR products, denoted as Product A, Product B, Product C, Product D, and Product E were generated to span the insert and adjacent 5′ and 3′ flanking DNA sequences in KK179 (Figure V-9). These products were analyzed to determine the nucleotide sequence of the insert in KK179, as well as that of the DNA flanking the 5′ and 3′ ends of the insert.

The PCR analyses for Product A, Product B and Product D were each conducted using 100 ng of genomic DNA template in a 50 µl reaction volume. The reaction volume contains a final concentration of 2 mM MgCl2, 0.2 µM of each primer, 0.2 mM of each dNTP, and 0.025 units/µl of TaKaRa Ex Taq™ DNA Polymerase (Takara Bio Inc., Shiga, Japan).[TM]

The PCR analyses for Product C and Product E were conducted using 80 ng of genomic DNA template in a 50 µl reaction volume. The reaction volume contains a final concentration of 1.5 mM $MgCl_2$, 0.2 µM of each primer, 0.08 mM of each dNTP, and 0.02 units/µl of Phusion Hot Start II High Fidelity DNA Polymerase (Finnzymes, Espoo, Finland).

The amplification of Product A and Product D was performed under the following cycling conditions: one cycle at 98°C for 30 seconds; 25 cycles at 98 C for 10 seconds, 55°C for 20 seconds, 63°C for two minutes and 50 seconds; one cycle at 63°C for five minutes.

[TM] TaKaRa Ex Taq DNA Polymerase is a trademark ofTakara Bio Inc., Shiga, Japan.

The amplification of Product B was performed under the following cycling conditions: one cycle at 98°C for 30 seconds; 35 cycles at 98°C for 10 seconds, 55°C for 30°seconds, and 63°C for one minute; one cycle at 63°C for five minutes.

The amplification of Product C was performed under the following cycling conditions: one cycle at 98°C for 30°seconds; 25°cycles at 98°C for 10 seconds, 63°C for one minute and 15 seconds; one cycle at 63°C for five minutes.

The amplification of Product E was performed under the following cycling conditions: one cycle at 98°C for 30 seconds; 25 cycles at 98°C for 10 seconds, 63°C for one minute and 15 seconds.

Aliquots of each PCR product were separated on a 1.0% (w/v) agarose gel and visualized by ethidium bromide staining to verify that the products were the expected size. Prior to sequencing, each verified PCR product was purified using the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA) and quantified using a Qubit fluorometer according to manufacturer's instruction. The purified PCR products were sequenced using multiple primers, including primers used for PCR amplification. All sequencing was performed by Monsanto's TGAC (The Genome Analysis Center) using BigDye terminator chemistry (Applied Biosystems, Foster City, CA).

A consensus sequence was generated by compiling multiple sequencing reactions performed on the overlapping PCR products. This consensus sequence was aligned to the PV-MSPQ12633 sequence to determine the integrity and organization of the integrated DNA and the 5′ and 3′ insert-to-flank DNA junctions in KK179.

B.10. PCR and DNA Sequence Analyses to examine the Integrity of the DNA Insertion site in KK179

To examine the KK179 insertion site in conventional alfalfa, PCR and sequence analyses were performed on genomic DNA from both KK179 and the conventional parental control alfalfa R2336 (Figure V-10). The primers used in this analysis were designed from the DNA sequences flanking the insert in KK179. A forward primer specific to the DNA sequence flanking the 5′ end of the insert was paired with a reverse primer specific to the DNA sequence flanking the 3′ end of the insert.

The PCR reactions were conducted using 80 ng of genomic DNA template in a 50 µl reaction volume. The reaction volume contains a final concentration of 1.5 mM $MgCl_2$, 0.2 µM of each primer, 0.08 mM of each dNTP, and 0.02 units/µl of Phusion Hot Start II High Fidelity DNA Polymerase (Finnzymes, Espoo, Finland).

The amplification was performed under the following cycling conditions: one cycle at 98°C for 30 seconds; 35 cycles at 98°C for 10 seconds, 55°C for 15 seconds, 72°C for 40 seconds; one cycle at 72°C for three minutes.

Aliquots of each PCR product were separated on a 1% (w/v) agarose gel and visualized by ethidium bromide staining to verify that the PCR products were the expected size prior

to sequencing. Only the verified PCR product from the conventional parental control R2336 was purified with the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA) and quantified using a Qubit fluorometer according to the manufacturer's instruction. The purified PCR product was sequenced using primers used for PCR amplification. All sequencing was performed by TGAC using BigDye terminator chemistry (Applied Biosystems , Foster City, CA).

A consensus sequence was generated by compiling multiple sequencing reactions performed on the verified PCR product. This consensus sequence was aligned to the 5′ and 3′ sequences flanking the KK179 insert to determine the integrity and organization of the insertion site.

References for Appendix B

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology 98:503-517.

Appendix C: Materials and Methods Used for Northern Blot Analyses of *CCOMT* Suppression in KK179

C.1. Materials

The mRNA used in the northern blot analysis was isolated from four replicate samples of root and forage tissue of the Syn1 generation of KK179 and the conventional control C_0.

Probe templates were used as positive hybridization controls in northern blot analyses. Prior to probe generation, DNA fragments from the caffeoyl coenzyme A 3-*O*-methyltransferase (*CCOMT)* (Monsanto Sequence Database) gene was generated from conventional alfalfa genomic DNA using a forward primer and a reverse primer specific to the 3′ UTR of the gene. The resulting PCR fragments of the *CCOMT* gene were cloned into pCR®-TOPO (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The sequence of the plasmid DNA was confirmed by sequencing and comparison to the expected sequences using pair-wise alignments. The plasmid DNA was then used as a template for PCR reactions to amplify the CCOMT probe templates. The actin probe template was generated by PCR from conventional alfalfa genomic DNA using a forward primer and a reverse primer specific to the partial-coding region of the alfalfa actin gene. RiboRuler™ RNA ladders (high range) from Fermentas Co (Glen Burnie, MD) were used for size estimations on northern blots and formaldehyde/agarose gels. The unique identities of the probes, as well as molecular weight markers are documented in the raw data.

C.2. Characterization of the Materials

The forage and root tissues for test and control substances used in this study were obtained from Forage Genetics International (FGI) (West Salem, WI). The synthesis records for these materials are retained with FGI. No certificate of analysis (COA) or verification of identity (VOI) certificates were generated for these materials. Prior to the start of the study, the identity of the test and control substances was confirmed by event-specific PCR; the raw data were archived with the study. The study director reviewed the chain of custody (COC) documentation, which confirmed the identity of the test and control substances prior to use of the materials in the study.

Prior to the start of the analysis, the *CCOMT* gene was sequenced by the Monsanto Genomics Sequencing Center and the sequences were aligned with the expected sequences to confirm the identities of the probes. The raw data are archived at Monsanto.

Extracted RNA and RNA markers were stored in a -80°C freezer. DNA probes were stored in a -20°C freezer. The test, control, and reference substances were deemed stable during storage because there were no signs of degradation on formaldehyde/agarose gels and probing with endogenous genes yielded interpretable signals on northern blots.

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™ RiboRuler RNA ladders is a trademark of Fermentas Co., Glen Burnie, MD.

C.3. Total RNA Extraction

KK179 and conventional control total RNA samples were isolated from alfalfa root and forage tissues. Prior to extraction, forage and root tissues were processed to a fine powder by mortar and pestle using liquid nitrogen. Total RNA was extracted using a TRIzol-based method. Briefly, 20 ml of TRIzol reagent is added to approximately 2 g of root or forage tissue sample. The sample is homogenized using a vortexer for one to two minutes and incubated at room temperature for 40-50 minutes. The samples were centrifuged at ~12,000 × g for five minutes at 4°C to remove the debris. The supernatant is transferred to a new tube. Approximately 6 ml of chloroform was added to the samples, mixed by vortexing for one to two minutes, and incubated at room temperature for five to 10 minutes, followed by centrifugation at ~12,000 × g for 10 minutes at 4°C. The upper aqueous phase was transferred to a clean tube. Approximately 10 ml of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the samples, mixed by hand for one to two minutes, and then centrifuged at ~12,000 × g for five minutes at 4°C for additional purification of RNA. The upper aqueous phase was transferred to a clean tube. Approximately 6 ml of chloroform was added to the samples, mixed by vortexing for one to two minutes, and incubated at room temperature for five to 10 minutes, followed by centrifugation at ~12,000 × g for 10 minutes at 4°C. The upper aqueous phase was transferred to a clean tube and the RNA was precipitated with approximately 10 ml of isopropyl alcohol. The samples were stored in a -80°C freezer for at least 30 minutes to 24 hours to precipitate the RNA. Samples were then centrifuged at ~12,000 × g for 15-20 minutes at 4°C. The RNA pellets were washed with 5 ml of 75% ethanol (prepared in diethylpyrocarboonate (DEPC) treated water) and centrifuged at ~12,000 × g for 20 minutes at 4°C to precipitate the RNA. The RNA pellets were air dried, then resuspended in 2ml of water. All extracted RNA was stored in a -80°C freezer.

C.4. Quantification of RNA

Total RNA was quantified using a Beckman Coulter DU-650 Spectrophotometer (Beckman Coulter, Inc. Atlanta, GA) according to manufacturer's instruction. PolyA$^+$ RNA was quantified using the Qubit Fluorometer (Invitrogen, Carlsbad, CA) according to manufacturer's instruction.

C.5. PolyA$^+$ RNA Isolation

PolyA$^+$ RNA from the KK179 and conventional control total RNA samples was extracted using the Poly(A) Purist MAG Kit (Ambion Co., Austin, TX) according to the manufacturer's instruction with minor modifications. Briefly, ~420 µg of total RNA was placed in a 2 ml microcentrifuge tube and Rnase-free water was added to obtain a final RNA concentration of ~600 µg/ml. An equal volume of 2X Binding Solution was added to the RNA samples and mixed thoroughly by brief vortexing. The RNA samples were transferred to the microcentrifuge tube containing the washed MagBeads and mixed thoroughly by inversion. The RNA/bead mixture was incubated in a 65°C water bath for five minutes and then incubated for ~ 1 hour at room temperature with gentle agitation by nutation. The tubes containing the RNA/bead mixture were placed onto the magnetic stand for ~ 2 minutes to pull the beads to the side of the tube. The supernatant was

discarded and the beads washed twice with an equal volume (to the starting RNA solution) of Wash Solution 1 followed by two washes with an equal volume (to the starting RNA solution) of Wash Solution 2, discarding all wash solution. Approximately 200 µl RNA Storage Solution, prewarmed in a 60-80°C water bath, was added to the captured MagBeads. The mixture was gently swirled by hand, and the tube was placed back onto the magnetic stand to capture the beads. The supernatant containing the eluted RNA was placed in a clean microcentrifuge tube. The elution step was repeated once to obtain a final volume of 400 µl. The RNA was precipitated by adding $1/10^{th}$ volume 5M ammonium acetate, 1 µl of 5 mg/ml glycogen and 2.5 volumes of 100% ethanol to the samples. The samples were incubated in a -80°C freezer for 30-40 minutes and then centrifuged at ~12,000 × g for 30 minutes at 4°C. The supernatant is discarded and the samples were washed with 70% ethanol. The pellets were briefly air dried, and then resuspended in an appropriate volume of prewarmed RNA Storage Solution and heated in a 65°C water bath until the RNA pellet dissolved. All extracted $polyA^+$ RNA is stored in 10 µl aliquots in a -80°C freezer.

C.6. Formaldehyde/Agarose Gel Electrophoresis

$PolyA^+$ RNA samples from KK179 and the conventional control were resolved in 0.8% formaldehyde/agarose gels.

C.7. Northern Blot Analyses

Northern blot analyses were performed by transferring $polyA^+$ RNA from formaldehyde/agarose gels onto nylon membranes and probing with radiolabeled DNA. *CCOMT* hybridization signals were stripped from the blots and the stripped blots were hybridized to a ^{32}P-labeled actin probe to show the relative amount of RNA loaded in each lane. Probes were prepared by random prime labeling according to manufacturer's instruction. Each probe was added at approximately $1\text{-}2 \times 10^6$ cpm/ml of the hybridization solution. Northern blots were hybridized and washed at 55°C based on the calculated Tm of the probes used. Multiple exposures of each blot were then generated using Kodak Biomax MS film (Eastman Kodak, Rochester, NY) in conjunction with at least one Kodak Biomax MS-intensifying screen in a -80°C freezer.

C.8. Positive Hybridization Controls and Probe Template

DNA probe templates were run on the formaldehyde/agarose gels to serve as positive controls for hybridization. On northern blots the probe templates migrated slightly differently than predicted by the RNA ladder. The difference in migration is most likely due to the inaccuracy of comparing a DNA probe to an RNA ladder. The following table lists the DNA probes, the positive hybridization controls, and the hybridization and wash temperatures for each northern blot analysis.

Table C-1. DNA Probes and Positive Hybridization Controls

Analysis	Probe	Positive hybridization controls	Hybridization and Wash Temperature
Expression of *CCOMT*	CCOMT	CCOMT probe template	55ºC
Expression of *actin*	actin	actin probe template	55ºC

Appendix D: Materials, Methods, and Results for Intended Changes to Lignin Levels in KK179 Forage

D.1. Materials

Forage was evaluated from KK179 (Seed Lot Number 11266289) and the conventional control C0-Syn1 (Seed Lot Number 11266292). The conventional control has background genetics similar to that of KK179. The conventional commercial reference varieties were 14 conventional alfalfa varieties (Table D-1).

Table D-1. Conventional Commercial Reference Varieties

Material Name	Seed Lot Number	Field Site
Pioneer 54V54	11266293	ILCY, WIDL
Croplan LegenDairy 5.0	11266294	IARL, ILCY, WIDL
Producer's Choice PGI 437	11266295	ILCY, KSLA
Pioneer 54H11	11266296	IARL, TXCL
Pioneer 54V46	11266297	KSLA
Pioneer 54V09	11266298	CAPR, TXCL
DKA50-18	11266299	IARL, KSLA
America's Alfalfa Archer III	11266301	CAPR
WL 319HQ	11266302	CAPR, KSLA, TXCL
Dow/Dairyland Hybriforce 400	11266303	ILCY, WIDL
AMPAC seed company Attention	11266304	CAPR
Vernal	11266305	WIDL
Ranger	11266306	IARL
Dow/Dairyland Hybriforce 2400	11266307	TXCL

D.2. Characterization of the Materials

The identities of forage samples from KK179, the conventional control C0-Syn1, and conventional commercial reference varieties were confirmed by verifying the chain of custody documentation prior to analysis. To further confirm the identities of KK179, the conventional C0-Syn1, and conventional commercial reference varieties, event-specific polymerase chain reaction (PCR) analyses were conducted on the forage from each site to confirm the presence or absence of KK179.

D.3. Field Production of the Samples

Forage samples from KK179, the conventional control C0-Syn1, and conventional commercial alfalfa varieties were collected from each of four replicated plots from six field sites [Tulare County, California (CAPR); Jefferson County, Iowa (IARL); Clinton County, Illinois (ILCY); Pawnee County, Kansas (KSLA); Armstrong County, Texas (TXCL); and Walworth County, Wisconsin (WIDL)]. The field plots were established in 2010 from plants that were grown in a greenhouse. Prior to transplanting in the field, the presence or absence of KK179 was verified using PCR. The field plots were planted in a randomized complete block design. All samples at the field sites were grown under normal agronomic field conditions for their respective geographic regions. Between 1

and 10% bloom, all whole plants, two to three inches above the soil surface, were harvested from the center of each individual plot. A subsample (about 680g) of plants was randomly collected from all harvested plants in each plot.

Forage samples were shipped frozen from the field sites to Monsanto Company (Saint Louis, Missouri). All samples were ground to a powder and stored in a freezer set to maintain -20°C located at Monsanto Company (Saint Louis, Missouri). Subsamples were lyophilized and shipped at ambient temperature to Samuel Roberts Noble Foundation (Ardmore, Oklahoma) for analysis of lignin monomer subunit composition. Subsamples were shipped on dry ice to Dairy One Forage Lab (Ithaca, New York) for lignin analysis.

D.4. Summary of Analytical Methods

Forage samples were analyzed for *p*-hydroxyphenyl lignin (H lignin), caffeoyl lignin, guaiacyl lignin (G lignin), 5-hydroxyguaiacyl lignin, and syringyl lignin (S lignin) subunits by the Samuel Roberts Noble Foundation to demonstrate that suppression of the caffeoyl coenzyme A 3-*O*-methyltransferase (*CCOMT*) gene by dsRNA produced by KK179 resulted in the intended reduction of the G lignin subunit, and thereby total lignin. Forage samples were also analyzed by Dairy One Forage Lab for acid detergent lignin (ADL) by an industry standard method used to provide a standard forage quality assessment.

D.4.1. Lignin Composition by Thioacidolysis (H, G and S Lignin) (Conducted at Samuel Roberts Noble Foundation)

The lyophilized, ground samples were extracted with chloroform/methanol (2:1, v/v), methanol (100%) and water three times each to obtain cell wall residue (CWR). The samples were then freeze-dried using a manifold freeze-drier. About 18 mg of dried samples were weighed into 12 ml glass tubes; 3 ml of thioacidolysis reaction mixture were added, and the reaction was conducted at 100°C for 4 hours. After solvent extraction, 3 ml of solvent were dried under nitrogen and derivatized with 150 µl pyridine and BSTFA mixture (1:1, v/v) before gas chromatography/mass spectroscopy (GC/MS) analyses (Lapierre et al., 1985; Lapierre et al., 1995). The limit of quantitation was 0.1 µmol/g CWR.

Internal Standards:

- Sigma-Aldrich, Docosane (Lot Number 18999MJ) 3.03 mg/ml in chloroform

D.4.2. Lignin (Acid Detergent Lignin) (Conductedat Dairy One Forage Lab)

Forage samples were dried and analyzed using an ANKOM based methodology that is semi-automated, uses filter bags and is comparable to the conventional crucible method (Weston et al., 2006). Briefly, samples were boiled in acid detergent fiber (ADF) solution, rinsed with water and acetone, and hydrolyzed in sulfuric acid. Following acid hydrolysis, samples were rinsed in water and soaked in acetone before being dried to a constant weight. The dried samples are weighed and then ashed in individual crucibles at

525ºC for 3 hours. After ashing, samples are cooled in a desiccator and weighed a final time. The limit of quantitation was 0.1%.

D.5. Data Processing and Statistical Analysis

After compositional analyses were performed, data spreadsheets containing individual values for each analysis were sent to Monsanto Company (St. Louis, Missouri) for review. Data were then transferred to Certus International (Chesterfield, Missouri) where they were converted into the appropriate units and statistically analyzed. The formulas that were used for re-expression of composition data for statistical analysis are listed in Table D-2.

In order to complete a statistical analysis for a compositional component in this study, at least 50% of the values for a component had to be greater than the assay limit of quantitation (LOQ). Components with more than 50% of observations below the assay LOQ were excluded from summaries and analysis. The following two components with more than 50% of the observations below the assay LOQ were excluded: caffeoyl lignin and 5-hydroxyguaiacyl lignin.

The data were assessed for potential outliers using a studentized PRESS residuals calculation. A PRESS residual is the difference between any value and its value predicted from a statistical model that excludes the data point. The studentized version scales these residuals so that the values tend to have a standard normal distribution when outliers are absent. Thus, most values are expected to be between ± 3. Extreme data points that are also outside of the ± 6 studentized PRESS residual range are considered for exclusion as outliers from the final analyses. In lignin units outliers as a proportion of HGS lignin were indentified in three conventional commercial reference varieties. All lignin unit component data from the three conventional commercial reference varieties were removed from the statistical analyses.

Alfalfa forage compositional components were statistically analyzed using a mixed model analysis. The six replicated field sites were analyzed as a combined data set. These combined-site analyses used model (1).

(1) $Y_{ijk} = U + T_i + L_j + B(L)_{jk} + LT_{ij} + e_{ijk}$,

where Y_{ijk} = unique individual observation, U = overall mean, T_i = substance effect, L_j = random location effect, $B(L)_{jk}$ = random block within location effect, LT_{ij} = random location by substance interaction effect, and e_{ijk} = residual error.

A supplementary individual site analysis used model (2).

(2) $Y_{ij} = U + T_i + B_j + e_{ij}$,

where Y_{ij} = unique individual observation, U = overall mean, T_i = substance effect, B_j = random block effect, and e_{ij} = residual error.

For each compositional component, a range of observed values and a 99% tolerance interval were calculated. A tolerance interval is an interval that one can claim, with a specified degree of confidence, contains at least a specified proportion, p, of an entire sampled population for the parameter measured. The calculated tolerance intervals are expected to contain, with 95% confidence, 99% of the quantities expressed in the population of conventional alfalfa. Each tolerance interval estimate was based upon the average observation for each unique reference material. Because negative quantities are not possible, negative calculated lower tolerance bounds were set to zero.

SAS® (Version 9.2) software was used to generate all summary statistics and perform all analyses.

Report tables present p-values from SAS as either <0.001 or the actual value truncated to three decimal places.

Table D-2. Re-expression Formulas for Statistical Analysis of Composition Data

Component	From (X)	To	Formula[1]
Lignin unit (HGS)	µmol/g CWR	% Total (HGS)	(100)Xj/ΣX, for each HGSj where ΣX is over all the HGS
[1]'X' is the individual sample value.			

® SAS is a registered trademark of the SAS Institute, Inc. Cary, North Carolina.

Table D-3. Statistical Summary of Alfalfa Forage Lignin Subunits for KK179 vs. Conventional Control (Conducted at Samuel Roberts Noble Foundation)

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)
Site CAPR						
Lignin Subunits (µmol/g CWR)						
Guaiacyl lignin unit	72.86 (11.69) (70.00 - 75.71)	92.84 (8.27) (66.63 - 113.14)	-19.99 (14.32) (-35.42 - -10.46)	-53.85, 13.88	0.205	8.83, 176.39 (25.34 - 153.11)
Hydroxyphenyl lignin unit	5.41 (0.62) (5.17 - 6.05)	4.40 (0.42) (2.78 - 5.26)	1.01 (0.66) (0.27 - 1.39)	-0.78, 2.79	0.196	1.59, 6.91 (0.29 - 8.26)
Syringyl lignin unit	73.27 (10.15) (63.21 - 87.67)	62.98 (6.72) (46.77 - 76.24)	10.29 (10.99) (6.87 - 15.10)	-19.01, 39.59	0.396	0, 120.96 (5.64 - 110.93)

Table D-3 (continued). Statistical Summary of Alfalfa Forage Lignin Subunits for KK179 vs. Conventional Control (Conducted at Samuel Roberts Noble Foundation)

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)
			Site CAPR			
Lignin Subunits (% Total HGS)						
Guaiacyl lignin unit	47.59 (1.64) (44.92 - 50.27)	57.82 (1.16) (56.88 - 58.92)	-10.23 (2.01) (-14.01 - -6.61)	-14.97, -5.48	0.001	46.69, 76.44 (50.02 - 76.69)
Hydroxyphenyl lignin unit	3.70 (0.35) (3.07 - 4.34)	2.75 (0.22) (2.39 - 3.29)	0.96 (0.40) (0.47 - 1.05)	-0.038, 1.95	0.056	0, 6.74 (0.18 - 6.23)
Syringyl lignin unit	48.70 (1.83) (45.39 - 52.01)	39.43 (1.30) (38.48 - 40.26)	9.27 (2.24) (5.56 - 13.54)	3.96, 14.57	0.004	17.39, 53.32 (17.07 - 46.14)
Ratio						
S:G Ratio	1.03 (0.065) (0.90 - 1.16)	0.68 (0.046) (0.65 - 0.70)	0.35 (0.080) (0.20 - 0.50)	0.16, 0.54	0.003	0.21, 0.96 (0.22 - 0.92)
			Site IARL			
Lignin Subunits (µmol/g CWR)						
Guaiacyl lignin unit	37.57 (6.10) (21.17 - 65.54)	37.48 (6.10) (33.11 - 43.31)	0.088 (8.63) (-13.83 - 27.03)	-19.44, 19.61	0.992	8.83, 176.39 (25.34 - 153.11)

Table D-3 (continued). Statistical Summary of Alfalfa Forage Lignin Subunits for KK179 vs. Conventional Control (Conducted at Samuel Roberts Noble Foundation)

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)
			Site IARL			
Lignin Subunits (µmol/g CWR)						
Hydroxyphenyl lignin unit	4.88 (1.08) (2.20 - 10.14)	2.69 (1.08) (2.40 - 3.06)	2.19 (1.52) (-0.20 - 7.24)	-1.25, 5.64	0.183	1.59, 6.91 (0.29 - 8.26)
Syringyl lignin unit	17.46 (2.63) (9.82 - 27.96)	13.74 (2.63) (12.20 - 15.36)	3.72 (3.72) (-2.38 - 13.42)	-4.69, 12.13	0.343	0, 120.96 (5.64 - 110.93)
Lignin Subunits (% Total HGS)						
Guaiacyl lignin unit	62.80 (0.81) (60.80 - 63.78)	69.50 (0.81) (68.47 - 70.56)	-6.70 (1.06) (-9.36 - -5.09)	-9.30, -4.11	<0.001	46.69, 76.44 (50.02 - 76.69)
Hydroxyphenyl lignin unit	7.55 (0.45) (6.39 - 9.78)	4.99 (0.45) (4.84 - 5.18)	2.56 (0.61) (1.43 - 4.60)	1.07, 4.05	0.005	0, 6.74 (0.18 - 6.23)
Syringyl lignin unit	29.65 (0.90) (26.98 - 31.80)	25.51 (0.90) (24.60 - 26.57)	4.14 (1.03) (0.99 - 6.92)	1.63, 6.65	0.006	17.39, 53.32 (17.07 - 46.14)

Table D-3 (continued). Statistical Summary of Alfalfa Forage Lignin Subunits for KK179 vs. Conventional Control (Conducted at Samuel Roberts Noble Foundation)

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)
			Site IARL			
Ratio						
S:G Ratio	0.47 (0.019) (0.43 - 0.52)	0.37 (0.019) (0.35 - 0.39)	0.11 (0.022) (0.049 - 0.17)	0.051, 0.16	0.003	0.21, 0.96 (0.22 - 0.92)
			Site ILCY			
Lignin Subunits (µmol/g CWR)						
Guaiacyl lignin unit	87.60 (12.13) (52.96 - 134.96)	104.06 (12.13) (77.34 - 131.40)	-16.46 (13.04) (-29.52 - 3.56)	-48.36, 15.44	0.253	8.83, 176.39 (25.34 - 153.11)
Hydroxyphenyl lignin unit	6.19 (1.03) (3.16 - 10.84)	4.14 (1.03) (2.92 - 5.49)	2.05 (1.38) (0.24 - 5.35)	-1.34, 5.44	0.188	1.59, 6.91 (0.29 - 8.26)
Syringyl lignin unit	67.00 (7.82) (49.44 - 85.83)	63.39 (7.82) (47.39 - 91.89)	3.61 (9.39) (-18.80 - 27.05)	-19.37, 26.59	0.713	0, 120.96 (5.64 - 110.93)
Lignin Subunits (% Total HGS)						
Guaiacyl lignin unit	53.58 (1.89) (49.12 - 61.66)	60.96 (1.89) (57.44 - 63.59)	-7.37 (2.68) (-13.10 - 4.22)	-13.43, -1.31	0.022	46.69, 76.44 (50.02 - 76.69)

Table D-3 (continued). Statistical Summary of Alfalfa Forage Lignin Subunits for KK179 vs. Conventional Control (Conducted at Samuel Roberts Noble Foundation)

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)
			Site ILCY			
Lignin Subunits (% Total HGS)						
Hydroxyphenyl lignin unit	3.69 (0.30) (2.99 - 4.95)	2.40 (0.30) (2.29 - 2.54)	1.29 (0.43) (0.58 - 2.55)	0.32, 2.25	0.014	0, 6.74 (0.18 - 6.23)
Syringyl lignin unit	42.73 (2.13) (33.39 - 47.77)	36.64 (2.13) (34.02 - 40.17)	6.09 (3.02) (-6.77 - 12.52)	-0.74, 12.91	0.074	17.39, 53.32 (17.07 - 46.14)
Ratio						
S:G Ratio	0.81 (0.063) (0.54 - 0.97)	0.60 (0.063) (0.54 - 0.70)	0.21 (0.089) (-0.16 - 0.41)	0.0083, 0.41	0.042	0.21, 0.96 (0.22 - 0.92)
			Site KSLA			
Lignin Subunits (µmol/g CWR)						
Guaiacyl lignin unit	62.88 (3.72) (59.88 - 66.12)	82.54 (3.72) (65.03 - 91.43)	-19.66 (5.25) (-31.46 - 1.09)	-31.54, -7.77	0.004	8.83, 176.39 (25.34 - 153.11)
Hydroxyphenyl lignin unit	4.38 (0.28) (4.14 - 4.60)	4.38 (0.28) (3.79 - 5.02)	0.0050 (0.39) (-0.70 - 0.35)	-0.88, 0.89	0.990	1.59, 6.91 (0.29 - 8.26)

Table D-3 (continued). Statistical Summary of Alfalfa Forage Lignin Subunits for KK179 vs. Conventional Control (Conducted at Samuel Roberts Noble Foundation)

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)
			Site KSLA			
Lignin Subunits (µmol/g CWR)						
Syringyl lignin unit	51.49 (3.43) (44.58 - 54.81)	52.06 (3.43) (40.48 - 62.77)	-0.58 (4.85) (-18.19 - 14.33)	-11.54, 10.39	0.908	0, 120.96 (5.64 - 110.93)
Lignin Subunits (% Total HGS)						
Guaiacyl lignin unit	53.00 (0.72) (52.15 - 54.91)	59.46 (0.72) (57.56 - 61.36)	-6.46 (1.02) (-9.21 - -2.65)	-8.77, -4.15	<0.001	46.69, 76.44 (50.02 - 76.69)
Hydroxyphenyl lignin unit	3.70 (0.25) (3.49 - 4.22)	3.19 (0.25) (2.82 - 3.76)	0.52 (0.36) (-0.19 - 1.33)	-0.30, 1.33	0.183	0, 6.74 (0.18 - 6.23)
Syringyl lignin unit	43.30 (0.82) (40.88 - 44.36)	37.35 (0.82) (35.81 - 39.56)	5.94 (1.16) (1.32 - 8.54)	3.33, 8.56	<0.001	17.39, 53.32 (17.07 - 46.14)
Ratio						
S:G Ratio	0.82 (0.024) (0.74 - 0.85)	0.63 (0.024) (0.58 - 0.69)	0.19 (0.034) (0.057 - 0.27)	0.11, 0.27	<0.001	0.21, 0.96 (0.22 - 0.92)

Table D-3 (continued). Statistical Summary of Intended Changes in Forage Lignin Subunits for KK179 vs. Conventional Control (Conducted at Samuel Roberts Noble Foundation)

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)
			Site TXCL			
Lignin Subunits (µmol/g CWR)						
Guaiacyl lignin unit	64.68 (9.26) (54.72 - 74.33)	91.11 (9.26) (71.43 - 105.30)	-26.44 (12.16) (-39.11 - -9.45)	-56.19, 3.32	0.072	8.83, 176.39 (25.34 - 153.11)
Hydroxyphenyl lignin unit	4.18 (0.91) (2.74 - 5.41)	4.55 (0.91) (4.24 - 4.94)	-0.37 (1.28) (-1.76 - 1.17)	-3.27, 2.53	0.777	1.59, 6.91 (0.29 - 8.26)
Syringyl lignin unit	59.13 (9.52) (45.79 - 70.15)	55.21 (9.52) (45.73 - 63.25)	3.92 (12.79) (-17.46 - 15.72)	-27.37, 35.22	0.769	0, 120.96 (5.64 - 110.93)
Lignin Subunits (% Total HGS)						
Guaiacyl lignin unit	50.67 (1.07) (49.59 - 52.63)	60.26 (1.07) (58.06 - 64.22)	-9.59 (1.51) (-14.63 - -5.43)	-13.01, -6.16	<0.001	46.69, 76.44 (50.02 - 76.69)
Hydroxyphenyl lignin unit	3.28 (0.53) (2.04 - 4.13)	3.08 (0.53) (2.59 - 4.05)	0.20 (0.75) (-0.85 - 1.02)	-1.49, 1.88	0.797	0, 6.74 (0.18 - 6.23)

Table D-3 (continued). Statistical Summary of Intended Changes in Forage Lignin Subunits for KK179 vs. Conventional Control (Conducted at Samuel Roberts Noble Foundation)

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)
			Site TXCL			
Lignin Subunits (% Total HGS)						
Syringyl lignin unit	46.05 (0.99) (44.04 - 47.67)	36.66 (0.99) (33.20 - 39.14)	9.39 (1.38) (4.90 - 13.61)	6.00, 12.78	<0.001	17.39, 53.32 (17.07 - 46.14)
Ratio						
S:G Ratio	0.91 (0.031) (0.84 - 0.95)	0.61 (0.031) (0.52 - 0.67)	0.30 (0.043) (0.16 - 0.43)	0.20, 0.40	<0.001	0.21, 0.96 (0.22 - 0.92)
			Site WIDL			
Lignin Subunits (µmol/g CWR)						
Guaiacyl lignin unit	84.34 (5.42) (77.36 - 88.36)	94.31 (5.42) (69.76 - 111.31)	-9.97 (7.66) (-24.67 - 18.60)	-27.30, 7.36	0.225	8.83, 176.39 (25.34 - 153.11)
Hydroxyphenyl lignin unit	5.24 (0.69) (4.34 - 6.22)	3.10 (0.69) (0.58 - 5.48)	2.15 (0.78) (-0.38 - 3.76)	0.23, 4.07	0.033	1.59, 6.91 (0.29 - 8.26)
Syringyl lignin unit	69.11 (4.76) (63.76 - 78.51)	55.09 (4.76) (34.94 - 64.85)	14.02 (6.73) (-0.86 - 43.57)	-1.20, 29.25	0.066	0, 120.96 (5.64 - 110.93)

Table D-3 (continued). Statistical Summary of Intended Changes in Forage Lignin Subunits for KK179 vs. Conventional Control (Conducted at Samuel Roberts Noble Foundation)

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)
			Site WIDL			
Lignin Subunits (% Total HGS)						
Guaiacyl lignin unit	53.18 (1.25) (51.32 - 54.77)	62.12 (1.25) (57.24 - 65.32)	-8.94 (1.76) (-14.01 - -2.47)	-12.92, -4.95	<0.001	46.69, 76.44 (50.02 - 76.69)
Hydroxyphenyl lignin unit	3.33 (0.42) (2.67 - 4.22)	2.04 (0.42) (0.34 - 3.33)	1.29 (0.49) (0.0067 - 2.33)	0.088, 2.49	0.039	0, 6.74 (0.18 - 6.23)
Syringyl lignin unit	43.49 (1.03) (41.89 - 45.60)	35.84 (1.03) (32.72 - 39.43)	7.65 (1.46) (2.46 - 12.88)	4.35, 10.94	<0.001	17.39, 53.32 (17.07 - 46.14)
Ratio						
S:G Ratio	0.82 (0.032) (0.76 - 0.89)	0.58 (0.032) (0.50 - 0.69)	0.24 (0.045) (0.076 - 0.39)	0.14, 0.34	<0.001	0.21, 0.96 (0.22 - 0.92)

[1]Total HGS is the sum of Hydroxyphenyl, Guaiacyl and Syringyl lignin subunits (µmol/g CWR); S:G Ratio = Syringyl lignin unit divided by Guaiacyl lignin unit. CWR = Cell Wall Residue
[2]Test refers to KK179.
[3]Mean (S.E.) = least-square mean (standard error)
[4]Control refers to the non-biotechnology derived, conventional control, C_0-Syn1.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties. Negative limits set to zero.

Table D-4. Statistical Summary of Alfalfa Forage Total Lignin (ADL) Levels for KK179 vs. Conventional Control (Conducted at Dairy One Forage Lab)

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)
Site CAPR						
Acid Detergent Lignin (% dw)	5.79 (0.44) (4.93 - 6.57)	8.38 (0.44) (7.30 - 10.10)	-2.59 (0.54) (-3.53 - -1.23)	-3.91, -1.27	0.002	1.39, 12.54 (1.70 - 10.03)
Site IARL						
Acid Detergent Lignin (% dw)	2.79 (0.46) (2.73 - 2.87)	3.70 (0.46) (2.23 - 5.20)	-0.91 (0.65) (-2.47 - 0.63)	-2.37, 0.55	0.193	1.39, 12.54 (1.70 - 10.03)
Site ILCY						
Acid Detergent Lignin (% dw)	6.87 (0.60) (6.43 - 7.60)	6.87 (0.60) (5.67 - 8.63)	0 (0.84) (-2.20 - 1.30)	-1.91, 1.91	1.000	1.39, 12.54 (1.70 - 10.03)
Site KSLA						
Acid Detergent Lignin (% dw)	5.35 (0.49) (3.47 - 6.53)	8.35 (0.49) (7.60 - 9.77)	-3.00 (0.70) (-4.33 - -1.07)	-4.58, -1.42	0.002	1.39, 12.54 (1.70 - 10.03)

Table D-4 (continued). Statistical Summary of Alfalfa Forage Total Lignin (ADL) Levels for KK179 vs. Conventional Control (Conducted at Dairy One Forage Lab)

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)
Site TXCL						
Acid Detergent Lignin (% dw)	4.65 (0.26) (4.07 - 5.03)	7.32 (0.26) (6.40 - 8.10)	-2.67 (0.37) (-3.17 - -1.40)	-3.58, -1.76	<0.001	1.39, 12.54 (1.70 - 10.03)
Site WIDL						
Acid Detergent Lignin (% dw)	6.92 (0.29) (6.70 - 7.07)	6.96 (0.29) (5.97 - 7.87)	-0.042 (0.37) (-1.17 - 0.87)	-0.94, 0.86	0.913	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 non-biotechnology derived, conventional control, C_0-Syn1.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties. Negative limits set to zero.

References for Appendix D

Lapierre, C., B. Monties and C. Rolando. 1985. Thioacidolysis of Lignin Comparison with Acidolysis. Journal of Wood Chemistry and Technology 5: 277-292.

Lapierre, C., B. Pollet and C. Rolando. 1995. New Insights into the Molecular Architecture of Hardwood Lignins by Chemical Degradative Methods. Research on Chemical Intermediates 21: 397-412.

Weston, T.R., V. Nayigihugu and B.W. Hess. 2006. Comparison of techniques for quantitative analysis of acid detergent lignin in roughages. Pages 242-244 in Proceedings, Western Section, American Society of Animal Science, American Society of Animal Science, Champaign, Illinois.

Appendix E: Materials, Methods, and Results for Compositional Analysis of KK179 Forage

E.1. Materials

Forage from KK179 (Seed Lot Number 11266289) and the conventional control C0-Syn1 (Seed Lot Number 11266292) was evaluated. The conventional control C0-Syn1has background genetics similar to that of KK179. The conventional commercial reference varieties were 14 conventional alfalfa varieties (Table E-1).

Table E-1. Conventional Commercial Reference Varieties

Material Name	Seed Lot Number	Field Site
Pioneer 54V54	11266293	ILCY, WIDL
Croplan LegenDairy 5.0	11266294	IARL, ILCY, WIDL
Producer's Choice PGI 437	11266295	ILCY, KSLA
Pioneer 54H11	11266296	IARL, TXCL
Pioneer 54V46	11266297	KSLA
Pioneer 54V09	11266298	CAPR, TXCL
DKA50-18	11266299	IARL, KSLA
America's Alfalfa Archer III	11266301	CAPR
WL 319HQ	11266302	CAPR, KSLA, TXCL
Dow/Dairyland Hybriforce 400	11266303	ILCY, WIDL
AMPAC seed company Attention	11266304	CAPR
Vernal	11266305	WIDL
Ranger	11266306	IARL
Dow/Dairyland Hybriforce 2400	11266307	TXCL

E.2. Characterization of the Materials

The identities of forage samples from KK179, the conventional control C0-Syn1, and conventional commercial reference varieties were confirmed by verifying the chain of custody documentation prior to analysis. To further confirm the identities of KK179, the conventional control C0-Syn1, and conventional commercial reference varieties, event-specific polymerase chain reaction (PCR) analyses were conducted on the forage from each site to confirm the presence or absence of KK179.

E.3. Field Production of the Samples

Forage samples from KK179, the conventional control C0-Syn1, and conventional commercial alfalfa varieties were collected from each of four replicated plots from six field sites [Tulare County, California (CAPR); Jefferson County, Iowa (IARL); Clinton County, Illinois (ILCY); Pawnee County, Kansas (KSLA); Armstrong County, Texas (TXCL); and Walworth County, Wisconsin (WIDL)]. The field plots were established in 2010 from plants that were grown in a greenhouse. Prior to transplanting in the field, the presence or absence of KK179 was verified using PCR. The field plots were planted in a randomized complete block design. All samples at the field sites were grown under normal agronomic field conditions for their respective geographic regions. Between 1

and 10% bloom, all whole plants that were two to three inches above the soil surface were harvested from the center of each individual plot. A subsample (about 680g) of plants was randomly collected from all harvested plants in each plot.

Forage samples were shipped frozen from the field sites to Monsanto Company (Saint Louis, Missouri). All samples were ground to a powder and stored in a freezer set to maintain -20°C located at Monsanto Company (Saint Louis, Missouri). Subsamples were shipped on dry ice to Covance Laboratories Inc. (Madison, Wisconsin) for compositional analysis. Subsamples were lyophilized and shipped on dry ice to Samuel Roberts Noble Foundation (Ardmore, Oklahoma) for analysis of saponins.

E.4. Summary of Analytical Methods

Compositional assessments by Covance and the Samuel Roberts Noble Foundation were conducted to establish the compositional equivalence of KK179 compared to conventional alfalfa using the principles and analytes outlined in the OECD consensus document for alflafa composition (OECD, 2005). Forage samples analyzed by Covance Laboratories Inc. included nutrients, proximates (ash, fat, moisture, and protein), carbohydrates by calculation, acid detergent fiber (ADF), neutral detergent fiber (NDF), acid detergent lignin (lignin), minerals (Ca, Cu, Fe, Mg, Mn, P, K, Na, and Zn), and amino acids (18 components). Anti-nutrients assessed by Covance included daidzein, glycitein, genistein, coumesterol, formononetin, and biochanin A. Secondary metabolites assessed by Covance included *p*-coumaric acid, ferulic acid, sinapic acid, total polyphenols, free phenylalanine, and canavanine. Forage samples were analyzed by Samuel Roberts Noble Foundation for total saponins, total bayogenin, total hederagenin, total medicagenic acid, total soyasapogenol B, total soyasapogenol E, and total zanhic acid.

E.4.1. Acid Detergent Fiber (Conducted at Covance Laboratories Inc.)

The ANKOM2000 Fiber Analyzer automated the process of removal of proteins, carbohydrates, and ash. Fats and pigments were removed with an acetone wash prior to analysis. The fibrous residue that was primarily cellulose and lignin and insoluble protein complexes remained in the ANKOM filter bag, and was determined gravimetrically (Goering and Van Soest, 1970; Komarek et al., 1993). The limit of quantitation was 0.100%.

E.4.2. Amino Acid Composition (Conducted at Covance Laboratories Inc.)

The following eighteen amino acids were analyzed:

- Total alanine
- Total arginine
- Total aspartic acid (including asparagine)
- Total cystine (including cysteine)
- Total glutamic acid (including glutamine)
- Total glycine

- Total histidine
- Total isoleucine
- Total leucine
- Total lysine
- Total methionine
- Total phenylalanine
- Total proline
- Total serine
- Total threonine
- Total tryptophan
- Total tyrosine
- Total valine

The samples were hydrolyzed in 6N hydrochloric acid for approximately 24 hours at approximately 106-110ºC. Phenol was added to the 6N hydrochloric acid to prevent halogenation of tyrosine. Cystine and cysteine are converted to S-2-carboxyethylthiocysteine by the addition of dithiodipropionic acid. Tryptophan was hydrolyzed from proteins by heating at approximately 110ºC in 4.2N sodium hydroxide for 20 hours.

The samples were analyzed by HPLC after pre-injection derivatization. The primary amino acids were derivatized with *o*-phthalaldehyde (OPA) and the secondary amino acids are derivatized with fluorenylmethyl chloroformate (FMOC) before injection (AOAC, 2011g; Barkholt and Jensen, 1989; Schuster, 1988). The limit of quantitation was 0.100 mg/g.

Reference Standards:

- Sigma-Aldrich, L-Alanine, 99.9%, Lot Number 1440397
- Sigma-Aldrich, L-Arginine Monohydrochloride, 100%, Lot Number 1361811
- Sigma-Aldrich, L-Aspartic Acid, 100.6%, Lot Number BCBB9274
- Sigma-Aldrich, L-Cystine, 99.9%, Lot Number 1418036
- Sigma-Aldrich, L-Glutamic Acid, 100.2%, 1423805
- Sigma-Aldrich, Glycine, 100%, Lot Number 1119375
- Sigma-Aldrich, L-Histidine Monohydrochloride Monohydrate, 99.9%, Lot Number BCBB1348
- Sigma-Aldrich, L-Isoleucine, 100%, Lot Number 1423806
- Sigma-Aldrich, L-Leucine, 98.6%, Lot Number BCBB1733
- Sigma-Aldrich, L-Lysine Monohydrochloride, 100.2%, 1362380
- Sigma-Aldrich, L-Methionine, 99.9%, Lot Number 1423807
- Sigma-Aldrich, L-Phenylalanine, 100%, Lot Number BCBB9200
- Sigma-Aldrich, L-Proline, 99.7%, Lot Number 1414414
- Sigma-Aldrich, L-Serine, 99.9%, Lot Number 1336081
- Sigma-Aldrich, L-Threonine, 100%, Lot Number 1402329
- Sigma-Aldrich, L-Tryptophan, 99.8%, BCBB1284

- Sigma-Aldrich, L-Tyrosine, 99.5%, Lot Number BCBB5393
- Sigma-Aldrich, L-Valine, 100%, Lot Number 1352709

E.4.3. Ash (Conducted at Covance Laboratories Inc.)

The sample was placed in an electric furnace at 550°C and ignited. The nonvolatile matter remaining was quantitated gravimetrically and calculated to determine percent ash (AOAC, 2011e). The limit of quantitation was 0.100%.

E.4.4. Biochanin A (Conducted at Covance Laboratories Inc.)

Samples were extracted using an acidified acetonitrile/water solution combined with mixing. The extracts were then quantitated using isocratic reverse phase high-performance liquid chromatography (HPLC) with UV detection (Chen et al., 2010; Saloniemi et al., 1995). The limit of quantitation was 10.0 ppm.

Reference Standards:

- Sigma-Aldrich, Biochanin A, 97.4%, Lot Number S39234-418

E.4.5. Canavanine, Free (Conducted at Covance Laboratories Inc.)

The samples were extracted with 0.02N HCL and purified with ultrafiltration. Analysis was performed with an automated amino acid analyzer (AOAC, 2011a). The limit of quantitation was 5.00 µg/g.

Reference Standards:

- Sigma-Aldrich, L-Canavanine Free Base from Jack Beans, 99%, Lot Number 116K7026

E.4.6. Carbohydrate (Conducted at Covance Laboratories Inc.)

The total carbohydrate level was calculated by difference using the fresh weight-derived data and the following equation (USDA, 1973):

% carbohydrates = 100 % - (% protein + % fat + % moisture + % ash)

The limit of quantitation was 0.100%.

E.4.7. Coumesterol and Formononetin (Conducted at Covance Laboratories Inc.)

The samples were extracted with 0.02N HCL and purified with ultrafiltration. Analysis was performed with an automated amino acid analyzer (Pettersson and Kiessling, 1984; Saloniemi et al., 1995). The limit of quantitation was 5.00 µg/g.

Reference Standards:

- Sigma Aldrich, Formononetin, 99.1%, Lot Number BCBD9644V
- Indofine Chemical Co., Coumestrol, 99.24%, Lot Number 1103121

E.4.8. Fat by Acid Hydrolysis (Conducted at Covance Laboratories Inc.)

The sample was hydrolyzed with hydrochloric acid. The fat was extracted using ether and hexane. The extract was dried down and filtered through a sodium sulfate column. The remaining extract was then evaporated, dried, and weighed (AOAC, 2010a; 2010b). The limit of quantitation was 0.100%.

E.4.9. Free Phenylalanine (Conducted at Covance Laboratories Inc.)

The sample was extracted in 0.1N HCl. Determination was by high-performance liquid chromatography (HPLC) with fluorescence cystine only detection. The phenylalanine was derivatized with *o*-phthalaldehyde before injection (Schuster, 1988). The limit of quantitation was 0.0200 mg/g.

Reference Standards:

- Sigma-Aldrich, Phenylalanine, 100%, Lot Number BCBB9200

E.4.10. Minerals/ICP Emission Spectrometry (Conducted at Covance Laboratories Inc.)

The following nine minerals were analyzed:

- Calcium
- Copper
- Iron
- Magnesium
- Manganese
- Phosphorus
- Potassium
- Sodium
- Zinc

The sample was dried, precharred, and ashed overnight in a muffle furnace set to maintain 500°C. The ashed sample was re-ashed with nitric acid, treated with hydrochloric acid, taken to dryness, and put into a solution of 5% hydrochloric acid. The amount of each element was determined at appropriate wavelengths by comparing the emission of the unknown sample, measured on the inductively coupled plasma spectrometer, with the emission of the standard solutions (AOAC, 2011c; 2011h).

Reference Standards:

Inorganic Ventures Reference Standards and Limits of Quantitation:

Mineral	Lot No.	Concentration (µg/mL)	LOQ (ppm)
Calcium	E2-MEB393070MCA, E2-MEB393072	200, 1000	20.0
Copper	E2-MEB393070MCA, E2-MEB393071MCA	2.00, 10.0	0.500
Iron	E2-MEB393070MCA, E2-MEB393073	10.0, 50.0	2.00
Magnesium	E2-MEB393070MCA, E2-MEB393071MCA	50.0, 250	20.0
Manganese	E2-MEB393070MCA, E2-MEB393071MCA	2.00, 10.0	0.300
Phosphorus	E2-MEB393070MCA, E2-MEB393072	200, 1000	20.0
Potassium	E2-MEB393070MCA, E2-MEB393072	200, 1000	100
Sodium	E2-MEB393070MCA, E2-MEB393072	200, 1000	100
Zinc	E2-MEB393070MCA, E2-MEB393071MCA	10.0, 50.0	0.400

E.4.11. Isoflavones (Conducted at Covance Laboratories Inc.)

The sample was extracted using a solution of hydrochloric acid and reagent alcohol heated on hot plates. The extract was brought to volume, diluted, and centrifuged. An aliquot of the supernatant was placed onto a C18 solid-phase extraction column. Unwanted components of the matrix were rinsed off with 20% methanol and then the isoflavones were eluted with 80% methanol. The sample was analyzed on a high-performance liquid chromatography system with ultraviolet detection and was compared to an external standard curve of known standards for quantitation (Pettersson and Kiessling, 1984; Seo and Morr, 1984). The limit of quantitation was 10.0 ppm.

Reference Standards:

- LC Labs, Daidzein, 99.7%, Lot Number DA-121
- LC Labs, Glycitein, 99.8%, Lot Number ARH-114
- LC Labs, Genistein, 99.7%, Lot Number CH-148

E.4.12. Acid Detergent Lignin (Conducted at Covance Laboratories Inc.)

The samples were analyzed using the conventional crucible method that is a manual method (Goering and Van Soest, 1970). The protein, carbohydrate, and ash contents were dissolved using a boiling detergent solution and filtered off. The fats and pigments were removed via an acetone wash leaving the lignocellulose fraction in a frit. The cellulose was then dissolved with sulfuric acid leaving the lignin fraction, which was determined gravimetrically. The limit of quantitation was calculated and reported on fresh weight basis. The limit of quantitation was 0.100%.

E.4.13. Moisture (Conducted at Covance Laboratories Inc.)

The sample was dried in a vacuum oven at approximately 100°C. The moisture weight loss was determined and converted to percent moisture (AOAC, 2011b; 2011d). The limit of quantitation was 0.100%.

E.4.14. Neutral Detergent Fiber (Conducted at Covance Laboratories Inc.)

The ANKOM2000 Fiber Analyzer automated the process of the removal of protein, carbohydrate, and ash. Fats and pigments were removed with an acetone wash prior to analysis. Hemicellulose, cellulose, lignin, and insoluble protein fraction were left in the filter bag and determined gravimetrically (AACC, 1998; Goering and Van Soest, 1970; Komarek et al., 1993). The limit of quantitation was 0.100%.

E.4.15. *p*-Coumaric Acid, Ferulic Acid, Sinapic Acid (Conducted at Covance Laboratories Inc.)

The sample was extracted with methanol followed by alkaline hydrolysis and buffering prior to injection on an analytical high-performance liquid chromatography (HPLC) system for quantification of sinapic acid, *p*-coumaric acid, and ferulic acid by ultraviolet

(UV) detection (Hagerman and Nicholson, 1982). The limit of quantitation was 33.0 ppm.

Reference Standards:

- Sigma-Aldrich, *p*-Coumaric acid, 99.5%, Lot Number 060m1774v
- Sigma-Aldrich, 3, 5-Dimethoxy-4-Hydroxycinnamic acid (Sinapic Acid), 99%, Lot Number 040M1943
- ACROS Organics, 4-Hydroxy-3-methoxycinnamic Acid (Ferulic Acid), 99.4%, Lot Number A0261354

E.4.16. Protein (Conducted at Covance Laboratories Inc.)

The protein and other organic nitrogen in the sample were converted to ammonia by digesting the sample with sulfuric acid containing a catalyst mixture. The acid digest was made alkaline. The ammonia was distilled and then titrated with a previously standardized acid. The percent nitrogen was calculated and converted to equivalent protein using the factor 6.25 (AOAC, 2010c; 2011f; AOCS, 2009). The limit of quantitation was 0.100%.

E.4.17. Saponin (Conducted at Samuel Roberts Noble Foundation)

The following seven saponins were analyzed:

- Total Bayogenin
- Total Hederagenin
- Total Medicagenic Acid
- Total Soyasapogenol B
- Total Soyasapogenol E
- Total Zanhic Acid
- Total Saponins

The lyophilized ground samples were extracted with 80% methanol solution that contained the Internal Standard (Huhman et al., 2005; Huhman and Sumner, 2002). Samples were analyzed with a Waters Acquity Ultra Performance Liquid Chromotography (UPLC) system (Waters Corporation, Milford, Massachusetts) fitted with a Quadrupole Time of Flight (QTOF) Premier mass spectrometer (Waters Corporation, Milford, Massachusetts). A reverse phase, 1.7-um UPLC BEH C18, 2.1 x 150 mm column was used for separations. BEH stands for Bridged Ethylene Hybrids (BEH Technolgy, Waters Corporation, Milford, Massachusetts). The mobile phase consisted of eluent A (0.1% [v/v] CH3COOH/water and Eluent B (acetonitrile) and separations achieved using a linear gradient of 95% to 30% A over 30 min, 30% to 5% A over 3.0 min, 95% to 95% A over 3.0 min. The flow rate was 0.56 mls/min and the column temperature was maintained at 60°C. Compounds were detected using ESI in conventional negative ion mode from 50-2000 m/z. The QTOF Premier was operated under the following instrument parameters: desolvation temperature of 385°C; desolvation nitrogen gas flow of 850 L/h; capillary voltage of 2.9 kV; cone voltage of 48

eV; and collision energy of 10 eV. The mass spectrometer system was calibrated using sodium formate, and raffinose was used for lockmass. Samples were normalized to the internal standard 7-OH coumarin by dividing individual peak area by the peak area of internal standard and multiplying by 1000. After normalization the saponins were separated by proposed aglycone class with peak areas of individual saponins summed. The units were response units/50 μg dry weight, where peak areas equals response units.

Internal Standard:

- Fluka, 7-OH Coumarin, 0.018 mg/ml, Lot Number 1256641

Reference Standards:

- Sigma, Esculin, 42.25 ng/inj (100 pmol), Lot Number 75H0706
- Fluka, 7-OH Coumarin, 48.6 ng/inj (300 pmol), Lot Number 1256641
- Sigma, Rutin, 76.3 ng/inj (125 pmol), Lot Number 10K0177
- Sigma, Naringin, 72.6 ng/inj (125 pmol), Lot Number 55H1139
- Sigma, Narigenin, 36.0 ng/inj (125 pmol), Lot Number 95H0996
- Sigma, Quercetin, 42.3 ng/inj (140 pmol) , Lot Number 85H0694
- Mark A. Berhow USDA Lab, Glc-Gal-GlcUA- Soyasaponin B, 2.62 ng/inj (2.7 pmol)
- Mark A. Berhow USDA Lab, Rha-Gal-GlcUA- Soyasaponin B, 47.10 ng/inj (50.0 pmol)
- Mark A. Berhow USDA Lab, Rha-Ara-GlcUA- Soyasaponin B, 21.7 ng/inj (23.7 pmol)
- Mark A. Berhow USDA Lab, Gal-GlcUA- Soyasaponin B, 10.5 ng/inj (13.1 pmol),
- Mark A. Berhow USDA Lab, Ara-GlcUA- Soyasaponin B, 1.9 ng/inj (2.5 pmol)

E.4.18. Total Polyphenols (Conducted at Covance Laboratories Inc.)

Polyphenols were extracted from the samples with methanol. The samples were reacted with Folin-Ciocalteu Reagent to produce a color that can be measured spectrophotometrically at 760 nm. Results are reported in units of mg/g gallic acid equivalents. The limit of quantitation was 1.00 mg/g.

E.5. Data Processing and Statistical Analysis

After compositional analyses were performed, data spreadsheets containing individual values for each analysis were sent to Monsanto Company (St. Louis, Missouri) for review. Data were then transferred to Certus International (Chesterfield, Missouri) where they were converted into the appropriate units and statistically analyzed. The formulas that were used for re-expression of composition data for statistical analysis are listed in Table D-2.

In order to complete a statistical analysis for a compositional component in this study, at least 50% of the values for a component had to be greater than the assay limit of quantitation (LOQ). Components with more than 50% of observations below the assay

LOQ were excluded from summaries and analysis. The following seven components with more than 50% of the observations below the assay LOQ were excluded: biochanin A, coumesterol, daidzein, formononetin, genistein, glycitein, and sinapic acid.

If less than 50% of the observations for a component were below the LOQ, individual analyses that were below the LOQ were assigned a value equal to one-half the LOQ. In this study 56 values for sodium were assigned a value of 50.0 ppm fw and 50 values for canavanine were assigned a value of 2.5 µg/g fw.

The data were assessed for potential outliers using a studentized PRESS residuals calculation. A PRESS residual is the difference between any value and its value predicted from a statistical model that excludes the data point. The studentized version scales these residuals so that the values tend to have a standard normal distribution when outliers are absent. Thus, most values are expected to be between ± 3. Extreme data points that are also outside of the ± 6 studentized PRESS residual range are considered for exclusion as outliers from the final analyses. One copper value from one commercial reference at the ILCY site was identified as an outlier, but the value was similar to other nearby data points and was not removed from the statistical analysis.

Alfalfa forage compositional components were statistically analyzed using a mixed model analysis. The six replicated field sites were analyzed as a combined data set. These combined-site analyses used model (1).

(1) $Y_{ijk} = U + T_i + L_j + B(L)_{jk} + LT_{ij} + e_{ijk}$,

where Y_{ijk} = unique individual observation, U = overall mean, T_i = substance effect, L_j = random location effect, $B(L)_{jk}$ = random block within location effect, LT_{ij} = random location by substance interaction effect, and e_{ijk} = residual error.

A supplementary individual site analysis used model (2).

(2) $Y_{ij} = U + T_i + B_j + e_{ij}$,

where Y_{ij} = unique individual observation, U = overall mean, T_i = substance effect, B_j = random block effect, and e_{ij} = residual error.

For each compositional component, a range of observed values and a 99% tolerance interval were calculated. A tolerance interval is an interval that one can claim, with a specified degree of confidence, contains at least a specified proportion, p, of an entire sampled population for the parameter measured. The calculated tolerance intervals are expected to contain, with 95% confidence, 99% of the quantities expressed in the population of conventional alfalfa. Each tolerance interval estimate was based upon the average observation for each unique reference material. Because negative quantities are not possible, negative calculated lower tolerance bounds were set to zero.

SAS (Version 9.2) software was used to generate all summary statistics and perform all analyses.

Report tables present p-values from SAS as either <0.001 or the actual value truncated to three decimal places.

Table E-2. Re-expression Formulas for Statistical Analysis of Composition Data

Component	From (X)[1]	To	Formula[2]
Proximates (excluding Moisture), Fiber	% fw	% dw	X/d
Copper, Iron, Manganese, Zinc	ppm fw	mg/kg dw	X/d
Calcium, Magnesium, Phosphorus, Potassium, Sodium	ppm fw	% dw	$X/(10^4 d)$
Ferulic Acid, *p*-Coumaric Acid	ppm fw	ppm dw	X/d
Canavanine	µg/g fw	ppm dw	X/d
Total Polyphenols	mg/g fw	mg/g dw	X/d
Free Phenylalanine	mg/g fw	ppm dw	$10^3(X/d)$
Amino Acids (AA)	mg/g fw	% dw	X/(10d)
Saponins	response units/50 µg dw	response units/ µg dw	X/50

[1] fw = fresh weight and dw = dry weight.
[2] 'X' is the individual sample value; 'd' is the fraction of the sample that is dry matter.

Table E-3. Statistical Summary of Site CAPR Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Proximate (% dw)						
Ash	8.82 (0.54) (8.73 - 9.18)	9.85 (0.36) (8.79 - 10.59)	-1.04 (0.58) (-1.80 - -0.32)	-2.59, 0.52	0.142	6.70, 13.54 (7.54 - 13.23)
Carbohydrates	69.61 (0.98) (68.88 - 70.34)	66.41 (0.69) (64.59 - 68.12)	3.20 (1.20) (1.55 - 5.75)	0.37, 6.03	0.031	50.57, 81.80 (54.35 - 74.91)
Moisture (% fw)	78.40 (0.92) (76.40 - 80.40)	79.90 (0.65) (79.10 - 81.40)	-1.50 (1.13) (-2.70 - 0.60)	-4.17, 1.17	0.225	65.06, 90.61 (66.10 - 85.30)
Protein	19.69 (0.55) (19.41 - 20.41)	21.86 (0.46) (20.72 - 22.47)	-2.17 (0.46) (-2.75 - -1.67)	-3.42, -0.92	0.008	9.26, 33.78 (14.52 - 30.07)
Total Fat	1.54 (0.40) (1.47 - 1.62)	1.92 (0.28) (1.31 - 2.67)	-0.38 (0.49) (-1.19 - 0.31)	-1.54, 0.78	0.463	0.73, 3.59 (0.53 - 4.21)
Fiber (% dw)						
Acid Detergent Fiber	29.98 (2.64) (27.91 - 32.12)	27.75 (1.58) (24.31 - 30.69)	2.23 (2.95) (-2.78 - 7.81)	-5.69, 10.15	0.489	6.16, 49.06 (7.07 - 39.11)

Table E-3 (continued). Statistical Summary of Site CAPR Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Fiber (% dw)						
Acid Detergent Lignin	6.50 (0.43) (5.92 - 7.07)	6.99 (0.31) (6.31 - 7.54)	-0.50 (0.53) (-1.63 - 0.012)	-1.75, 0.76	0.380	2.13, 11.99 (3.38 - 9.67)
Neutral Detergent Fiber	36.38 (1.98) (37.14 - 38.14)	36.43 (1.65) (30.91 - 40.74)	-0.043 (1.63) (-3.60 - 1.72)	-4.55, 4.46	0.980	12.04, 58.18 (18.97 - 49.82)
Amino Acid (% dw)						
Alanine	1.06 (0.022) (1.06 - 1.07)	1.17 (0.015) (1.12 - 1.21)	-0.11 (0.026) (-0.095 - -0.063)	-0.17, -0.047	0.004	0.49, 1.79 (0.80 - 1.66)
Arginine	0.98 (0.027) (0.95 - 0.99)	1.10 (0.017) (1.09 - 1.11)	-0.12 (0.031) (-0.16 - -0.094)	-0.20, -0.047	0.008	0.44, 1.59 (0.70 - 1.44)
Aspartic acid	2.69 (0.13) (2.44 - 2.94)	3.06 (0.091) (2.95 - 3.14)	-0.36 (0.16) (-0.64 - -0.012)	-0.74, 0.010	0.055	0.44, 5.63 (1.96 - 5.15)
Cystine	0.22 (0.0097) (0.20 - 0.23)	0.23 (0.0069) (0.21 - 0.24)	-0.010 (0.012) (-0.023 - -0.014)	-0.039, 0.018	0.411	0.12, 0.32 (0.16 - 0.31)

Table E-3 (continued). Statistical Summary of Site CAPR Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Amino Acid (% dw)						
Glutamic acid	1.82 (0.044) (1.77 - 1.88)	2.05 (0.031) (2.01 - 2.11)	-0.23 (0.054) (-0.31 - -0.14)	-0.36, -0.10	0.003	0.81, 3.01 (1.31 - 2.80)
Glycine	0.94 (0.020) (0.90 - 0.94)	1.01 (0.015) (0.99 - 1.05)	-0.076 (0.019) (-0.10 - -0.046)	-0.13, -0.025	0.014	0.49, 1.44 (0.70 - 1.33)
Histidine	0.43 (0.013) (0.42 - 0.43)	0.46 (0.0096) (0.43 - 0.48)	-0.026 (0.012) (-0.038 - -0.0067)	-0.059, 0.0074	0.097	0.26, 0.63 (0.34 - 0.61)
Isoleucine	0.86 (0.025) (0.83 - 0.87)	0.94 (0.017) (0.91 - 0.98)	-0.080 (0.027) (-0.097 - -0.039)	-0.15, -0.0084	0.035	0.43, 1.36 (0.63 - 1.27)
Leucine	1.42 (0.043) (1.38 - 1.43)	1.56 (0.027) (1.51 - 1.63)	-0.15 (0.048) (-0.16 - -0.076)	-0.27, -0.022	0.029	0.70, 2.25 (1.03 - 2.05)
Lysine	1.13 (0.054) (1.10 - 1.12)	1.24 (0.034) (1.15 - 1.34)	-0.11 (0.059) (-0.14 - -0.046)	-0.27, 0.049	0.129	0.55, 1.82 (0.82 - 1.73)

Table E-3 (continued). Statistical Summary of Site CAPR Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Amino Acid (% dw)						
Methionine	0.21 (0.018) (0.20 - 0.21)	0.24 (0.011) (0.21 - 0.27)	-0.034 (0.020) (-0.019 - -0.0094)	-0.086, 0.018	0.159	0.068, 0.42 (0.14 - 0.45)
Phenylalanine	0.96 (0.028) (0.95 - 0.97)	1.06 (0.020) (1.01 - 1.11)	-0.10 (0.035) (-0.11 - -0.045)	-0.18, -0.018	0.023	0.48, 1.53 (0.71 - 1.39)
Proline	0.85 (0.034) (0.83 - 0.84)	0.95 (0.021) (0.91 - 1.01)	-0.10 (0.037) (-0.13 - -0.064)	-0.20, -0.0018	0.047	0.43, 1.41 (0.65 - 1.24)
Serine	0.84 (0.022) (0.80 - 0.88)	0.92 (0.015) (0.90 - 0.96)	-0.087 (0.027) (-0.16 - -0.023)	-0.15, -0.024	0.014	0.45, 1.35 (0.66 - 1.25)
Threonine	0.84 (0.024) (0.82 - 0.85)	0.94 (0.014) (0.91 - 0.97)	-0.10 (0.026) (-0.12 - -0.064)	-0.17, -0.029	0.016	0.45, 1.33 (0.63 - 1.23)
Tryptophan	0.34 (0.028) (0.34 - 0.35)	0.38 (0.018) (0.35 - 0.39)	-0.033 (0.032) (-0.056 - -0.00099)	-0.11, 0.047	0.337	0.20, 0.56 (0.25 - 0.50)

Table E-3 (continued). Statistical Summary of Site CAPR Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Amino Acid (% dw)						
Tyrosine	0.70 (0.017) (0.69 - 0.70)	0.78 (0.012) (0.76 - 0.80)	-0.081 (0.021) (-0.098 - -0.053)	-0.13, -0.033	0.005	0.35, 1.09 (0.52 - 1.01)
Valine	1.03 (0.027) (1.00 - 1.06)	1.13 (0.019) (1.09 - 1.18)	-0.098 (0.033) (-0.12 - -0.028)	-0.18, -0.019	0.021	0.52, 1.64 (0.79 - 1.55)
Mineral						
Calcium (% dw)	1.42 (0.092) (1.29 - 1.55)	1.58 (0.065) (1.42 - 1.72)	-0.16 (0.11) (-0.36 - 0.13)	-0.43, 0.11	0.196	0.55, 2.56 (0.95 - 2.07)
Copper (mg/kg dw)	9.98 (1.09) (9.19 - 10.77)	10.79 (0.77) (10.10 - 11.93)	-0.81 (1.33) (-1.62 - 0.67)	-3.96, 2.34	0.561	1.87, 14.98 (4.54 - 19.67)
Iron (mg/kg dw)	256.68 (78.54) (236.86 - 316.33)	348.49 (51.22) (174.40 - 516.75)	-91.81 (85.46) (-279.88 - 5.44)	-320.55, 136.92	0.337	41.59, 446.31 (105.45 - 691.43)
Magnesium (% dw)	0.14 (0.0085) (0.12 - 0.15)	0.15 (0.0060) (0.15 - 0.16)	-0.016 (0.0089) (-0.028 - -0.00009)	-0.040, 0.0070	0.129	0.027, 0.41 (0.11 - 0.34)

Table E-3 (continued). Statistical Summary of Site CAPR Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Mineral						
Manganese (mg/kg dw)	35.15 (4.47) (36.36 - 37.65)	40.72 (3.17) (30.92 - 48.33)	-5.57 (4.58) (-11.97 - -5.81)	-18.07, 6.94	0.288	17.53, 69.85 (23.24 - 98.04)
Phosphorus (% dw)	0.26 (0.010) (0.27 - 0.28)	0.30 (0.0091) (0.29 - 0.31)	-0.038 (0.0063) (-0.040 - -0.033)	-0.055, -0.021	0.003	0.14, 0.46 (0.18 - 0.43)
Potassium (% dw)	2.19 (0.078) (2.18 - 2.20)	2.36 (0.055) (2.31 - 2.48)	-0.17 (0.096) (-0.13 - -0.11)	-0.40, 0.054	0.114	1.82, 3.04 (1.85 - 3.35)
Sodium (% dw)	0.16 (0.013) (0.15 - 0.16)	0.14 (0.0093) (0.11 - 0.15)	0.025 (0.013) (0.0075 - 0.039)	-0.0086, 0.059	0.108	0, 0.24 (0.016 - 0.20)
Zinc (mg/kg dw)	27.03 (1.05) (25.38 - 26.99)	29.30 (0.94) (27.27 - 30.81)	-2.28 (0.71) (-3.26 - -1.89)	-4.23, -0.32	0.031	8.89, 47.44 (17.08 - 47.48)

[1]dw = dry weight; fw = fresh weight
[2]Mean (S.E.) = least-square mean (standard error)
[3]Control refers to the non-biotechnology derived, conventional control (C_0-Syn1).
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties. Negative limits set to zero.

Table E-4. Statistical Summary of Site CAPR Alfalfa Forage Secondary Metabolites for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Metabolite						
Canavanine (ppm dw)	44.03 (9.98) (32.97 - 55.10)	69.09 (7.06) (58.60 - 79.23)	-25.05 (12.22) (-26.84 - -23.61)	-53.96, 3.85	0.079	0, 137.35 (11.47 - 151.33)
Ferulic Acid (ppm dw)	1509.08 (114.39) (1411.02 - 1607.14)	1350.71 (80.89) (1103.96 - 1596.77)	158.37 (140.10) (80.87 - 503.18)	-172.92, 489.65	0.295	854.88, 2061.10 (1103.32 - 1906.86)
Free Phenylalanine (ppm dw)	165.37 (43.57) (111.86 - 218.88)	204.65 (30.81) (154.07 - 278.22)	-39.28 (53.36) (-59.34 - -42.20)	-165.45, 86.89	0.485	0, 627.23 (133.05 - 579.05)
Total Polyphenols (mg/g dw)	8.51 (0.39) (8.31 - 8.72)	7.45 (0.25) (7.20 - 7.66)	1.06 (0.46) (0.65 - 1.40)	-0.070, 2.18	0.061	4.86, 11.15 (6.17 - 11.17)
p-Coumaric Acid (ppm dw)	567.23 (57.51) (466.10 - 668.37)	514.33 (40.66) (442.08 - 645.16)	52.91 (70.43) (-31.51 - 226.29)	-113.64, 219.45	0.477	188.81, 949.95 (326.19 - 945.58)

[1]dw = dry weight; fw = fresh weight
[2]Mean (S.E.) = least-square mean (standard error)
[3]Control refers to the non-biotechnology derived, conventional control (C_0-Syn1).
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties. Negative limits set to zero.

Table E-5. Statistical Summary of Site IARL Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Proximate (% dw)						
Ash	10.05 (0.36) (9.88 - 10.39)	10.63 (0.36) (10.29 - 11.39)	-0.58 (0.51) (-1.52 - 0.095)	-1.74, 0.57	0.281	6.70, 13.54 (7.54 - 13.23)
Carbohydrates	59.32 (0.78) (57.73 - 62.20)	61.15 (0.78) (59.94 - 62.42)	-1.83 (0.89) (-3.45 - 1.12)	-4.01, 0.34	0.084	50.57, 81.80 (54.35 - 74.91)
Moisture (% fw)	84.13 (0.24) (83.60 - 84.60)	83.38 (0.24) (83.00 - 83.70)	0.75 (0.34) (0.20 - 1.60)	-0.025, 1.53	0.056	65.06, 90.61 (66.10 - 85.30)
Protein	27.34 (0.72) (24.51 - 29.03)	25.71 (0.72) (24.73 - 27.30)	1.64 (0.59) (-0.58 - 3.19)	0.19, 3.08	0.032	9.26, 33.78 (14.52 - 30.07)
Total Fat	3.25 (0.30) (2.58 - 3.98)	2.59 (0.30) (1.72 - 3.38)	0.66 (0.28) (-0.21 - 1.07)	-0.011, 1.34	0.052	0.73, 3.59 (0.53 - 4.21)
Fiber (% dw)						
Acid Detergent Fiber	16.65 (1.42) (15.71 - 17.85)	15.76 (1.42) (10.96 - 19.18)	0.89 (1.58) (-3.20 - 6.12)	-2.96, 4.75	0.591	6.16, 49.06 (7.07 - 39.11)

Table E-5 (continued). Statistical Summary of Site IARL Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Fiber (% dw)						
Acid Detergent Lignin	3.22 (0.17) (2.72 - 3.69)	3.85 (0.17) (3.58 - 4.23)	-0.62 (0.24) (-1.51 - -0.16)	-1.21, -0.041	0.039	2.13, 11.99 (3.38 - 9.67)
Neutral Detergent Fiber	22.65 (2.23) (18.57 - 30.78)	22.89 (2.23) (18.94 - 26.83)	-0.24 (3.16) (-8.11 - 11.84)	-7.38, 6.90	0.941	12.04, 58.18 (18.97 - 49.82)
Amino Acid (% dw)						
Alanine	1.40 (0.034) (1.31 - 1.52)	1.36 (0.034) (1.30 - 1.39)	0.038 (0.032) (-0.042 - 0.13)	-0.041, 0.12	0.281	0.49, 1.79 (0.80 - 1.66)
Arginine	1.28 (0.029) (1.23 - 1.35)	1.23 (0.029) (1.19 - 1.28)	0.052 (0.022) (0.0092 - 0.11)	-0.0012, 0.10	0.053	0.44, 1.59 (0.70 - 1.44)
Aspartic acid	4.05 (0.17) (3.74 - 4.65)	3.85 (0.17) (3.47 - 4.08)	0.20 (0.16) (-0.29 - 0.57)	-0.19, 0.59	0.253	0.44, 5.63 (1.96 - 5.15)
Cystine	0.26 (0.013) (0.23 - 0.30)	0.26 (0.013) (0.24 - 0.29)	0.0044 (0.010) (-0.018 - 0.019)	-0.021, 0.029	0.685	0.12, 0.32 (0.16 - 0.31)

Table E-5 (continued). Statistical Summary of Site IARL Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Amino Acid (% dw)						
Glutamic acid	2.39 (0.045) (2.24 - 2.55)	2.31 (0.045) (2.27 - 2.36)	0.079 (0.038) (-0.026 - 0.22)	-0.013, 0.17	0.080	0.81, 3.01 (1.31 - 2.80)
Glycine	1.15 (0.020) (1.10 - 1.21)	1.12 (0.020) (1.10 - 1.14)	0.026 (0.020) (-0.022 - 0.085)	-0.022, 0.075	0.230	0.49, 1.44 (0.70 - 1.33)
Histidine	0.52 (0.0099) (0.49 - 0.55)	0.50 (0.0099) (0.49 - 0.51)	0.015 (0.012) (-0.012 - 0.059)	-0.015, 0.044	0.270	0.26, 0.63 (0.34 - 0.61)
Isoleucine	1.08 (0.021) (1.02 - 1.15)	1.05 (0.021) (1.03 - 1.07)	0.032 (0.028) (-0.048 - 0.12)	-0.037, 0.10	0.293	0.43, 1.36 (0.63 - 1.27)
Leucine	1.80 (0.031) (1.73 - 1.90)	1.74 (0.031) (1.71 - 1.78)	0.061 (0.030) (-0.011 - 0.16)	-0.012, 0.13	0.085	0.70, 2.25 (1.03 - 2.05)
Lysine	1.44 (0.034) (1.37 - 1.55)	1.40 (0.034) (1.34 - 1.44)	0.038 (0.021) (-0.0052 - 0.12)	-0.014, 0.091	0.124	0.55, 1.82 (0.82 - 1.73)

Table E-5 (continued). Statistical Summary of Site IARL Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Amino Acid (% dw)						
Methionine	0.37 (0.015) (0.34 - 0.39)	0.34 (0.015) (0.30 - 0.37)	0.031 (0.017) (0.0086 - 0.062)	-0.010, 0.072	0.117	0.068, 0.42 (0.14 - 0.45)
Phenylalanine	1.22 (0.020) (1.16 - 1.27)	1.18 (0.020) (1.15 - 1.21)	0.034 (0.019) (-0.027 - 0.083)	-0.013, 0.080	0.127	0.48, 1.53 (0.71 - 1.39)
Proline	1.09 (0.030) (1.02 - 1.18)	1.07 (0.030) (1.05 - 1.11)	0.020 (0.022) (-0.024 - 0.10)	-0.033, 0.072	0.401	0.43, 1.41 (0.65 - 1.24)
Serine	1.09 (0.023) (1.03 - 1.16)	1.01 (0.023) (0.95 - 1.05)	0.076 (0.018) (0.019 - 0.13)	0.030, 0.12	0.006	0.45, 1.35 (0.66 - 1.25)
Threonine	1.07 (0.017) (1.02 - 1.12)	1.02 (0.017) (0.99 - 1.05)	0.048 (0.016) (0.0064 - 0.10)	0.0075, 0.088	0.027	0.45, 1.33 (0.63 - 1.23)
Tryptophan	0.45 (0.012) (0.41 - 0.48)	0.43 (0.012) (0.40 - 0.45)	0.015 (0.016) (-0.027 - 0.038)	-0.025, 0.055	0.399	0.20, 0.56 (0.25 - 0.50)

Table E-5 (continued). Statistical Summary of Site IARL Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Amino Acid (% dw)						
Tyrosine	0.90 (0.023) (0.86 - 0.94)	0.86 (0.023) (0.84 - 0.89)	0.040 (0.028) (-0.0025 - 0.10)	-0.028, 0.11	0.200	0.35, 1.09 (0.52 - 1.01)
Valine	1.31 (0.024) (1.24 - 1.38)	1.27 (0.024) (1.24 - 1.32)	0.040 (0.033) (-0.073 - 0.13)	-0.041, 0.12	0.274	0.52, 1.64 (0.79 - 1.55)
Mineral						
Calcium (% dw)	1.67 (0.041) (1.63 - 1.71)	1.76 (0.041) (1.69 - 1.86)	-0.093 (0.058) (-0.20 - -0.0096)	-0.22, 0.038	0.142	0.55, 2.56 (0.95 - 2.07)
Copper (mg/kg dw)	9.38 (0.86) (8.54 - 11.23)	8.57 (0.86) (8.20 - 8.83)	0.80 (0.98) (0.073 - 2.40)	-1.60, 3.21	0.444	1.87, 14.98 (4.54 - 19.67)
Iron (mg/kg dw)	169.80 (36.93) (123.38 - 207.36)	310.65 (36.93) (202.40 - 386.50)	-140.85 (52.22) (-263.13 - 1.26)	-258.99, -22.71	0.024	41.59, 446.31 (105.45 - 691.43)
Magnesium (% dw)	0.30 (0.0095) (0.29 - 0.31)	0.31 (0.0095) (0.30 - 0.32)	-0.011 (0.014) (-0.023 - -0.0023)	-0.041, 0.020	0.452	0.027, 0.41 (0.11 - 0.34)

Table E-5 (continued). Statistical Summary of Site IARL Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Mineral						
Manganese (mg/kg dw)	31.84 (2.81) (30.52 - 34.57)	39.98 (2.81) (34.37 - 42.39)	-8.14 (3.97) (-11.42 - 0.20)	-17.12, 0.84	0.070	17.53, 69.85 (23.24 - 98.04)
Phosphorus (% dw)	0.37 (0.018) (0.33 - 0.40)	0.35 (0.018) (0.33 - 0.38)	0.016 (0.025) (-0.035 - 0.071)	-0.040, 0.072	0.535	0.14, 0.46 (0.18 - 0.43)
Potassium (% dw)	2.45 (0.11) (2.26 - 2.62)	2.53 (0.11) (2.41 - 2.71)	-0.077 (0.15) (-0.45 - 0.18)	-0.42, 0.27	0.624	1.82, 3.04 (1.85 - 3.35)
Sodium (% dw)	0.050 (0.010) (0.030 - 0.069)	0.041 (0.010) (0.029 - 0.075)	0.0087 (0.014) (-0.044 - 0.039)	-0.023, 0.041	0.557	0, 0.24 (0.016 - 0.20)
Zinc (mg/kg dw)	36.08 (1.99) (33.90 - 39.22)	37.26 (1.99) (34.12 - 40.42)	-1.18 (2.32) (-5.64 - 2.31)	-6.86, 4.51	0.630	8.89, 47.44 (17.08 - 47.48)

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

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

[3]Control refers to the non-biotechnology derived, conventional control (C0-Syn1).

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

Table E-6. Statistical Summary of Site IARL Alfalfa Forage Secondary Metabolites for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Metabolite						
Canavanine (ppm dw)	15.76 (0.24) (15.24 - 16.23)	15.04 (0.24) (14.71 - 15.34)	0.72 (0.33) (0.19 - 1.53)	-0.035, 1.48	0.059	0, 137.35 (11.47 - 151.33)
Ferulic Acid (ppm dw)	1591.83 (78.25) (1402.60 - 1779.22)	1390.17 (78.25) (1181.82 - 1580.84)	201.66 (94.83) (22.23 - 361.57)	-30.37, 433.70	0.077	854.88, 2061.10 (1103.32 - 1906.86)
Free Phenylalanine (ppm dw)	378.02 (21.12) (345.73 - 409.20)	386.96 (21.12) (370.30 - 422.94)	-8.94 (26.59) (-29.72 - 38.90)	-74.01, 56.13	0.748	0, 627.23 (133.05 - 579.05)
Total Polyphenols (mg/g dw)	9.68 (0.22) (8.96 - 10.19)	8.86 (0.22) (8.47 - 9.22)	0.82 (0.31) (0.048 - 1.72)	0.11, 1.52	0.027	4.86, 11.15 (6.17 - 11.17)
p-Coumaric Acid (ppm dw)	748.40 (40.83) (625.77 - 870.13)	732.14 (40.83) (660.61 - 784.43)	16.26 (57.74) (-79.99 - 128.95)	-114.36, 146.89	0.784	188.81, 949.95 (326.19 - 945.58)

[1]dw = dry weight
[2]Mean (S.E.) = least-square mean (standard error)
[3]Control refers to the non-biotechnology derived, conventional control (C_0-Syn1).
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties
Negative limits set to zero.

Table E-7. Statistical Summary of Site ILCY Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Proximate (% dw)						
Ash	9.85 (0.31) (8.81 - 10.83)	10.33 (0.31) (9.80 - 11.22)	-0.48 (0.28) (-1.35 - -0.025)	-1.17, 0.21	0.139	6.70, 13.54 (7.54 - 13.23)
Carbohydrates	69.37 (1.34) (66.84 - 71.14)	67.53 (1.34) (61.73 - 71.28)	1.84 (1.89) (-2.74 - 8.36)	-2.44, 6.13	0.355	50.57, 81.80 (54.35 - 74.91)
Moisture (% fw)	79.73 (0.43) (78.10 - 81.30)	80.45 (0.43) (80.30 - 80.60)	-0.73 (0.49) (-2.20 - 0.70)	-1.93, 0.48	0.192	65.06, 90.61 (66.10 - 85.30)
Protein	18.87 (1.15) (17.41 - 21.12)	20.06 (1.15) (16.56 - 25.00)	-1.18 (1.62) (-6.67 - 2.15)	-4.86, 2.49	0.485	9.26, 33.78 (14.52 - 30.07)
Total Fat	1.99 (0.36) (0.84 - 2.62)	2.22 (0.36) (1.31 - 3.23)	-0.23 (0.50) (-1.30 - 0.93)	-1.45, 0.99	0.662	0.73, 3.59 (0.53 - 4.21)
Fiber (% dw)						
Acid Detergent Fiber	27.25 (1.40) (20.53 - 29.59)	32.57 (1.40) (30.62 - 33.67)	-5.32 (1.84) (-10.08 - -2.75)	-9.82, -0.82	0.027	6.16, 49.06 (7.07 - 39.11)

Table E-7 (continued). Statistical Summary of Site ILCY Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Fiber (% dw)						
Acid Detergent Lignin	5.88 (0.31) (5.51 - 6.32)	6.81 (0.31) (5.89 - 7.50)	-0.94 (0.41) (-1.99 - -0.36)	-1.94, 0.069	0.062	2.13, 11.99 (3.38 - 9.67)
Neutral Detergent Fiber	34.67 (1.47) (30.00 - 38.58)	39.92 (1.47) (38.26 - 43.32)	-5.25 (1.62) (-9.74 - 0.21)	-9.21, -1.29	0.017	12.04, 58.18 (18.97 - 49.82)
Amino Acid (% dw)						
Alanine	0.99 (0.027) (0.91 - 1.06)	1.01 (0.027) (0.94 - 1.07)	-0.020 (0.038) (-0.096 - 0.074)	-0.11, 0.066	0.610	0.49, 1.79 (0.80 - 1.66)
Arginine	0.89 (0.024) (0.83 - 0.94)	0.90 (0.024) (0.87 - 0.96)	-0.0090 (0.033) (-0.043 - 0.039)	-0.089, 0.071	0.792	0.44, 1.59 (0.70 - 1.44)
Aspartic acid	2.68 (0.11) (2.36 - 3.13)	2.50 (0.11) (2.42 - 2.61)	0.18 (0.15) (-0.25 - 0.71)	-0.16, 0.52	0.258	0.44, 5.63 (1.96 - 5.15)
Cystine	0.20 (0.0081) (0.19 - 0.23)	0.20 (0.0081) (0.18 - 0.21)	0.0071 (0.011) (-0.021 - 0.018)	-0.019, 0.033	0.550	0.12, 0.32 (0.16 - 0.31)

Table E-7 (continued). Statistical Summary of Site ILCY Alfalfa Forage Nutrients for KK179 vs Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Amino Acid (% dw)						
Glutamic acid	1.66 (0.046) (1.59 - 1.78)	1.70 (0.046) (1.59 - 1.80)	-0.043 (0.065) (-0.16 - 0.14)	-0.19, 0.10	0.524	0.81, 3.01 (1.31 - 2.80)
Glycine	0.84 (0.022) (0.80 - 0.90)	0.88 (0.022) (0.82 - 0.93)	-0.033 (0.032) (-0.096 - 0.053)	-0.11, 0.038	0.320	0.49, 1.44 (0.70 - 1.33)
Histidine	0.41 (0.0095) (0.38 - 0.43)	0.40 (0.0095) (0.38 - 0.43)	0.0035 (0.013) (-0.012 - 0.032)	-0.027, 0.034	0.800	0.26, 0.63 (0.34 - 0.61)
Isoleucine	0.76 (0.023) (0.73 - 0.82)	0.80 (0.023) (0.74 - 0.85)	-0.038 (0.032) (-0.094 - 0.050)	-0.11, 0.034	0.260	0.43, 1.36 (0.63 - 1.27)
Leucine	1.27 (0.037) (1.19 - 1.36)	1.33 (0.037) (1.23 - 1.41)	-0.061 (0.052) (-0.15 - 0.075)	-0.18, 0.057	0.270	0.70, 2.25 (1.03 - 2.05)
Lysine	1.04 (0.028) (0.99 - 1.11)	1.05 (0.028) (0.97 - 1.14)	-0.0074 (0.040) (-0.11 - 0.11)	-0.097, 0.082	0.856	0.55, 1.82 (0.82 - 1.73)

Table E-7 (continued). Statistical Summary of Site ILCY Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Amino Acid (% dw)						
Methionine	0.26 (0.023) (0.17 - 0.32)	0.23 (0.023) (0.22 - 0.24)	0.032 (0.032) (-0.053 - 0.10)	-0.041, 0.10	0.350	0.068, 0.42 (0.14 - 0.45)
Phenylalanine	0.86 (0.023) (0.82 - 0.90)	0.91 (0.023) (0.84 - 0.96)	-0.050 (0.032) (-0.10 - 0.022)	-0.12, 0.022	0.153	0.48, 1.53 (0.71 - 1.39)
Proline	0.84 (0.025) (0.81 - 0.87)	0.81 (0.025) (0.75 - 0.88)	0.031 (0.035) (-0.064 - 0.11)	-0.047, 0.11	0.390	0.43, 1.41 (0.65 - 1.24)
Serine	0.83 (0.023) (0.77 - 0.90)	0.83 (0.023) (0.79 - 0.87)	0.0042 (0.032) (-0.055 - 0.099)	-0.068, 0.077	0.898	0.45, 1.35 (0.66 - 1.25)
Threonine	0.79 (0.022) (0.74 - 0.84)	0.80 (0.022) (0.76 - 0.85)	-0.013 (0.032) (-0.058 - 0.061)	-0.085, 0.059	0.695	0.45, 1.33 (0.63 - 1.23)
Tryptophan	0.35 (0.010) (0.32 - 0.38)	0.35 (0.010) (0.32 - 0.37)	0.0032 (0.015) (-0.047 - 0.035)	-0.030, 0.036	0.831	0.20, 0.56 (0.25 - 0.50)

Table E-7 (continued). Statistical Summary of Site ILCY Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Amino Acid (% dw)						
Tyrosine	0.64 (0.015) (0.60 - 0.68)	0.65 (0.015) (0.63 - 0.67)	-0.0087 (0.022) (-0.024 - 0.025)	-0.058, 0.040	0.698	0.35, 1.09 (0.52 - 1.01)
Valine	0.95 (0.023) (0.89 - 0.99)	0.99 (0.023) (0.91 - 1.04)	-0.040 (0.033) (-0.090 - -0.00058)	-0.11, 0.035	0.263	0.52, 1.64 (0.79 - 1.55)
Mineral						
Calcium (% dw)	1.45 (0.085) (1.27 - 1.62)	1.46 (0.085) (1.25 - 1.66)	-0.013 (0.12) (-0.12 - 0.062)	-0.30, 0.27	0.912	0.55, 2.56 (0.95 - 2.07)
Copper (mg/kg dw)	7.04 (0.57) (5.21 - 8.50)	6.05 (0.57) (5.18 - 7.32)	0.99 (0.77) (0.028 - 1.51)	-0.88, 2.86	0.243	1.87, 14.98 (4.54 - 19.67)
Iron (mg/kg dw)	212.81 (25.68) (173.97 - 230.88)	212.29 (25.68) (163.92 - 313.78)	0.52 (35.24) (-82.89 - 52.13)	-85.70, 86.74	0.988	41.59, 446.31 (105.45 - 691.43)
Magnesium (% dw)	0.17 (0.010) (0.13 - 0.20)	0.18 (0.010) (0.17 - 0.19)	-0.0085 (0.012) (-0.040 - 0.019)	-0.038, 0.021	0.514	0.027, 0.41 (0.11 - 0.34)

Table E-7 (continued). Statistical Summary of Site ILCY Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Mineral						
Manganese (mg/kg dw)	77.86 (7.33) (59.82 - 106.47)	69.83 (7.33) (58.16 - 77.32)	8.02 (10.37) (-15.31 - 37.75)	-15.89, 31.94	0.461	17.53, 69.85 (23.24 - 98.04)
Phosphorus (% dw)	0.29 (0.0074) (0.28 - 0.31)	0.30 (0.0074) (0.29 - 0.31)	-0.0078 (0.010) (-0.032 - 0.0061)	-0.033, 0.018	0.480	0.14, 0.46 (0.18 - 0.43)
Potassium (% dw)	2.37 (0.055) (2.25 - 2.58)	2.51 (0.055) (2.44 - 2.60)	-0.14 (0.077) (-0.35 - 0.082)	-0.32, 0.031	0.096	1.82, 3.04 (1.85 - 3.35)
Sodium (% dw)	0.090 (0.013) (0.065 - 0.14)	0.091 (0.013) (0.074 - 0.13)	-0.0014 (0.019) (-0.056 - 0.068)	-0.043, 0.041	0.942	0, 0.24 (0.016 - 0.20)
Zinc (mg/kg dw)	25.77 (1.88) (18.40 - 32.73)	20.32 (1.88) (17.38 - 22.50)	5.45 (2.58) (-1.34 - 11.08)	-0.87, 11.77	0.079	8.89, 47.44 (17.08 - 47.48)

[1]dw = dry weight; fw = fresh weight
[2]Mean (S.E.) = least-square mean (standard error).
[3]Control refers to the non-biotechnology derived, conventional control (C_0-Syn1).
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties. Negative limits set to zero.

Table E-8. Statistical Summary of Site ILCY Alfalfa Forage Secondary Metabolites for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Metabolite						
Canavanine (ppm dw)	12.37 (0.26) (11.42 - 13.37)	12.79 (0.26) (12.69 - 12.89)	-0.42 (0.30) (-1.27 - 0.48)	-1.15, 0.31	0.210	0, 137.35 (11.47 - 151.33)
Ferulic Acid (ppm dw)	1617.62 (75.96) (1508.02 - 1840.80)	1605.38 (75.96) (1515.31 - 1809.28)	12.24 (107.43) (-301.26 - 292.08)	-230.77, 255.26	0.911	854.88, 2061.10 (1103.32 - 1906.86)
Free Phenylalanine (ppm dw)	301.46 (23.08) (232.62 - 362.69)	297.57 (23.08) (245.36 - 317.44)	3.89 (32.03) (-41.81 - 45.25)	-74.49, 82.27	0.907	0, 627.23 (133.05 - 579.05)
Total Polyphenols (mg/g dw)	8.43 (0.36) (7.86 - 9.00)	8.74 (0.36) (8.11 - 10.21)	-0.31 (0.51) (-2.13 - 0.87)	-1.46, 0.84	0.555	4.86, 11.15 (6.17 - 11.17)
p-Coumaric Acid (ppm dw)	696.28 (24.51) (661.76 - 736.32)	715.16 (24.51) (649.75 - 819.59)	-18.88 (31.57) (-108.36 - 44.01)	-96.12, 58.37	0.571	188.81, 949.95 (326.19 - 945.58)

[1]dw = dry weight
[2]Mean (S.E.) = least-square mean (standard error)
[3]Control refers to the non-biotechnology derived, conventional control (C_0-Syn1).
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties
Negative limits set to zero.

Table E-9. Statistical Summary of Site KSLA Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Proximate (% dw)						
Ash	11.97 (0.36) (11.27 - 13.26)	12.43 (0.36) (12.11 - 12.88)	-0.46 (0.50) (-1.04 - 0.83)	-1.69, 0.77	0.398	6.70, 13.54 (7.54 - 13.23)
Carbohydrates	64.61 (0.56) (63.04 - 65.40)	64.29 (0.56) (62.84 - 65.77)	0.32 (0.80) (-2.72 - 2.22)	-1.49, 2.12	0.698	50.57, 81.80 (54.35 - 74.91)
Moisture (% fw)	76.90 (0.28) (76.30 - 77.20)	77.53 (0.28) (77.00 - 78.20)	-0.63 (0.37) (-1.10 - 0.10)	-1.53, 0.28	0.141	65.06, 90.61 (66.10 - 85.30)
Protein	21.08 (0.56) (20.57 - 21.40)	20.94 (0.56) (19.91 - 22.16)	0.14 (0.79) (-1.59 - 1.31)	-1.64, 1.92	0.860	9.26, 33.78 (14.52 - 30.07)
Total Fat	2.35 (0.32) (1.90 - 2.77)	2.31 (0.32) (1.08 - 2.92)	0.043 (0.42) (-0.71 - 1.46)	-0.98, 1.07	0.921	0.73, 3.59 (0.53 - 4.21)
Fiber (% dw)						
Acid Detergent Fiber	27.84 (1.43) (25.19 - 30.13)	28.97 (1.43) (23.70 - 31.42)	-1.14 (1.14) (-4.13 - 1.49)	-3.92, 1.65	0.356	6.16, 49.06 (7.07 - 39.11)

Table E-9 (continued). Statistical Summary of Site KSLA Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Fiber (% dw)						
Acid Detergent Lignin	6.43 (0.35) (5.47 - 7.27)	7.44 (0.35) (6.65 - 8.17)	-1.02 (0.49) (-2.08 - 0.61)	-2.13, 0.097	0.068	2.13, 11.99 (3.38 - 9.67)
Neutral Detergent Fiber	34.37 (0.83) (31.79 - 37.37)	36.31 (0.83) (35.86 - 37.09)	-1.94 (1.17) (-4.22 - 1.08)	-4.59, 0.71	0.132	12.04, 58.18 (18.97 - 49.82)
Amino Acid (% dw)						
Alanine	1.11 (0.032) (1.06 - 1.17)	1.09 (0.032) (1.03 - 1.14)	0.021 (0.045) (-0.083 - 0.070)	-0.082, 0.12	0.654	0.49, 1.79 (0.80 - 1.66)
Arginine	0.98 (0.040) (0.96 - 1.00)	0.99 (0.040) (0.90 - 1.06)	-0.0086 (0.057) (-0.10 - 0.079)	-0.14, 0.12	0.883	0.44, 1.59 (0.70 - 1.44)
Aspartic acid	2.72 (0.081) (2.55 - 3.02)	2.61 (0.081) (2.41 - 2.72)	0.10 (0.11) (-0.14 - 0.31)	-0.16, 0.37	0.369	0.44, 5.63 (1.96 - 5.15)
Cystine	0.21 (0.013) (0.18 - 0.25)	0.21 (0.013) (0.19 - 0.23)	0.00044 (0.018) (-0.041 - 0.062)	-0.040, 0.041	0.981	0.12, 0.32 (0.16 - 0.31)

Table E-9 (continued). Statistical Summary of Site KSLA Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Amino Acid (% dw)						
Glutamic acid	1.89 (0.051) (1.83 - 1.97)	1.87 (0.051) (1.75 - 1.95)	0.020 (0.073) (-0.12 - 0.13)	-0.14, 0.18	0.789	0.81, 3.01 (1.31 - 2.80)
Glycine	0.98 (0.025) (0.97 - 1.02)	0.98 (0.025) (0.93 - 1.04)	0.0034 (0.035) (-0.071 - 0.048)	-0.076, 0.083	0.924	0.49, 1.44 (0.70 - 1.33)
Histidine	0.43 (0.012) (0.41 - 0.45)	0.43 (0.012) (0.41 - 0.45)	-0.0062 (0.017) (-0.022 - 0.0075)	-0.046, 0.033	0.730	0.26, 0.63 (0.34 - 0.61)
Isoleucine	0.87 (0.023) (0.83 - 0.91)	0.86 (0.023) (0.82 - 0.90)	0.0069 (0.033) (-0.040 - 0.056)	-0.068, 0.081	0.839	0.43, 1.36 (0.63 - 1.27)
Leucine	1.45 (0.041) (1.42 - 1.53)	1.45 (0.041) (1.36 - 1.53)	0.0010 (0.057) (-0.10 - 0.085)	-0.13, 0.13	0.986	0.70, 2.25 (1.03 - 2.05)
Lysine	1.12 (0.034) (1.09 - 1.16)	1.15 (0.034) (1.10 - 1.19)	-0.031 (0.048) (-0.062 - -0.0039)	-0.14, 0.077	0.536	0.55, 1.82 (0.82 - 1.73)

Table E-9 (continued). Statistical Summary of Site KSLA Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Amino Acid (% dw)						
Methionine	0.22 (0.020) (0.19 - 0.25)	0.23 (0.020) (0.18 - 0.30)	-0.0029 (0.028) (-0.089 - 0.064)	-0.066, 0.061	0.918	0.068, 0.42 (0.14 - 0.45)
Phenylalanine	1.00 (0.029) (0.97 - 1.04)	0.99 (0.029) (0.93 - 1.05)	0.0049 (0.041) (-0.063 - 0.057)	-0.088, 0.097	0.907	0.48, 1.53 (0.71 - 1.39)
Proline	0.88 (0.024) (0.84 - 0.92)	0.88 (0.024) (0.85 - 0.91)	-0.0029 (0.034) (-0.075 - 0.038)	-0.081, 0.075	0.933	0.43, 1.41 (0.65 - 1.24)
Serine	0.86 (0.021) (0.84 - 0.90)	0.86 (0.021) (0.82 - 0.89)	0.0036 (0.030) (-0.030 - 0.034)	-0.064, 0.071	0.906	0.45, 1.35 (0.66 - 1.25)
Threonine	0.87 (0.024) (0.86 - 0.90)	0.87 (0.024) (0.81 - 0.91)	0.0042 (0.034) (-0.049 - 0.052)	-0.074, 0.082	0.905	0.45, 1.33 (0.63 - 1.23)
Tryptophan	0.38 (0.017) (0.34 - 0.42)	0.38 (0.017) (0.36 - 0.39)	0.00096 (0.024) (-0.050 - 0.065)	-0.054, 0.056	0.969	0.20, 0.56 (0.25 - 0.50)

Table E-9 (continued). Statistical Summary of Site KSLA Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Amino Acid (% dw)						
Tyrosine	0.71 (0.034) (0.68 - 0.73)	0.70 (0.034) (0.63 - 0.77)	0.0084 (0.048) (-0.053 - 0.060)	-0.10, 0.12	0.864	0.35, 1.09 (0.52 - 1.01)
Valine	1.05 (0.027) (1.01 - 1.09)	1.04 (0.027) (0.98 - 1.09)	0.0063 (0.039) (-0.051 - 0.070)	-0.081, 0.094	0.874	0.52, 1.64 (0.79 - 1.55)
Mineral						
Calcium (% dw)	2.04 (0.048) (1.98 - 2.09)	1.95 (0.048) (1.85 - 2.10)	0.093 (0.037) (-0.017 - 0.15)	0.0021, 0.18	0.046	0.55, 2.56 (0.95 - 2.07)
Copper (mg/kg dw)	9.13 (0.52) (8.31 - 9.96)	9.18 (0.52) (8.60 - 9.59)	-0.053 (0.74) (-0.98 - 1.35)	-1.72, 1.61	0.943	1.87, 14.98 (4.54 - 19.67)
Iron (mg/kg dw)	362.07 (58.09) (269.62 - 473.91)	451.45 (58.09) (375.69 - 547.83)	-89.38 (82.16) (-278.21 - 67.61)	-275.23, 96.47	0.304	41.59, 446.31 (105.45 - 691.43)
Magnesium (% dw)	0.26 (0.0067) (0.24 - 0.27)	0.26 (0.0067) (0.25 - 0.27)	-0.0077 (0.0078) (-0.0094 - -0.0063)	-0.027, 0.011	0.359	0.027, 0.41 (0.11 - 0.34)

Table E-9 (continued). Statistical Summary of Site KSLA Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Mineral						
Manganese (mg/kg dw)	50.02 (1.51) (47.68 - 53.48)	49.82 (1.51) (46.79 - 53.15)	0.20 (1.50) (-4.49 - 3.28)	-3.48, 3.88	0.899	17.53, 69.85 (23.24 - 98.04)
Phosphorus (% dw)	0.25 (0.0060) (0.24 - 0.26)	0.25 (0.0060) (0.23 - 0.26)	0.0036 (0.0083) (-0.0061 - 0.018)	-0.017, 0.024	0.682	0.14, 0.46 (0.18 - 0.43)
Potassium (% dw)	2.21 (0.041) (2.16 - 2.28)	2.23 (0.041) (2.18 - 2.29)	-0.019 (0.054) (-0.12 - 0.071)	-0.15, 0.11	0.732	1.82, 3.04 (1.85 - 3.35)
Sodium (% dw)	0.17 (0.019) (0.13 - 0.22)	0.14 (0.019) (0.12 - 0.15)	0.035 (0.026) (0.0023 - 0.083)	-0.028, 0.099	0.223	0, 0.24 (0.016 - 0.20)
Zinc (mg/kg dw)	24.75 (0.88) (23.28 - 27.57)	22.94 (0.88) (21.91 - 24.13)	1.81 (1.18) (-0.85 - 3.92)	-1.08, 4.71	0.176	8.89, 47.44 (17.08 - 47.48)

[1]dw = dry weight; fw = fresh weight
[2]Mean (S.E.) = least-square mean (standard error)
[3]Control refers to the non-biotechnology derived, conventional control (C_0-Syn1).
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties. Negative limits set to zero.

Table E-10. Statistical Summary of Site KSLA Alfalfa Forage Secondary Metabolites for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Metabolite						
Canavanine (ppm dw)	80.13 (7.01) (64.19 - 87.83)	111.94 (7.01) (87.61 - 134.50)	-31.81 (7.01) (-53.36 - -23.42)	-48.97, -14.65	0.003	0, 137.35 (11.47 - 151.33)
Ferulic Acid (ppm dw)	1584.79 (68.95) (1392.41 - 1729.26)	1438.26 (68.95) (1334.86 - 1582.61)	146.54 (97.51) (-190.20 - 394.40)	-74.04, 367.11	0.167	854.88, 2061.10 (1103.32 - 1906.86)
Free Phenylalanine (ppm dw)	225.68 (24.61) (172.15 - 253.48)	252.06 (24.61) (174.67 - 327.98)	-26.39 (34.02) (-101.34 - 75.77)	-109.64, 56.86	0.467	0, 627.23 (133.05 - 579.05)
Total Polyphenols (mg/g dw)	7.25 (0.28) (6.62 - 8.34)	7.27 (0.28) (6.93 - 7.55)	-0.020 (0.40) (-0.93 - 1.41)	-0.93, 0.88	0.960	4.86, 11.15 (6.17 - 11.17)
p-Coumaric Acid (ppm dw)	630.59 (26.98) (544.30 - 716.16)	595.81 (26.98) (545.87 - 626.13)	34.78 (38.15) (-81.78 - 170.29)	-51.53, 121.09	0.385	188.81, 949.95 (326.19 - 945.58)

[1]dw = dry weight.
[2]Mean (S.E.) = least-square mean (standard error)
[3]Control refers to the non-biotechnology derived, conventional control (C_0-Syn1).
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties
Negative limits set to zero.

Table E-11. Statistical Summary of Site TXCL Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Proximate (% dw)						
Ash	12.12 (0.30) (11.33 - 12.93)	12.27 (0.30) (11.53 - 12.95)	-0.15 (0.42) (-1.32 - 1.06)	-1.10, 0.79	0.725	6.70, 13.54 (7.54 - 13.23)
Carbohydrates	65.05 (0.68) (62.89 - 66.54)	64.63 (0.68) (63.47 - 65.69)	0.42 (0.90) (-2.80 - 3.07)	-1.79, 2.62	0.659	50.57, 81.80 (54.35 - 74.91)
Moisture (% fw)	74.18 (0.50) (73.70 - 74.50)	72.60 (0.50) (70.50 - 74.40)	1.58 (0.71) (0.10 - 3.60)	-0.021, 3.17	0.052	65.06, 90.61 (66.10 - 85.30)
Protein	20.42 (0.46) (18.86 - 21.17)	20.58 (0.46) (19.56 - 21.90)	-0.17 (0.65) (-1.55 - 1.61)	-1.63, 1.30	0.801	9.26, 33.78 (14.52 - 30.07)
Total Fat	2.43 (0.29) (1.45 - 2.99)	2.54 (0.29) (1.63 - 3.15)	-0.11 (0.29) (-0.54 - 0.49)	-0.82, 0.60	0.713	0.73, 3.59 (0.53 - 4.21)
Fiber (% dw)						
Acid Detergent Fiber	26.32 (1.02) (24.36 - 29.88)	26.16 (1.02) (24.75 - 27.66)	0.16 (1.32) (-2.63 - 3.36)	-3.06, 3.38	0.907	6.16, 49.06 (7.07 - 39.11)

Table E-11 (continued). Statistical Summary of Site TXCL Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Fiber (% dw)						
Acid Detergent Lignin	7.43 (0.66) (5.86 - 10.31)	6.64 (0.66) (5.88 - 7.28)	0.80 (0.66) (-0.11 - 3.39)	-0.82, 2.41	0.273	2.13, 11.99 (3.38 - 9.67)
Neutral Detergent Fiber	32.47 (1.21) (28.34 - 36.00)	31.92 (1.21) (30.64 - 33.71)	0.55 (0.97) (-2.30 - 2.82)	-1.83, 2.93	0.591	12.04, 58.18 (18.97 - 49.82)
Amino Acid (% dw)						
Alanine	1.22 (0.049) (1.19 - 1.28)	1.22 (0.049) (1.09 - 1.39)	-0.0012 (0.070) (-0.19 - 0.13)	-0.16, 0.16	0.986	0.49, 1.79 (0.80 - 1.66)
Arginine	1.02 (0.036) (0.97 - 1.05)	1.02 (0.036) (0.95 - 1.08)	0.0031 (0.051) (-0.031 - 0.063)	-0.11, 0.12	0.952	0.44, 1.59 (0.70 - 1.44)
Aspartic acid	2.11 (0.060) (1.97 - 2.23)	2.27 (0.060) (2.04 - 2.42)	-0.16 (0.084) (-0.30 - 0.19)	-0.35, 0.031	0.090	0.44, 5.63 (1.96 - 5.15)
Cystine	0.21 (0.0065) (0.19 - 0.22)	0.20 (0.0065) (0.18 - 0.22)	0.0073 (0.0088) (-0.0093 - 0.024)	-0.014, 0.029	0.437	0.12, 0.32 (0.16 - 0.31)

Table E-11 (continued). Statistical Summary of Site TXCL Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Amino Acid (% dw)						
Glutamic acid	1.86 (0.065) (1.80 - 1.92)	1.96 (0.065) (1.83 - 2.06)	-0.10 (0.092) (-0.17 - 0.011)	-0.31, 0.11	0.302	0.81, 3.01 (1.31 - 2.80)
Glycine	1.03 (0.026) (1.00 - 1.05)	1.05 (0.026) (0.98 - 1.10)	-0.021 (0.037) (-0.077 - 0.042)	-0.10, 0.062	0.583	0.49, 1.44 (0.70 - 1.33)
Histidine	0.44 (0.011) (0.43 - 0.44)	0.45 (0.011) (0.43 - 0.48)	-0.012 (0.014) (-0.053 - 0.0095)	-0.047, 0.023	0.421	0.26, 0.63 (0.34 - 0.61)
Isoleucine	0.90 (0.028) (0.87 - 0.95)	0.91 (0.028) (0.84 - 0.97)	-0.0062 (0.039) (-0.070 - 0.051)	-0.095, 0.082	0.877	0.43, 1.36 (0.63 - 1.27)
Leucine	1.51 (0.046) (1.47 - 1.57)	1.53 (0.046) (1.42 - 1.62)	-0.023 (0.065) (-0.11 - 0.057)	-0.17, 0.12	0.734	0.70, 2.25 (1.03 - 2.05)
Lysine	1.19 (0.035) (1.16 - 1.24)	1.20 (0.035) (1.12 - 1.30)	-0.0061 (0.049) (-0.14 - 0.11)	-0.12, 0.11	0.904	0.55, 1.82 (0.82 - 1.73)

Table E-11 (continued). Statistical Summary of Site TXCL Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Amino Acid (% dw)						
Methionine	0.24 (0.034) (0.19 - 0.29)	0.21 (0.034) (0.15 - 0.31)	0.025 (0.049) (-0.12 - 0.14)	-0.085, 0.13	0.623	0.068, 0.42 (0.14 - 0.45)
Phenylalanine	1.04 (0.029) (1.01 - 1.07)	1.06 (0.029) (0.99 - 1.12)	-0.019 (0.041) (-0.057 - 0.034)	-0.11, 0.075	0.663	0.48, 1.53 (0.71 - 1.39)
Proline	0.92 (0.039) (0.88 - 0.94)	1.03 (0.039) (0.93 - 1.21)	-0.11 (0.055) (-0.28 - -0.0051)	-0.23, 0.015	0.078	0.43, 1.41 (0.65 - 1.24)
Serine	0.85 (0.031) (0.80 - 0.89)	0.89 (0.031) (0.83 - 0.93)	-0.037 (0.044) (-0.10 - 0.031)	-0.14, 0.063	0.425	0.45, 1.35 (0.66 - 1.25)
Threonine	0.90 (0.031) (0.87 - 0.93)	0.93 (0.031) (0.86 - 0.99)	-0.031 (0.044) (-0.055 - 0.041)	-0.13, 0.068	0.495	0.45, 1.33 (0.63 - 1.23)
Tryptophan	0.40 (0.016) (0.38 - 0.43)	0.40 (0.016) (0.36 - 0.45)	0.0054 (0.023) (-0.026 - 0.035)	-0.047, 0.058	0.819	0.20, 0.56 (0.25 - 0.50)

Table E-11 (continued). Statistical Summary of Site TXCL Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Amino Acid (% dw)						
Tyrosine	0.75 (0.025) (0.71 - 0.78)	0.71 (0.025) (0.66 - 0.78)	0.040 (0.035) (-0.017 - 0.066)	-0.040, 0.12	0.282	0.35, 1.09 (0.52 - 1.01)
Valine	1.08 (0.033) (1.04 - 1.13)	1.09 (0.033) (1.01 - 1.14)	-0.0079 (0.047) (-0.065 - 0.044)	-0.11, 0.099	0.870	0.52, 1.64 (0.79 - 1.55)
Mineral						
Calcium (% dw)	2.34 (0.091) (2.12 - 2.62)	2.35 (0.091) (2.05 - 2.53)	-0.012 (0.13) (-0.41 - 0.28)	-0.30, 0.28	0.927	0.55, 2.56 (0.95 - 2.07)
Copper (mg/kg dw)	11.09 (0.76) (8.52 - 13.16)	9.67 (0.76) (8.75 - 11.64)	1.42 (1.01) (-0.67 - 4.04)	-1.32, 4.16	0.228	1.87, 14.98 (4.54 - 19.67)
Iron (mg/kg dw)	338.53 (28.04) (221.18 - 417.97)	308.71 (28.04) (270.51 - 356.46)	29.82 (35.02) (-60.07 - 115.21)	-55.86, 115.50	0.427	41.59, 446.31 (105.45 - 691.43)
Magnesium (% dw)	0.24 (0.015) (0.21 - 0.28)	0.25 (0.015) (0.23 - 0.29)	-0.013 (0.018) (-0.048 - 0.037)	-0.056, 0.030	0.486	0.027, 0.41 (0.11 - 0.34)

Table E-11 (continued). Statistical Summary of Site TXCL Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Mineral						
Manganese (mg/kg dw)	61.36 (2.91) (49.41 - 71.48)	59.33 (2.91) (57.63 - 61.25)	2.02 (4.05) (-9.18 - 11.63)	-7.89, 11.94	0.635	17.53, 69.85 (23.24 - 98.04)
Phosphorus (% dw)	0.23 (0.0056) (0.22 - 0.24)	0.21 (0.0056) (0.20 - 0.23)	0.014 (0.0079) (-0.00067 - 0.030)	-0.0040, 0.032	0.113	0.14, 0.46 (0.18 - 0.43)
Potassium (% dw)	2.45 (0.067) (2.29 - 2.65)	2.47 (0.067) (2.37 - 2.54)	-0.017 (0.079) (-0.085 - 0.10)	-0.21, 0.18	0.837	1.82, 3.04 (1.85 - 3.35)
Sodium (% dw)	0.052 (0.010) (0.040 - 0.069)	0.033 (0.010) (0.018 - 0.058)	0.019 (0.014) (-0.013 - 0.049)	-0.013, 0.051	0.217	0, 0.24 (0.016 - 0.20)
Zinc (mg/kg dw)	23.52 (3.82) (19.89 - 27.30)	22.92 (3.82) (20.58 - 25.09)	0.59 (5.40) (-5.21 - 6.72)	-11.62, 12.80	0.915	8.89, 47.44 (17.08 - 47.48)

[1]dw = dry weight; fw = fresh weight
[2]Mean (S.E.) = least-square mean (standard error)
[3]Control refers to the non-biotechnology derived, conventional control (C_0-Syn1).
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties. Negative limits set to zero.

Table E-12. Statistical Summary of Site TXCL Alfalfa Forage Secondary Metabolites for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Metabolite						
Canavanine (ppm dw)	44.54 (7.37) (29.96 - 53.52)	84.96 (7.37) (70.11 - 109.49)	-40.42 (10.42) (-79.53 - -22.46)	-63.99, -16.85	0.003	0, 137.35 (11.47 - 151.33)
Ferulic Acid (ppm dw)	1831.12 (68.79) (1737.25 - 1884.17)	1763.27 (68.79) (1594.89 - 2007.38)	67.86 (76.96) (-148.06 - 248.86)	-120.45, 256.17	0.411	854.88, 2061.10 (1103.32 - 1906.86)
Free Phenylalanine (ppm dw)	290.05 (26.29) (244.11 - 332.05)	346.46 (26.29) (298.89 - 457.63)	-56.41 (20.60) (-125.58 - -6.99)	-106.82, -6.01	0.033	0, 627.23 (133.05 - 579.05)
Total Polyphenols (mg/g dw)	8.61 (0.36) (7.99 - 9.35)	8.22 (0.36) (7.41 - 9.12)	0.39 (0.46) (-1.13 - 1.65)	-0.74, 1.52	0.429	4.86, 11.15 (6.17 - 11.17)
p-Coumaric Acid (ppm dw)	676.49 (34.94) (600.00 - 726.56)	700.57 (34.94) (644.53 - 797.05)	-24.08 (43.71) (-112.64 - 40.43)	-131.03, 82.87	0.601	188.81, 949.95 (326.19 - 945.58)

[1]dw = dry weight
[2]Mean (S.E.) = least-square mean (standard error)
[3]Control refers to the non-biotechnology derived, conventional control (C_0-Syn1).
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties
Negative limits set to zero.

Table E-13. Statistical Summary of Site WIDL Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Proximate (% dw)						
Ash	9.05 (0.27) (8.43 - 10.13)	9.21 (0.27) (8.99 - 9.63)	-0.16 (0.38) (-0.79 - 1.01)	-1.03, 0.71	0.685	6.70, 13.54 (7.54 - 13.23)
Carbohydrates	72.45 (0.51) (70.80 - 73.90)	71.82 (0.51) (70.54 - 72.91)	0.63 (0.73) (-2.11 - 2.08)	-1.01, 2.27	0.408	50.57, 81.80 (54.35 - 74.91)
Moisture (% fw)	75.83 (0.42) (74.80 - 77.40)	75.08 (0.42) (74.20 - 75.90)	0.75 (0.56) (-0.20 - 2.50)	-0.62, 2.12	0.230	65.06, 90.61 (66.10 - 85.30)
Protein	16.75 (0.41) (15.50 - 17.65)	16.97 (0.41) (15.98 - 18.18)	-0.23 (0.58) (-1.51 - 1.68)	-1.53, 1.08	0.705	9.26, 33.78 (14.52 - 30.07)
Total Fat	1.86 (0.18) (1.60 - 2.14)	2.07 (0.18) (2.01 - 2.17)	-0.22 (0.26) (-0.47 - 0.13)	-0.80, 0.36	0.421	0.73, 3.59 (0.53 - 4.21)
Fiber (% dw)						
Acid Detergent Fiber	34.76 (1.41) (31.41 - 37.26)	30.88 (1.41) (27.69 - 36.11)	3.88 (1.99) (-4.71 - 9.57)	-0.63, 8.39	0.083	6.16, 49.06 (7.07 - 39.11)

Table E-13 (continued). Statistical Summary of Site WIDL Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Fiber (% dw)						
Acid Detergent Lignin	7.88 (0.39) (6.83 - 8.58)	7.51 (0.39) (6.15 - 8.26)	0.37 (0.53) (-0.77 - 2.24)	-0.93, 1.66	0.516	2.13, 11.99 (3.38 - 9.67)
Neutral Detergent Fiber	42.99 (1.53) (37.47 - 48.67)	39.31 (1.53) (36.67 - 40.64)	3.68 (1.61) (-1.88 - 8.04)	-0.26, 7.63	0.062	12.04, 58.18 (18.97 - 49.82)
Amino Acid (% dw)						
Alanine	0.87 (0.024) (0.84 - 0.90)	0.93 (0.024) (0.87 - 1.03)	-0.055 (0.033) (-0.19 - 0.014)	-0.13, 0.021	0.133	0.49, 1.79 (0.80 - 1.66)
Arginine	0.76 (0.020) (0.73 - 0.78)	0.82 (0.020) (0.75 - 0.90)	-0.060 (0.029) (-0.17 - 0.014)	-0.12, 0.0046	0.065	0.44, 1.59 (0.70 - 1.44)
Aspartic acid	2.25 (0.070) (2.12 - 2.51)	2.15 (0.070) (2.09 - 2.23)	0.10 (0.099) (-0.035 - 0.28)	-0.13, 0.32	0.341	0.44, 5.63 (1.96 - 5.15)
Cystine	0.17 (0.0084) (0.15 - 0.19)	0.18 (0.0084) (0.15 - 0.20)	-0.0082 (0.010) (-0.030 - 0.017)	-0.034, 0.017	0.461	0.12, 0.32 (0.16 - 0.31)

Table E-13 (continued). Statistical Summary of Site WIDL Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Amino Acid (% dw)						
Glutamic acid	1.47 (0.040) (1.40 - 1.52)	1.54 (0.040) (1.39 - 1.70)	-0.075 (0.057) (-0.30 - 0.065)	-0.20, 0.054	0.222	0.81, 3.01 (1.31 - 2.80)
Glycine	0.77 (0.016) (0.75 - 0.79)	0.78 (0.016) (0.73 - 0.85)	-0.015 (0.023) (-0.099 - 0.022)	-0.067, 0.038	0.545	0.49, 1.44 (0.70 - 1.33)
Histidine	0.36 (0.0056) (0.35 - 0.38)	0.38 (0.0056) (0.36 - 0.40)	-0.013 (0.0068) (-0.033 - 0.00032)	-0.029, 0.0040	0.113	0.26, 0.63 (0.34 - 0.61)
Isoleucine	0.70 (0.018) (0.67 - 0.73)	0.72 (0.018) (0.66 - 0.78)	-0.018 (0.026) (-0.12 - 0.024)	-0.078, 0.041	0.497	0.43, 1.36 (0.63 - 1.27)
Leucine	1.14 (0.031) (1.09 - 1.18)	1.19 (0.031) (1.09 - 1.31)	-0.051 (0.043) (-0.22 - 0.023)	-0.15, 0.047	0.272	0.70, 2.25 (1.03 - 2.05)
Lysine	0.93 (0.021) (0.93 - 0.93)	0.98 (0.021) (0.92 - 1.08)	-0.053 (0.027) (-0.15 - 0.0086)	-0.12, 0.014	0.100	0.55, 1.82 (0.82 - 1.73)

Table E-13 (continued). Statistical Summary of Site WIDL Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Amino Acid (% dw)						
Methionine	0.17 (0.017) (0.15 - 0.21)	0.18 (0.017) (0.15 - 0.22)	-0.015 (0.021) (-0.062 - 0.011)	-0.066, 0.036	0.506	0.068, 0.42 (0.14 - 0.45)
Phenylalanine	0.77 (0.020) (0.75 - 0.80)	0.81 (0.020) (0.74 - 0.89)	-0.038 (0.029) (-0.15 - 0.018)	-0.10, 0.027	0.223	0.48, 1.53 (0.71 - 1.39)
Proline	0.73 (0.015) (0.71 - 0.76)	0.75 (0.015) (0.71 - 0.80)	-0.022 (0.021) (-0.092 - 0.051)	-0.069, 0.024	0.308	0.43, 1.41 (0.65 - 1.24)
Serine	0.73 (0.022) (0.68 - 0.80)	0.75 (0.022) (0.68 - 0.81)	-0.012 (0.032) (-0.13 - 0.055)	-0.083, 0.060	0.721	0.45, 1.35 (0.66 - 1.25)
Threonine	0.69 (0.017) (0.66 - 0.73)	0.72 (0.017) (0.67 - 0.79)	-0.029 (0.024) (-0.13 - 0.019)	-0.083, 0.026	0.266	0.45, 1.33 (0.63 - 1.23)
Tryptophan	0.31 (0.0090) (0.30 - 0.33)	0.29 (0.0090) (0.27 - 0.31)	0.015 (0.013) (-0.011 - 0.062)	-0.014, 0.044	0.264	0.20, 0.56 (0.25 - 0.50)

Table E-13 (continued). Statistical Summary of Site WIDL Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Amino Acid (% dw)						
Tyrosine	0.57 (0.013) (0.55 - 0.58)	0.58 (0.013) (0.53 - 0.63)	-0.013 (0.019) (-0.053 - 0.024)	-0.056, 0.030	0.504	0.35, 1.09 (0.52 - 1.01)
Valine	0.85 (0.026) (0.79 - 0.90)	0.87 (0.026) (0.81 - 0.96)	-0.023 (0.036) (-0.16 - 0.046)	-0.11, 0.059	0.543	0.52, 1.64 (0.79 - 1.55)
Mineral						
Calcium (% dw)	1.14 (0.031) (1.12 - 1.15)	1.22 (0.031) (1.09 - 1.31)	-0.082 (0.044) (-0.17 - 0.035)	-0.18, 0.018	0.096	0.55, 2.56 (0.95 - 2.07)
Copper (mg/kg dw)	5.78 (0.18) (5.14 - 6.33)	5.74 (0.18) (5.42 - 5.93)	0.047 (0.25) (-0.65 - 0.91)	-0.52, 0.61	0.854	1.87, 14.98 (4.54 - 19.67)
Iron (mg/kg dw)	258.80 (29.54) (200.00 - 357.52)	262.86 (29.54) (210.08 - 366.93)	-4.05 (37.93) (-46.89 - 41.91)	-96.85, 88.74	0.918	41.59, 446.31 (105.45 - 691.43)
Magnesium (% dw)	0.23 (0.0061) (0.22 - 0.25)	0.22 (0.0061) (0.21 - 0.24)	0.0043 (0.0086) (-0.025 - 0.022)	-0.015, 0.024	0.627	0.027, 0.41 (0.11 - 0.34)

Table E-13 (continued). Statistical Summary of Site WIDL Alfalfa Forage Nutrients for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Mineral						
Manganese (mg/kg dw)	56.24 (3.32) (49.40 - 65.93)	55.01 (3.32) (44.94 - 62.15)	1.24 (4.65) (-8.50 - 5.20)	-10.13, 12.60	0.799	17.53, 69.85 (23.24 - 98.04)
Phosphorus (% dw)	0.30 (0.0075) (0.28 - 0.32)	0.29 (0.0075) (0.27 - 0.31)	0.011 (0.0098) (-0.025 - 0.035)	-0.013, 0.035	0.287	0.14, 0.46 (0.18 - 0.43)
Potassium (% dw)	2.39 (0.063) (2.30 - 2.44)	2.34 (0.063) (2.23 - 2.56)	0.055 (0.060) (-0.13 - 0.21)	-0.092, 0.20	0.393	1.82, 3.04 (1.85 - 3.35)
Sodium (% dw)	0.021 (0.012) (0.020 - 0.022)	0.020 (0.012) (0.019 - 0.021)	0.00065 (0.017) (-0.00016 - 0.0022)	-0.040, 0.042	0.970	0, 0.24 (0.016 - 0.20)
Zinc (mg/kg dw)	29.54 (0.95) (28.89 - 30.27)	28.13 (0.95) (25.82 - 32.16)	1.42 (1.34) (-2.53 - 4.45)	-1.61, 4.44	0.316	8.89, 47.44 (17.08 - 47.48)

[1]dw = dry weight; fw = fresh weight
[2]Mean (S.E.) = least-square mean (standard error)
[3]Control refers to the non-biotechnology derived, conventional control (C_0-Syn1).
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties. Negative limits set to zero.

Table E-14. Statistical Summary of Site WIDL Alfalfa Forage Secondary Metabolites for KK179 vs. Conventional Control (Conducted at Covance Laboratories)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Metabolite						
Canavanine (ppm dw)	43.03 (3.39) (38.76 - 46.25)	49.63 (3.39) (39.92 - 59.92)	-6.60 (4.79) (-21.16 - 5.32)	-17.44, 4.24	0.201	0, 137.35 (11.47 - 151.33)
Ferulic Acid (ppm dw)	1443.73 (38.85) (1389.38 - 1479.17)	1367.10 (38.85) (1314.74 - 1403.10)	76.63 (54.94) (33.41 - 117.65)	-47.65, 200.92	0.196	854.88, 2061.10 (1103.32 - 1906.86)
Free Phenylalanine (ppm dw)	224.65 (15.68) (190.36 - 267.26)	214.48 (15.68) (155.04 - 246.15)	10.17 (20.04) (-55.79 - 58.45)	-38.87, 59.21	0.629	0, 627.23 (133.05 - 579.05)
Total Polyphenols (mg/g dw)	6.90 (0.43) (6.35 - 7.35)	7.38 (0.43) (6.57 - 8.22)	-0.48 (0.60) (-1.87 - 0.77)	-1.84, 0.88	0.447	4.86, 11.15 (6.17 - 11.17)
p-Coumaric Acid (ppm dw)	526.67 (25.69) (458.33 - 566.37)	483.23 (25.69) (469.64 - 503.88)	43.44 (35.63) (-22.99 - 88.28)	-43.74, 130.63	0.268	188.81, 949.95 (326.19 - 945.58)

[1]dw = dry weight
[2]Mean (S.E.) = least-square mean (standard error)
[3]Control refers to the non-biotechnology derived, conventional control (C_0-Syn1).
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties
Negative limits set to zero.

Table E -15. Statistical Summary of Alfalfa Forage Anti-Nutrients for KK179 vs. Conventional Control (Conducted at Samuel Roberts Noble Foundation)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Saponins (response units/µg)						
			Site CAPR			
Total Bayogenin	8.30 (2.52) (3.00 - 13.97)	4.98 (1.66) (3.58 - 8.16)	3.33 (2.85) (-1.44 - 5.81)	-3.84, 10.49	0.292	0.92, 8.86 (1.46 - 11.28)
Total Hederagenin	4.16 (0.64) (3.01 - 5.80)	3.25 (0.53) (2.14 - 4.59)	0.91 (0.54) (-0.11 - 1.21)	-0.55, 2.37	0.163	0.85, 7.20 (0.90 - 10.31)
Total Medicagenic Acid	38.13 (8.20) (31.41 - 45.08)	26.51 (5.15) (9.43 - 40.45)	11.63 (9.45) (4.64 - 11.57)	-12.14, 35.39	0.269	0, 44.42 (2.04 - 48.33)

Table E-15 (continued). Statistical Summary of Alfalfa Forage Anti-Nutrients for KK179 vs. C_0-Syn1 (Conducted at Samuel Roberts Noble Foundation)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Saponins (response units/µg)						
			Site CAPR			
Total Soyasapogenol B	26.47 (5.31) (15.68 - 40.48)	24.88 (3.97) (17.41 - 31.77)	1.59 (5.27) (-10.25 - 8.72)	-12.28, 15.46	0.775	7.83, 44.92 (9.22 - 43.87)
Total Soyasapogenol E	3.42 (0.77) (1.84 - 5.02)	2.74 (0.49) (2.12 - 3.16)	0.68 (0.90) (-1.30 - 1.87)	-1.56, 2.91	0.481	0, 6.59 (0.91 - 7.53)
Total Zanhic Acid	7.14 (2.29) (2.58 - 12.08)	5.03 (1.52) (3.66 - 8.39)	2.11 (2.56) (-1.45 - 3.69)	-4.36, 8.57	0.446	0.32, 12.06 (1.75 - 13.20)
Total Saponins	87.15 (16.16) (57.51 - 122.44)	67.38 (11.60) (38.88 - 96.50)	19.76 (16.73) (-2.99 - 25.94)	-23.88, 63.40	0.293	21.87, 108.47 (17.38 - 103.19)
			Site IARL			
Total Bayogenin	3.55 (0.41) (3.06 - 4.44)	2.80 (0.57) (2.20 - 3.40)	0.74 (0.70) (-0.034 - 1.11)	-0.92, 2.41	0.326	0.92, 8.86 (1.46 - 11.28)

Table E-15 (continued). Statistical Summary of Alfalfa Forage Anti-Nutrients for KK179 vs. C_0-Syn1 (Conducted at Samuel Roberts Noble Foundation)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Saponins (response units/µg)						
			Site IARL			
Total Hederagenin	2.37 (0.25) (1.83 - 3.09)	1.91 (0.35) (1.58 - 2.23)	0.47 (0.43) (-0.40 - 0.73)	-0.54, 1.48	0.309	0.85, 7.20 (0.90 - 10.31)
Total Medicagenic Acid	18.75 (1.98) (15.66 - 21.15)	20.09 (2.98) (14.90 - 24.08)	-1.33 (3.28) (-8.41 - 2.34)	-9.78, 7.12	0.701	0, 44.42 (2.04 - 48.33)
Total Soyasapogenol B	12.72 (1.53) (9.68 - 18.28)	9.61 (2.16) (7.05 - 12.16)	3.11 (2.65) (-0.61 - 4.31)	-3.16, 9.38	0.278	7.83, 44.92 (9.22 - 43.87)
Total Soyasapogenol E	1.40 (0.12) (1.20 - 1.73)	1.10 (0.17) (0.84 - 1.35)	0.30 (0.21) (-0.15 - 0.38)	-0.19, 0.79	0.187	0, 6.59 (0.91 - 7.53)
Total Zanhic Acid	2.80 (0.41) (2.25 - 4.21)	3.15 (0.59) (2.62 - 3.67)	-0.35 (0.72) (-1.29 - -0.27)	-2.05, 1.35	0.642	0.32, 12.06 (1.75 - 13.20)

Table E-15 (continued). Statistical Summary of Alfalfa Forage Anti-Nutrients for KK179 vs. C_0-Syn1 (Conducted at Samuel Roberts Noble Foundation)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Saponins (response units/µg)						
			Site IARL			
Total Saponins	41.59 (4.25) (36.00 - 52.91)	38.05 (6.01) (29.20 - 46.90)	3.54 (7.36) (-10.90 - 8.59)	-13.86, 20.93	0.645	21.87, 108.47 (17.38 - 103.19)
			Site ILCY			
Total Bayogenin	3.79 (0.55) (2.81 - 4.52)	5.23 (0.55) (2.87 - 6.77)	-1.45 (0.59) (-2.25 - -0.061)	-2.89, -0.0043	0.049	0.92, 8.86 (1.46 - 11.28)
Total Hederagenin	2.59 (0.40) (1.89 - 3.66)	3.10 (0.40) (2.37 - 4.68)	-0.50 (0.48) (-1.82 - 0.83)	-1.68, 0.67	0.333	0.85, 7.20 (0.90 - 10.31)
Total Medicagenic Acid	27.83 (4.68) (19.21 - 36.75)	30.73 (4.68) (19.96 - 51.04)	-2.90 (5.40) (-14.28 - 9.18)	-16.12, 10.31	0.610	0, 44.42 (2.04 - 48.33)
Total Soyasapogenol B	16.69 (2.17) (14.30 - 22.25)	21.58 (2.17) (13.56 - 26.38)	-4.89 (2.34) (-11.46 - 0.75)	-10.61, 0.83	0.081	7.83, 44.92 (9.22 - 43.87)

Table E-15 (continued). Statistical Summary of Alfalfa Forage Anti-Nutrients for KK179 vs. C_0-Syn1 (Conducted at Samuel Roberts Noble Foundation)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Saponins (response units/µg)						
			Site ILCY			
Total Soyasapogenol E	1.94 (0.26) (1.47 - 2.72)	2.58 (0.26) (1.82 - 3.18)	-0.64 (0.23) (-1.12 - -0.28)	-1.21, -0.073	0.032	0, 6.59 (0.91 - 7.53)
Total Zanhic Acid	3.49 (0.42) (2.80 - 3.88)	4.35 (0.42) (2.67 - 5.75)	-0.86 (0.52) (-1.87 - 0.13)	-2.14, 0.42	0.150	0.32, 12.06 (1.75 - 13.20)
Total Saponins	56.32 (7.09) (42.54 - 68.38)	67.57 (7.09) (43.25 - 94.51)	-11.25 (7.52) (-30.05 - 1.39)	-29.65, 7.16	0.185	21.87, 108.47 (17.38 - 103.19)
			Site KSLA			
Total Bayogenin	4.83 (0.49) (3.83 - 6.60)	6.30 (0.49) (5.45 - 6.71)	-1.48 (0.59) (-2.39 - -0.12)	-2.91, -0.039	0.045	0.92, 8.86 (1.46 - 11.28)
Total Hederagenin	2.44 (0.53) (2.09 - 3.13)	4.04 (0.53) (2.97 - 4.62)	-1.59 (0.68) (-2.10 - -0.88)	-3.26, 0.073	0.057	0.85, 7.20 (0.90 - 10.31)

Table E-15 (continued). Statistical Summary of Alfalfa Forage Anti-Nutrients for KK179 vs. C_0-Syn1 (Conducted at Samuel Roberts Noble Foundation)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Saponins (response units/µg)						
			Site KSLA			
Total Medicagenic Acid	12.60 (3.67) (9.73 - 19.65)	18.16 (3.67) (15.48 - 20.96)	-5.57 (5.15) (-8.70 - -1.31)	-18.16, 7.03	0.321	0, 44.42 (2.04 - 48.33)
Total Soyasapogenol B	24.34 (2.47) (18.83 - 28.51)	30.71 (2.47) (23.91 - 34.73)	-6.38 (2.62) (-10.72 - -0.70)	-12.79, 0.037	0.050	7.83, 44.92 (9.22 - 43.87)
Total Soyasapogenol E	4.05 (0.30) (3.30 - 4.90)	4.01 (0.30) (3.43 - 4.31)	0.045 (0.35) (-1.01 - 0.70)	-0.82, 0.91	0.902	0, 6.59 (0.91 - 7.53)
Total Zanhic Acid	3.66 (0.42) (2.60 - 4.67)	5.49 (0.42) (4.88 - 6.55)	-1.83 (0.41) (-2.62 - -0.21)	-2.83, -0.82	0.004	0.32, 12.06 (1.75 - 13.20)
Total Saponins	51.92 (7.16) (40.39 - 67.46)	68.72 (7.16) (62.00 - 76.11)	-16.79 (8.43) (-22.46 - -8.65)	-37.41, 3.83	0.093	21.87, 108.47 (17.38 - 103.19)

Table E-15 (continued). Statistical Summary of Alfalfa Forage Anti-Nutrients for KK179 vs. C_0-Syn1 (Conducted at Samuel Roberts Noble Foundation)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Saponins (response units/µg)						
			Site TXCL			
Total Bayogenin	7.45 (0.76) (5.90 - 8.43)	9.09 (0.76) (7.46 - 11.28)	-1.64 (1.08) (-2.85 - 0.18)	-4.07, 0.79	0.161	0.92, 8.86 (1.46 - 11.28)
Total Hederagenin	3.66 (0.64) (2.51 - 4.92)	4.71 (0.64) (3.28 - 6.85)	-1.05 (0.90) (-3.51 - 0.55)	-3.10, 0.99	0.273	0.85, 7.20 (0.90 - 10.31)
Total Medicagenic Acid	17.77 (3.55) (9.09 - 35.00)	17.59 (3.55) (14.06 - 20.31)	0.19 (5.02) (-7.33 - 15.92)	-11.17, 11.54	0.971	0, 44.42 (2.04 - 48.33)
Total Soyasapogenol B	27.51 (3.31) (21.98 - 31.25)	31.83 (3.31) (25.02 - 41.93)	-4.32 (3.87) (-12.47 - 3.21)	-13.80, 5.15	0.306	7.83, 44.92 (9.22 - 43.87)
Total Soyasapogenol E	3.80 (1.10) (2.42 - 4.49)	5.00 (1.10) (3.29 - 8.89)	-1.20 (1.21) (-4.99 - 1.20)	-4.16, 1.75	0.358	0, 6.59 (0.91 - 7.53)

Table E-15 (continued). Statistical Summary of Alfalfa Forage Anti-Nutrients for KK179 vs. C_0-Syn1 (Conducted at Samuel Roberts Noble Foundation)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Saponins (response units/µg)						
			Site TXCL			
Total Zanhic Acid	6.73 (0.92) (3.08 - 9.33)	6.77 (0.92) (6.16 - 7.89)	-0.037 (1.18) (-3.39 - 2.76)	-2.93, 2.86	0.976	0.32, 12.06 (1.75 - 13.20)
Total Saponins	66.92 (8.31) (44.97 - 91.65)	74.99 (8.31) (59.72 - 95.91)	-8.07 (11.75) (-31.55 - 19.44)	-34.65, 18.51	0.509	21.87, 108.47 (17.38 - 103.19)
			Site WIDL			
Total Bayogenin	4.16 (0.56) (2.54 - 5.52)	5.09 (0.56) (3.70 - 6.54)	-0.93 (0.79) (-2.10 - -0.035)	-2.73, 0.86	0.270	0.92, 8.86 (1.46 - 11.28)
Total Hederagenin	2.79 (0.40) (1.70 - 3.67)	3.42 (0.40) (1.99 - 4.22)	-0.63 (0.56) (-1.42 - 0.29)	-1.90, 0.65	0.294	0.85, 7.20 (0.90 - 10.31)
Total Medicagenic Acid	23.23 (5.18) (14.06 - 34.94)	26.30 (5.18) (15.02 - 42.64)	-3.07 (7.32) (-22.95 - 12.43)	-19.63, 13.49	0.684	0, 44.42 (2.04 - 48.33)

Table E-15 (continued). Statistical Summary of Alfalfa Forage Anti-Nutrients for KK179 vs. C_0-Syn1 (Conducted at Samuel Roberts Noble Foundation)

Analytical Component (Units)[1]	KK179 Mean (S.E.)[2] (Range)	Control[3] Mean (S.E.) (Range)	Difference (Test minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[4] (Range)
Saponins (response units/µg)						
			Site WIDL			
Total Soyasapogenol B	26.38 (2.10) (21.42 - 29.70)	26.31 (2.10) (19.10 - 31.46)	0.073 (2.97) (-1.95 - 2.31)	-6.65, 6.80	0.980	7.83, 44.92 (9.22 - 43.87)
Total Soyasapogenol E	2.40 (0.23) (2.08 - 3.10)	2.80 (0.23) (2.00 - 3.45)	-0.39 (0.31) (-0.73 - 0.081)	-1.15, 0.36	0.249	0, 6.59 (0.91 - 7.53)
Total Zanhic Acid	4.96 (0.81) (2.79 - 6.54)	5.90 (0.81) (3.77 - 8.69)	-0.93 (1.14) (-3.97 - 1.86)	-3.52, 1.65	0.434	0.32, 12.06 (1.75 - 13.20)
Total Saponins	63.92 (7.99) (44.58 - 81.55)	69.81 (7.99) (45.57 - 91.36)	-5.89 (11.29) (-32.96 - 10.64)	-31.43, 19.66	0.614	21.87, 108.47 (17.38 - 103.19)

[1] Response units equals peak area counts
[2]Mean (S.E.) = least-square mean (standard error)
[3]Control refers to the non-biotechnology derived, conventional control, C_0-Syn1.
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties. Negative limits set to zero.

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Appendix F: Materials and Methods for Seed Germination and Dormancy Assessment of KK179

F.1. Materials

Seed germination and dormancy characteristics were assessed on seed from KK179, the conventional control, and four conventional commercial reference varieties produced in a greenhouse at a single location in West Salem, WI in 2010. Both non-scarified and scarified seed were assessed. Uniform, mechanical scarification of the seed was performed using a Forsberg manual seed scarifier.

F.2. Characterization of the Materials

The presence or absence of KK179 in the starting seed from KK179, the conventional control (C_0-Syn1), and the conventional commercial reference varieties was verified by event-specific polymerase chain reaction (PCR) analyses.

F.3. Germination Testing Facility and Experimental Methods

Seed germination and dormancy evaluations were conducted at BioDiagnostics, Inc. in River Falls, WI. The principal investigator was qualified to conduct seed germination and dormancy testing consistent with the standards established by the Association of Official Seed Analysts (AOSA), a seed trade association (AOSA, 2007, 2010a; b).

Seed of KK179, the conventional control, and four conventional commercial reference varieties was tested under three different temperature regimes. Three germination chambers were maintained under dark conditions with one of the following temperature regimes: constant temperature of approximately 10 °C, 20 °C (AOSA recommended temperature for testing alfalfa), or 30 °C. The temperature inside each germination chamber was monitored and recorded every 15 minutes throughout the duration of the study.

Approximately 100 seeds of each treatment of KK179, the conventional control, and the conventional commercial reference varieties were placed on pre-moistened germination towels using a vacuum planting system. Additional pre-moistened germination towels were placed on top of the seed. The bottom edge of the towels was folded to prevent loss of seeds, and the towels were then rolled up in a wax cover. The labeled, rolled germination towels were then placed into an appropriately labeled bucket. Each replication consisted of 12 labeled germination towels, placed into a single, labeled bucket, for a total of four buckets per temperature regime. Buckets were then placed in the appropriate germination chambers.

Each temperature regime constituted a separate experiment. The experiments were established in a two-way factorial design with four replications per temperature regime. The two factors assessed were material (*i.e.*, KK179, conventional control, or a conventional commercial reference variety) and treatment (*i.e.*, scarified or non-scarified).

A description of each germination characteristic evaluated and the timing of evaluations are presented in Table VII-1. Seed placed in the AOSA-recommended temperature regime (*i.e.*, 20 °C) was evaluated according to AOSA standards as normal germinated, abnormal germinated, hard, dead, or firm-swollen (AOSA, 2010a; b). AOSA only provides guidelines (AOSA, 2010a) for testing seed under optimal temperatures, whereas additional temperature regimes were included to test diverse environmental conditions. Therefore, seed placed in temperature regimes of 10 °C and 30 °C were evaluated as germinated, hard, dead, or firm-swollen. Because temperature extremes could affect the development of seedlings, AOSA standards were not applied and no distinction was made between normal or abnormal germinated seed. Therefore, any seedling with a radical of 1 mm or more was classified as germinated.

The calculation of percent seed in each assessment category was based on the actual number of seeds evaluated (*e.g.*, 99 or 101). Across temperature regimes, the total number of seeds evaluated from each germination towel was approximately 100.

Within both AOSA and the additional temperature regimes, hard and firm-swollen seeds remaining at the final evaluation date were subjected to a tetrazolium (Tz) test for evaluation of viability according to AOSA standards (AOSA, 2007). The number of nonviable hard and nonviable firm-swollen seed was added to the number of dead seed counted on both collection dates to determine the total percent dead seed. Total counts for percent viable hard and viable firm-swollen seed were determined from the Tz test.

F.4. Statistical Analysis

Analysis of variance (ANOVA) was conducted using SAS® (SAS, 2008). Within each temperature regime, the experiment was established in a two-way factorial design with four replications. The two factors assessed were material (*i.e.*, KK179, conventional control, or a conventional commercial reference variety) and treatment (*i.e.*, scarified or non-scarified). KK179 was compared to the conventional control for germination characteristics. The seed germination characteristics analyzed included percent of germinated seed, percent of viable hard seed, percent of dead seed, and percent of viable firm-swollen seed. The percent of germinated seed were categorized as either normal germinated or abnormal germinated for the AOSA temperature regime. The level of statistical significance was predetermined to be 5% (α=0.05). KK179 was not statistically compared to the conventional commercial reference varieties, nor were comparisons made across temperature regimes. The minimum and maximum mean values (reference range) were determined from the conventional commercial reference varieties. Results from the analyses are presented in Table VII-2 and Table VII-3.

® SAS is a registered trademark of the SAS Institute, Inc., Cary North Carolina.

Table F-1. Starting Seed of KK179, Control and Conventional Commercial Alfalfa Reference Varieties Used in Dormancy Assessment

Material Name	Genotype / Phenotype	Treatment[1]	Monsanto Lot Number
KK179	Reduced Lignin; Syn1 population	Non-scarified	11287366
C_0-Syn1	Conventional; Syn1 population	Non-scarified	11287367
Producer's Choice PGI 437	Conventional	Non-scarified	11287370
Pioneer 54V54	Conventional	Non-scarified	11287368
Croplan LegenDairy 5.0	Conventional	Non-scarified	11287369
Vernal	Conventional	Non-scarified	11287371
KK179	Reduced Lignin; Syn1 population	Scarified	11287372
C_0-Syn1	Conventional; Syn1 population	Scarified	11287373
Producer's Choice PGI 437	Conventional	Scarified	11287376
Pioneer 54V54	Conventional	Scarified	11287374
Croplan LegenDairy 5.0	Conventional	Scarified	11287375
Vernal	Conventional	Scarified	11287377

[1]Scarification scratches the seed coat to facilitate imbibition of water. Scarification was performed using a Forsberg manual seed scarifier. Each 7 g seed lot was scarified with 25 revolutions of the handle.

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Appendix G: Materials, Methods, and Individual Site Results from Phenotypic, Agronomic, and Environmental Interaction Assessment of KK179 under Field Conditions during Forage Production

G.1. Materials

Agronomic, phenotypic, and environmental interaction characteristics were assessed for KK179, the conventional control, and 12 conventional commercial reference varieties grown under similar agronomic conditions of forage production (Table G-1).

G.2. Characterization of the Materials

The presence or absence of KK179 in the starting seed of KK179, the conventional control (C_0-Syn1), and three of the conventional commercial reference varieties, which were produced in the same production with KK179 and the conventional control, was verified by event-specific PCR analyses.

G.3. Field Sites and Plot Design

Field trials were established in 2010 at 10 sites that provided a range of environmental and agronomic conditions representative of U.S. and Canadian alfalfa growing regions (Table VII-4). The Principal Investigator at each site was familiar with the growth, production, and evaluation of the alfalfa characteristics.

At the nine U.S. sites, seed of KK179, the conventional control, four conventional commercial reference varieties, and one other material were planted in a randomized complete block design with four replications. The planted plot dimensions varied between sites, due to variability in available planting equipment (Table G-2). Each planted plot at CADV consisted of six rows spaced seven inches apart and 10 feet in length. Each planted plot at IABN consisted of five planted rows spaced six inches apart and 19 feet in length. Rows were then thinned to 16 feet in length. Each planted plot at IDNP consisted of five rows spaced six inches apart and 13 feet in length. Each planted plot at PACO consisted of five rows spaced six inches apart and 17 feet in length. Rows were then thinned to 16 feet in length. Each planted plot at WATC and WIWS consisted of five rows spaced six inches apart and 16 feet in length. At these sites, all rows were used for data collection.

Planted plots at ILCL, NYNR, and KSLA consisted of 10 rows. At ILCL, rows were spaced six inches apart, with an additional six inches between planter passes, and 12 feet in length. At KSLA, rows were spaced six inches apart, with an additional 6 inches between planter passes, and 19 feet in length. Row ends were then culled to 18 feet in length. At NYNR, rows were spaced seven inches apart, with approximately an additional 8.5 inches between planter passes, and 12 feet in length. At these sites, rows #1-6 were used for phenotypic and qualitative environmental interactions data collection and rows #7-9 were used for quantitative environmental interactions data collection.

At the Canadian site, CANA, seed of KK179, the conventional control, and five conventional commercial reference varieties were planted in a randomized complete block design with four replications. Each plot at CANA consisted of seven planted rows spaced approximately 6.7 inches and approximately 19.7 feet in length. In 2010, data were collected out of rows # 2-6. In 2011, all rows were used for data collection.

At some sites, the harvested plot area was different from the planted plot area. Harvested plot areas used to calculate forage yield are presented in Table G-2.

At each site, the entire trial area (*i.e.*, all replicates) was surrounded by a border approximately 3-10 feet of a conventional commercial alfalfa variety. Alleys between replicates were planted with approximately 3-8 feet of a conventional commercial alfalfa variety. The purpose of the planted border and alleys was to create a continuous alfalfa stand across the entire trial area to ensure collection of more robust arthropod abundance data within each plot.

G.4. Planting and Field Operations

Planting information, soil description, and cropping history of the trial area are listed in Table G-2. Prior to planting, the Principal Investigator at each site prepared a proper seedbed according to local agronomic practices, including tillage, fertilization and pH adjustment, and pest management. During each growing season, the trial area was scouted for agronomic conditions and pest populations, including pest arthropods, diseases, and weeds. Fertilizer, irrigation, agricultural chemicals and other management treatments were applied as necessary. All maintenance operations were performed uniformly across the entire trial area.

G.5. Study Duration and Management

This study was conducted over two years (Spring 2010 through Spring 2012). Cuttings at each site were managed according to standard local agronomic practices for forage production of alfalfa and were timed to harvest forage at 1-10% bloom throughout both the 2010 and 2011 growing seasons. The number of cuttings per site for each growing season is listed in Table G-3.

Table G-1. Starting Seed for Phenotypic, Agronomic, and Environmental Interaction Assessment during Forage Production

Site	Material Name	Genotype / Phenotype	Monsanto Lot Number
All	C_0-Syn 1	Conventional; Syn 1 population	11265474
All	KK179	Reduced Lignin; Syn 1 population	11265471
CADV	WL 319HQ	Conventional	11265484
CADV	Pioneer 54V09	Conventional	11265480
CADV	Dow/Dairyland Hybriforce 2400	Conventional	11265770
CADV	Producer's Choice PGI 437	Conventional	11265477
CANA	Croplan LegenDairy 5.0	Conventional	11265476
CANA	Pioneer 54V46	Conventional	11265479
CANA	Dow/Dairyland Hybriforce 400	Conventional	11265485
CANA	Pioneer 54H11	Conventional	11265478
CANA	Pioneer 54V09	Conventional	11265480
IABN	Croplan LegenDairy 5.0	Conventional	11265476
IABN	Pioneer 54V54	Conventional	11265475
IABN	Dow/Dairyland Hybriforce 400	Conventional	11265485
IABN	Pioneer 54H11	Conventional	11265478
IDNP	WL 319HQ	Conventional	11265484
IDNP	DKA50-18	Conventional	11265481
IDNP	Dow/Dairyland Hybriforce 2400	Conventional	11265770
IDNP	Producer's Choice PGI 437	Conventional	11265477
ILCL	Croplan LegenDairy 5.0	Conventional	11265476
ILCL	DKA50-18	Conventional	11265481
ILCL	Pioneer 54V54	Conventional	11265475
ILCL	Vernal	Conventional	11265487
KSLA	WL 319HQ	Conventional	11265484
KSLA	Pioneer 54V09	Conventional	11265480
KSLA	Ranger	Conventional	11265488
KSLA	Producer's Choice PGI 437	Conventional	11265477

Table G-1. Starting Seed for Phenotypic, Agronomic, and Environmental Interaction Assessment during Forage Production (continued)

Site	Material Name	Genotype / Phenotype	Monsanto Lot Number
NYNR	WL 319HQ	Conventional	11265484
NYNR	DKA50-18	Conventional	11265481
NYNR	Dow/Dairyland Hybriforce 2400	Conventional	11265770
NYNR	Vernal	Conventional	11265487
PACO	Croplan LegenDairy 5.0	Conventional	11265476
PACO	Pioneer 54V46	Conventional	11265479
PACO	Ranger	Conventional	11265488
PACO	Producer's Choice PGI 437	Conventional	11265477
WATC	WL 319HQ	Conventional	11265484
WATC	Pioneer 54V54	Conventional	11265475
WATC	Ranger	Conventional	11265488
WATC	Pioneer 54H11	Conventional	11265478
WIWS	Croplan LegenDairy 5.0	Conventional	11265476
WIWS	DKA50-18	Conventional	11265481
WIWS	Dow/Dairyland Hybriforce 400	Conventional	11265485
WIWS	Vernal	Conventional	11265487

Note: The study also included one additional experimental material that was outside of the scope of the objectives of this evaluation of KK179.

Table G-2. Field and Planting Information

Site[1]	Harvested plot area (ft^2)	Rows (#/plot)	Planting rate (lb/A)[1]	Soil series	OM[2] (%)	2009 Crop
CADV	35	6	18.0	Clay loam	1.8	Wheat
CANA[3]	54.9 / 76.9	7	9.6	Brant silt loam	1.3	Soybeans
IABN	40	5	18.0	Nicollet loam	4.0	Oats
IDNP	32.5	5	15.0	Power silt loam	1.0	Fallow
ILCL	66	10	19.2	Cisne silt loam	2.7	Fallow
KSLA	99	10	16.4	Loam silt loam	2.6	Wheat/soybeans
NYNR[4]	50.4 / 78.4	10	13.8	Elnora sand	2.5	Wheat
PACO	40	5	20.1	Hagerstown silt loam	1.2	Forage grasses
WATC	40	5	14.6	Touchet silt loam	1.3	Fallow
WIWS	40	5	21.4	Toddville silt loam	3.7	Oats

[1]A broad range of seeding rates is acceptable to produce adequate stands of alfalfa. Stands naturally self-thin over time, primarily due to competition between seedlings, and seedling survival further decreases with increasing seeding rate (Tesar and Marble, 1988). Plants can also compensate for low germination by increasing the number of stems per plant. Stand and yield are ultimately influenced by the number of stems, not the number of plants, per unit area.
[2]OM = organic matter
[3]CANA harvested a smaller area in 2010 and cutting #1 of 2011 due to potential impacts to outer rows from heavy rain immediately after planting. By 2011, it was apparent that the impact of the heavy rain on the stand was minor and the entire plot area was harvested.
[4]NYNR harvested six of 10 rows in 2010 due to a spotty stand. In 2011, the stand had filled in and all rows were harvested.

Table G-3. Study Site Management

	Dates[1] by Site									
Activity	CADV	CANA	IABN	IDNP	ILCL	KSLA	NYNR	PACO	WATC	WIWS
First Year										
Planting	3/29/10	6/2/10	4/20/10	4/26/10	4/19/10	4/21/10	4/23/10	4/12/10	4/10/10	4/20/10
Cutting #1	6/8/10	7/19/10	6/30/10	7/7/10	6/25/10	6/21/10	7/1/10	6/18/10	7/14/10	7/14/10
Cutting #2	7/16/10	8/10/10	7/29/10	8/13/10	8/6/10	7/20/10	8/3/10	7/28/10	8/16/10	8/27/10
Cutting #3	9/3/10	9/23/10	9/8/10	10/1/10	9/20/10	8/19/10	9/22/10	8/31/10	9/15/10	10/22/10
Cutting #4	11/6/10	NA	NA	NA	NA	9/20/10	NA	NA	NA	NA
Second Year										
Cutting #1	4/22/11	6/10/11	6/6/11	6/6/11	5/17/11	5/23/11	6/3/11	5/24/11	5/28/11	6/2/11
Cutting #2	5/27/11	7/8/11	7/7/11	7/12/11	6/20/11	6/22/11	7/6/11	6/24/11	7/1/11	7/6/11
Cutting #3	7/5/11	7/28/11	8/9/11	8/16/11	7/21-22/11[2]	7/22/11	8/18/11	7/27/11	8/5/11	8/5/11
Cutting #4	8/3/11	8/25/11	9/16/11	9/21/11	9/1/11	8/22/11	10/14/11	9/1/11	9/9/11	9/14/11
Cutting #5	9/9/11	9/29/11	NA	NA	NA	9/21/11	NA	10/5/11	10/25/11	NA

NA Indicates activity not applicable for this site.
[1]Month/day/year.
[2]Cutting #3 of 2011 at ILCL occurred over two dates- reps 1 and 2 were cut on 7/21/11 and reps 3 and 4 were cut on 7/22/11.

G.6. Phenotypic Observations

The descriptions of the phenotypic characteristics assessed are listed in Table VII-1. After planting, KK179 was compared to the conventional control for seedling emergence and early season vigor. For each crop growth cycle and cutting of each year, KK179 was compared to the conventional control for lodging, crop growth stage (using Mean Stage by Count method; see Table G-4), forage yield, and regrowth after cutting (except for the final cut of each season). At the end of each growing season, KK179 was compared to the conventional control for fall plant height, as an indicator of fall dormancy, and total forage yield. KK179 was also compared to the conventional control for winter survival, through the assessment of spring vigor, spring stand recovery, and spring stand count.

Phenotypic characteristics were assessed over each complete year. The first year was considered to be from planting in 2010 through the winter survival assessment in the spring of 2011. The second year was considered to be from the first cutting of 2011 through the winter survival assessment in the spring of 2012. For each year, assessments were made within each crop growth cycle (*i.e.*, the period of forage growth between cuttings) and at each cutting.

Table G-4. Definition of Morphological Stages of Development for Individual Alfalfa Stems Used to Calculate Crop Growth Stage

Stage number	Stage name	Stage definition
0	Early vegetative	Stem length ≤ 15 cm; no buds, flowers, or seed pods
1	Mid-vegetative	Stem length 16 to 30 cm; no buds, flowers, or seed pod
2	Late vegetative	Stem length ≥ 31 cm; no buds, flowers, or seed pods
3	Early bud	1 to 2 nodes with buds; no flowers or seed pods
4	Late bud	≥ 3 nodes with buds; no flowers or seed pods
5	Early flower	One node with one open flower (standard open); no seed pods
6	Late flower	≥ 2 nodes with open flowers; no seed pods

Crop growth stage was determined using the Mean Stage by Count (MSC) method (Kalu and Fick 1981). Crop growth stage data were collected at each site during each crop growth cycle, prior to harvest. Approximately 35-45 stems from each plot were non-systematically selected and ranked by growth stage. The MSC was calculated for each plot using the following formula: MSC = [Σ(stage number * number of stems in stage)]/total number of stems evaluated.

G.7. Environmental Observations

Environmental interactions (*i.e.*, interactions between the crop plants and their receiving environment) were used to characterize KK179 by evaluating plant response to abiotic stressors, disease damage, and arthropod-related damage using qualitative methods described in section G.8. In addition, specific arthropod damage and pest- and beneficial-arthropod abundance were evaluated using the quantitative methods described in Section G.9.

G.8. Plant Response to Abiotic Stress, Disease Damage, and Arthropod-Related Damage

KK179 and the conventional control were evaluated at all sites for plant response to abiotic stressors, disease damage, and arthropod damage. Three abiotic stressors, three diseases, and three arthropod pests were evaluated three to four times throughout the first growing season (2010) and four to five times throughout the second growing season (2011). The timing of each observation ranged from 10 days before the cut to the day of the cut.

The Principal Investigator at each site chose abiotic stressors, diseases, and arthropod pests that were either actively causing plant injury in the study area or were likely to occur during a given observation period. The type of abiotic stressors, diseases, and arthropod pests assessed vary between observation times at a site, between sites, and between years.

Abiotic stressors, disease damage, and arthropod damage data were collected from all rows (or rows #1-6 at sites with arthropod collections) of each plot using a continuous 0- 9 scale of increasing severity. Data were collected numerically using the rating scale below. Due to the non-specific nature of the scale used, the data were placed into one of the following qualitative categories of plant damage severity: none (0), slight (1-3), moderate (4-6), or severe (7-9) and then expressed as a range of responses observed across the four replications at a site.

Rating	Severity of plant damage
0	none (no symptoms observed)
1 – 3	slight (symptoms not damaging to plant development)
4 – 6	moderate (intermediate between slight and severe)
7 – 9	severe (symptoms damaging to plant development)

For each stressor evaluated, the range of responses for KK179 was then compared to the range of responses for the conventional control. If the range of injury symptoms across all four replications overlapped between KK179 and the conventional control, the KK179 and the conventional control were considered not different in plant response. The stressors evaluated, the number of times KK179 and the conventional control were compared for each stressor, and the number of times those comparisons showed differences are reported.

G.9. Specific Arthropod Damage and Arthropod Abundance

Alfalfa weevil and potato leafhopper damage were assessed quantitatively at three sites, ILCL, KSLA, and NYNR, three to four times throughout the first growing season (2010) and four to five times throughout the second growing season (2011). Alfalfa weevil damage was assessed at each site one to nine days before each cutting. At each assessment time, 10 non-systematically selected 6 × 6 inch areas within rows #8 and 9 of each plot were chosen for examination. The following rating scale was used for each evaluation of alfalfa weevil damage:

Rating	Severity of plant damage
0	No damage
1	< 50% foliage have pin holes (usually terminal leaves)
2	51-90 % foliage have pinholes with minor feeding damage between the veins
3	< 50 % foliage with major feeding damage between the veins
4	51-90% foliage with skeletonized appearance
5	>90 % foliage with skeletonized appearance

For potato leafhopper damage, 10 non-systematically selected 6 × 6 inch areas within rows #8 and 9 of each plot were chosen for examination from one to nine days before each cutting. The following rating scale was used for each evaluation of potato leafhopper damage:

Rating	Severity of plant damage
0	No damage
1	< 50% foliage with yellowing
2	51-90% foliage with yellowing
3	< 50% foliage with yellowing and puckering
4	51-90% foliage with yellowing and puckering (stunting of plant is evident)
5	>90% foliage with yellowing and puckering (severe stunting of plant)

Pest- and beneficial-arthropods were collected at ILCL, KSLA, and NYNR zero to 10 days prior to each cutting, which occurred three to four times during the first growing season (2010) and four to five times during the second growing season (2011). Arthropods were collected using a vertical beat sheet sampling method (Drees and Rice, 1985). The beat sheet was approximately a 36 × 36 inch sheet constructed of a stiff material with a collecting trough at the bottom. A total of six sub-samples were collected from rows #7-9 from each plot. The sheet was placed between rows #6 and 7, and the collecting trough was positioned near the base of the plants in row #7. Plants were shaken vigorously along the length of the beat sheet to dislodge arthropods from the plants. Another sub-sample was collected from the same row, approximately two feet from the first sub-sample. Two sub-samples were collected from each rows #8 and 9 in

the same manner. The six sub-samples were combined into one pre-labeled container and placed on freezer ice packs. The samples were first sent to Monsanto Company, St. Louis, MO and then sent to Kansas State University, Manhattan, KS for arthropod identification and enumeration.

From each collection in each year, a maximum of five pest- and five beneficial-arthropods were enumerated. For each individual collection (*e.g.*, collection #1 at KSLA), four non-systematically selected samples were examined to determine presence and relative abundance of up to five pest- and beneficial-arthropods to be enumerated for that particular collection and site. Thus, the suite of pest- and beneficial-arthropods assessed often varied between collections from a site and between sites due to differences in temporal activity and geographical distribution of arthropod taxa.

G.10. Data Assessment

Experienced scientists familiar with the experimental design and evaluation criteria were involved in all components of data collection, summarization, and analysis. Study personnel assessed that measurements were taken properly, data were consistent with expectations based on experience with the crop, and the experiment was carefully monitored. Prior to analysis, the overall dataset was evaluated for evidence of biologically relevant changes and for possible evidence of an unexpected plant response. Any unexpected observations or issues that would impact the objectives were noted. Data were then subjected to statistical analysis.

G.11. Environmental Interactions Evaluation Criteria for Qualitative Data

The qualitative environmental interactions data, including plant responses to abiotic stressors, disease damage, and arthropod damage were categorical and, therefore, were not subjected to statistical analysis. KK179 and the conventional control were considered different in plant responses if the range of injury symptoms across all four replications did not overlap between KK179 and the conventional control. Any observed differences between KK179 and the conventional control were assessed for biological significance in the context of the range of the conventional commercial reference varieties at a site and for consistency with other observation times and sites. Differences that were not consistently observed at other observation times and sites were not considered to be biologically meaningful in terms of plant pest potential or an effect on the environment.

G.12. Statistical Analyses

G.12.1 Statistical Analysis of Agronomic and Phenotypic Data

An analysis of variance was conducted according to a randomized complete block design using SAS® to compare KK179 and the conventional control for the phenotypic characteristics listed in Table VII-1. For each year, comparisons of KK179 and the conventional control were conducted within sites (individual-site analysis) and in a combined-site analysis in which the data were pooled among the sites. KK179 was also compared to the conventional control for a subset of phenotypic characteristics in a combined-year analysis in which data were pooled across years. The level of statistical

significance was predetermined to be 5% (α=0.05). KK179 and the conventional control were not statistically compared to the conventional commercial reference materials. Minimum and maximum mean values were determined for each characteristic from the 12 unique conventional commercial reference varieties that were included at all sites.

G.12.2 Statistical Analysis for Specific Arthropod Damage

An analysis of variance was conducted according to a randomized complete block design using SAS® to compare KK179 and the conventional control for potato leafhopper damage and alfalfa weevil damage assessed at three sites (ILCL, KSLA, and NYNR). For each year, comparisons of KK179 and the conventional control were conducted within sites (individual-site analysis). Since specific arthropod damage data were collected for the same two arthropods at the three sites, a combined-site analysis in which the data were pooled among the sites was also conducted for each year. The level of statistical significance was predetermined to be 5% (α=0.05). KK179 and the conventional control were not statistically compared to the conventional commercial reference varieties. Minimum and maximum mean values were determined for each characteristic from the nine unique conventional commercial reference varieties that were included at the three sites.

G.12.3 Statistical Analysis for Arthropod Abundance

An analysis of variance was conducted according to a randomized complete block design using SAS® to compare KK179 and the conventional control for pest- and beneficial-arthropod abundance assessed at three sites (ILCL, KSLA, and NYNR). Variations in temporal activity and geographical distribution of arthropod taxa occur between sites. Therefore, comparisons of KK179 and the conventional control were conducted only within sites (individual-site analysis) for both years. The level of statistical significance was predetermined to be 5% (α=0.05). KK179 and the conventional control were not statistically compared to the conventional commercial reference materials. Minimum and maximum mean values were determined for arthropod abundance from the conventional commercial reference varieties at each site and collection time.

Data excluded from analysis for 2010 through 2012 and the reasons for exclusion are listed in Table G-5.

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Table G-5. Data Missing or Excluded from Analysis

Site	Plots	Entry type	Data	Reason
First Year				
All	All	All	Forage yield #1	No yield data were collected from the first cutting at each site due to variability in establishment year.
CADV, IABN, IDNP, WATC, WIWS, CADV	All	All	Forage quality sample weights	Not all sites weighed the quality sample that was cut prior to harvest; therefore, this weight was excluded from the plot weight of forage yield per cut.
CANA, IABN, IDNP, ILCL, NYNR, PACO, WATC	All	All	Regrowth after cutting #3	Rating was not required following the last harvest of the season.
CADV, IDNP, WATC	All	All	Fall plant height-Yr 1: 1st rating	Fall plant height data were collected twice at western sites, as is regionally appropriate. Plants had not yet started going dormant at the first rating.
CADV	All	All	Percent stand	Data collected by PI were not requested in the protocol.
CADV	All	All	Regrowth after cutting #1 and #4	Regrowth after cutting #1 was not collected at the appropriate time and regrowth after cutting #4 was not required following the last harvest of the season.

Table G-5 (continued). Data Missing or Excluded from Analysis

Site	Plots	Entry type	Data	Reason
CADV	107	Control	Forage yield #4	The forage weight for plot 107 was lost due to a harvester malfunction.
CANA	All	All	Spring stand count- Yr 2	Plants per foot were counted instead of stems per foot, as requested.
IABN	All	All	Abiotic Stressor Observation #1, rating for stressor #3	Plots were evaluated for winter injury when plants had yet to be exposed to winter.
IABN	All	All	Abiotic Stressor, Disease Damage, and Arthropod Damage Observation #3	Ratings were not made at the appropriate time as specified in the protocol.
KSLA	305	Reference	Arthropod collection #1	Collection jar broke and the sample was lost.
NYNR	All	All	Spring stand rating and spring vigor rating- Yr 2	Data reporting was inconsistent.
NYNR	102	Reference	Spring stand count- subsample 2	Entry on paper was not readable.
PACO	All	All	Mean stage by count #2	Data were not collected at this site.
PACO	All	All	Disease Damage Observation #1	PI did not complete evaluation or follow protocol instructions.
WATC	All	All	2nd Seedling emergence rating; Early season vigor; Mean stage by count #1; Lodging #1	Seedling emergence was collected twice and the second rating was unnecessary. Timing of data collection for Early season vigor and Mean stage by count #1 did not follow the protocol and Lodging #1data were not collected.
WATC	101, 102, 103	References, additional material	All	Frost event in April 2010 killed most plants in these plots.

Table G-5 (continued). Data Missing or Excluded from Analysis

Site	Plots	Entry type	Data	Reason
WIWS	All	All	Lodging #2	Heavy rains immediately preceding the rating caused forage in all plots to be weighed down. The rating was therefore not an accurate assessment of lodging.
All	All	All	All mean stage by count evaluations	Any plots at any site for which fewer than 35 stems were evaluated per plot.
Second Year				
CADV, CANA, KSLA, PACO	All	All	Regrowth after cutting #5	Rating was not required following the last harvest of the season.
IABN, IDNP, ILCL, NYNR, WIWS	All	All	Regrowth after cutting #4	Rating was not required following the last harvest of the season.
CADV	All	All	Forage yield #2[1]	Plot weight data could not be reconstructed. Additionally, the data collector is no longer employed by FGI and is thus unavailable to provide clarification.
CADV	201	Control	Forage yield #1 and #3[1]	There was gopher damage to a significant number of plants in the plot.
CADV	207	Reference	Forage yield #5[1]	Plot weight was not collected due to an error in harvesting equipment operation.
CADV	All	All	Forage yield #6	Plot weight data were collected that were not requested.
CADV	301, 302, 304, 305, 305, 306	All	Arthropod Damage Observation #4	Entries on paper were not readable.
CADV	All	All	Abiotic Stressor, Disease Damage, and Arthropod Damage Observation #2	PI did not complete data evaluation or follow protocol instructions.

Table G-5 (continued). Data Missing or Excluded from Analysis

Site	Plots	Entry type	Data	Reason
CADV	301	Test	Spring vigor, Spring stand recovery, and Spring stand count	There was significant gopher damage to the plot.
CANA	All	All	Fall plant height Yr 2	Data were not collected at the appropriate time.
CANA	101	Test	Arthropod Damage Observation #5, stressor 1 and 2	Raw data were not available.
IDNP	307	Reference	Mean stage by count #4	Raw data were not available.
KSLA	All	All	Mean stage by count #5	The data were lost due to an electronic notebook malfunction.
KSLA	403, 407	Control, reference	Fall plant height-Yr 2, plant number 9	An extra plant was measured.
KSLA	203, 304, 405	References, additional material	Arthropod collection #3	Collection samples were of poor quality and could not be enumerated.
NYNR	All	All	Mean stage by count # 1-4	PI did not take representative stem samples.
NYNR	All	All	Fall plant height-Yr 2	Data were not collected at the appropriate time.
PACO	All	All	Lodging #1	Data were not collected at the appropriate time.
PACO	All	All	Mean stage by count # 1 and 2	Data were not collected at this site.
PACO	303	Test	Forage yield #3[1]	There was groundhog damage to a significant number of plants in the plot.
PACO	All	All	Fall plant height- Yr 2, plant numbers 6-10	The protocol only required five plants to be measured.

Table G-5 (continued). Data Missing or Excluded from Analysis

Site	Plots	Entry type	Data	Reason
PACO	All	All	Abiotic Stressor, Disease Damage, and Arthropod Damage Observation #1	PI did not complete data evaluation or follow protocol instructions.
WATC	All	All	Mean stage by count #2	Insect (Lygus) stripping of buds interfered with crop growth stage evaluation.
WATC	402	Reference	Forage yield #3[1]	Gopher mound caused excessive soil to enter sample, affecting plot weight.
All	All	All	All mean stage by count evaluations	Any plots at any site for which fewer than 35 stems were evaluated per plot.

[1]Forage yield in the second year was determined by adding a plot weight with the sample weight. For all instances of excluded plot weights, the corresponding sample weight was also excluded.

G.13. Individual Field Site Plant Growth and Development Results and Discussion

G.13.1. Individual Field Site Plant Growth and Development Results and Discussion- First Year (2010-2011)

In the individual-site analysis for the first year, 12 statistically significant differences were detected between KK179 and the conventional control (Table G-6). No statistical comparisons could be made in some instances due to lack of variability in the data.

At the beginning of the 2010 season, one statistically significant difference was detected between KK179 and the conventional control for seedling emergence. Seedling emergence was lower for KK179 than the conventional control at ILCL (20.0 vs. 25.5 plants/ft).

At cutting #1, three statistically significant differences were detected between KK179 and the conventional control. KK179 had a lower lodging rating than the conventional control at ILCL (8.5 vs. 9.0 rating). KK179 was at a later growth stage than the conventional control at IABN (2.7 vs. 2.3). KK179 showed greater regrowth after cutting than the conventional control at WATC (9.3 vs. 8.0 rating).

At cutting #2, three statistically significant differences were detected between KK179 and the conventional control. KK179 had a lower lodging rating than the conventional control at IABN (7.8 vs. 8.5 rating). KK179 was at a slightly earlier growth stage than the conventional control at NYNR (3.58 vs. 3.64; means are rounded in Table G-6). KK179 had significantly greater forage yield than the conventional control at WATC (11.0 vs. 8.2 fresh weight ton/acre).

At cutting #3, two statistically significant differences were detected between KK179 and the conventional control. KK179 was at a later growth stage than the conventional control at WATC (3.0 vs. 2.8) and was at an earlier growth stage than the conventional control at WIWS (0.7 vs. 1.1).

At cutting #4, one statistically significant difference was detected between KK179 and the conventional control. KK179 was at an earlier growth stage than the conventional control at KSLA (2.0 vs. 2.1).

At the end of the 2010 season, one statistically significant difference was detected between KK179 and the conventional control for total forage yield, which is the sum of all cuttings over the season. KK179 had significantly greater total forage yield than the conventional control at WATC (18.1 vs. 14.9 fresh weight ton/acre).

In the assessment of winter survival conducted in spring 2011, one statistically significant difference was detected between KK179 and the conventional control. KK179 had lower spring vigor than the conventional control at WATC (8.5 vs. 10.0 rating).

None of the statistical differences detected in the individual-site analysis were detected in the combined-site analysis, suggesting these differences were not indicative of a consistent plant response associated with the trait and were not considered biologically

meaningful in terms of plant pest/weed potential of KK179 compared to the conventional control (Figure VII-1, Step 2, answer "no").

G.13.2. Individual Field Site Plant Growth and Development Results and Discussion- Second Year (2011-2012)

In the individual-site analysis for the second year, 17 statistically significant differences were detected between KK179 and the conventional control (Table G-7). No statistical comparisons could be made in some instances due to lack of variability in the data.

At cutting #1, two statistically significant differences were detected between KK179 and the conventional control. KK179 was at an earlier growth stage than the conventional control at ILCL (2.9 vs. 3.2). KK179 showed reduced regrowth after cutting than the conventional control at WATC (9.0 vs. 10.0 rating).

At cutting #2, three statistically significant differences were detected between KK179 and the conventional control. KK179 had a higher lodging rating than the conventional control at IABN (5.8 vs. 4.3 rating). KK179 was at an earlier growth stage than the conventional control at CADV (2.0 vs. 2.5) and at KSLA (3.6 vs. 4.2).

At cutting #3, four statistically significant differences were detected between KK179 and the conventional control. KK179 was at an earlier growth stage than the conventional control at WIWS (3.2 vs. 3.7). KK179 had a greater forage yield for cutting #3 than the conventional control at PACO (9.1 vs. 8.4 fresh weight ton/acre). KK179 showed reduced regrowth after cutting than the conventional control at IABN (7.0 vs. 8.3 rating) and greater regrowth after cutting than the conventional control at PACO (10.0 vs. 9.5 rating).

At cutting #4, one statistically significant difference was detected between KK179 and the conventional control. KK179 had a lower forage yield for cutting #4 than the conventional control at IABN (5.4 vs. 6.0 fresh weight ton/acre).

At cutting #5, one statistically significant difference was detected between KK179 and the conventional control. KK179 had a lower forage yield for cutting #5 than the conventional control at WATC (4.8 vs. 5.4 fresh weight ton/acre).

At the end of the 2011 season, one statistically significant difference was detected between KK179 and the conventional control. Plants of KK179 were shorter than the conventional control at WATC (8.8 vs. 9.7 in).

In the assessment of winter survival conducted in spring 2012, five significant differences were detected between KK179 and the conventional control. KK179 had greater spring vigor than the conventional control at PACO (6.5 vs. 5.5 rating) and lower spring vigor than the conventional control at WATC (9.3 vs. 10.0 rating). KK179 had a reduced spring stand recovery than the conventional control at IABN (7.8 vs. 9.5 rating), showing greater gaps in the stand across the plot. KK179 had a higher spring stand count than the conventional control at IDNP (54.8 vs. 33.8 stems/ft) and a lower spring stand count than the conventional control at NYNR (59.3 vs. 69.7 stems/ft).

None of the statistical differences detected in the individual-site analysis were detected in the combined-site analysis, suggesting these differences were not indicative of a consistent plant response associated with the trait and were not considered biologically meaningful in terms of plant pest/weed potential of KK179 compared to the conventional control (Figure VII-1, Step 2, answer “no”).

Table G-6. Individual-Site Phenotypic Comparison of KK179 to the Conventional Control in the First Year (2010-2011) of Forage Production

Site	Seedling emergence (# plants/foot)		Early season vigor (1-10 rating)		Cutting #1					
					Lodging (0-9 rating)		Crop growth stage (stage number 0-6)		Regrowth after cutting[1] (1-10 rating)	
	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)
CADV	5.4 (0.63)	7.3 (1.71)	† 1.0 (0.00)	1.0 (0.00)	† 9.0 (0.00)	9.0 (0.00)	2.6 (0.08)	2.4 (0.04)	–	–
CANA	28.8 (3.88)	24.0 (1.83)	5.3 (0.25)	6.0 (0.41)	† 9.0 (0.00)	9.0 (0.00)	1.8 (0.21)	1.4 (0.13)	9.0 (0.00)	9.0 (0.00)
IABN	24.7 (1.60)	21.5 (2.93)	5.8 (0.48)	6.3 (0.48)	9.0 (0.00)	8.5 (0.50)	* 2.7 (0.04)	2.3 (0.12)	7.5 (0.50)	7.3 (0.48)
IDNP	13.4 (2.20)	10.4 (2.50)	4.8 (0.48)	4.5 (0.65)	8.5 (0.50)	8.5 (0.50)	3.2 (0.06)	3.3 (0.11)	8.8 (0.25)	8.8 (0.75)
ILCL	* 20.0 (0.53)	25.5 (0.59)	5.3 (0.48)	5.8 (0.25)	* 8.5 (0.29)	9.0 (0.00)	2.0 (0.27)	1.9 (0.21)	9.0 (0.58)	8.0 (0.00)
KSLA	12.5 (0.22)	12.3 (0.49)	† 5.0 (0.00)	5.0 (0.00)	† 9.0 (0.00)	9.0 (0.00)	3.7 (0.05)	3.5 (0.03)	9.3 (0.48)	8.5 (0.65)
NYNR	15.8 (0.37)	12.5 (3.66)	3.0 (0.71)	4.3 (2.02)	† 9.0 (0.00)	9.0 (0.00)	3.4 (0.02)	3.5 (0.03)	† 10.0 (0.00)	10.0 (0.00)
PACO	28.0 (3.62)	30.7 (3.36)	5.0 (0.00)	4.8 (0.25)	† 9.0 (0.00)	9.0 (0.00)	2.4 (0.16)	2.1 (0.17)	10.0 (0.00)	9.3 (0.48)
WATC	12.9 (1.82)	13.5 (2.33)	–	–	–	–	–	–	* 9.3 (0.48)	8.0 (0.41)
WIWS	33.0 (4.40)	26.8 (2.66)	8.3 (0.25)	8.3 (0.48)	5.8 (0.75)	4.8 (0.48)	3.5 (0.04)	3.9 (0.11)	9.3 (0.48)	9.3 (0.48)

Table G-6 (continued). Individual-Site Phenotypic Comparison of KK179 to the Conventional Control in the First Year (2010-2011) of Forage Production

Site	Cutting #2							
	Lodging (0-9 rating)		Crop growth stage (stage number 0-6)		Forage yield (fresh weight ton/acre)		Regrowth after cutting[1] (1-10 rating)	
	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)
CADV	† 9.0 (0.00)	9.0 (0.00)	3.3 (0.51)	3.3 (0.35)	6.6 (0.66)	6.9 (0.14)	8.8 (0.25)	9.0 (0.00)
CANA	† 9.0 (0.00)	9.0 (0.00)	1.2 (0.11)	1.1 (0.05)	1.1 (0.26)	1.2 (0.10)	† 8.0 (0.00)	8.0 (0.00)
IABN	* 7.8 (0.48)	8.5 (0.50)	2.5 (0.12)	2.4 (0.15)	5.9 (0.51)	5.2 (0.45)	7.3 (0.85)	7.3 (1.03)
IDNP	7.5 (0.65)	7.3 (1.44)	3.4 (0.28)	3.2 (0.36)	10.2 (0.81)	10.5 (0.86)	8.3 (0.48)	8.3 (0.63)
ILCL	8.5 (0.50)	9.0 (0.00)	3.2 (0.25)	3.2 (0.26)	5.3 (0.49)	4.7 (0.28)	7.3 (1.31)	6.8 (0.85)
KSLA	† 9.0 (0.00)	9.0 (0.00)	3.6 (0.03)	3.5 (0.04)	7.5 (0.25)	8.0 (0.36)	10.0 (0.00)	9.8 (0.25)
NYNR	† 9.0 (0.00)	9.0 (0.00)	* 3.6 (0.01)	3.6 (0.02)	5.6 (0.41)	5.5 (0.44)	† 10.0 (0.00)	10.0 (0.00)
PACO	6.0 (0.00)	6.3 (0.25)	–	–	8.9 (0.37)	8.2 (0.49)	9.8 (0.25)	9.0 (0.41)
WATC	8.3 (0.25)	8.0 (0.00)	3.9 (0.10)	3.9 (0.08)	* 11.0 (1.10)	8.2 (1.03)	8.8 (0.25)	9.0 (0.41)
WIWS	–	–	3.0 (0.17)	3.1 (0.06)	8.1 (0.38)	8.0 (0.26)	9.5 (0.29)	10.0 (0.00)

Table G-6 (continued). Individual-Site Phenotypic Comparison of KK179 to the Conventional Control in the First Year (2010-2011) of Forage Production

Site	Cutting #3							
	Lodging (0-9 rating)		Crop growth stage (stage number 0-6)		Forage yield (fresh weight ton/acre)		Regrowth after cutting[1] (1-10 rating)	
	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)
CADV	8.0 (0.58)	7.8 (0.48)	2.1 (0.12)	2.2 (0.05)	4.7 (0.23)	4.9 (0.38)	7.0 (0.41)	7.8 (0.25)
CANA	† 9.0 (0.00)	9.0 (0.00)	1.2 (0.39)	1.9 (0.12)	1.4 (0.28)	1.7 (0.37)	–	–
IABN	8.3 (0.48)	8.5 (0.29)	1.7 (0.27)	1.6 (0.25)	3.7 (0.76)	3.2 (0.70)	–	–
IDNP	8.5 (0.50)	7.8 (0.48)	2.0 (0.08)	1.7 (0.19)	7.9 (0.18)	8.5 (0.38)	–	–
ILCL	† 9.0 (0.00)	9.0 (0.00)	2.2 (0.12)	2.2 (0.06)	2.9 (0.21)	2.8 (0.23)	–	–
KSLA	† 9.0 (0.00)	9.0 (0.00)	3.5 (0.03)	3.5 (0.04)	8.7 (0.14)	9.1 (0.64)	9.0 (0.00)	8.5 (0.29)
NYNR	† 9.0 (0.00)	9.0 (0.00)	3.4 (0.02)	3.5 (0.03)	4.5 (0.15)	4.3 (0.20)	–	–
PACO	† 9.0 (0.00)	9.0 (0.00)	1.5 (0.09)	1.5 (0.07)	3.6 (0.19)	3.6 (0.18)	–	–
WATC	8.5 (0.29)	8.0 (0.00)	* 3.0 (0.07)	2.8 (0.15)	7.1 (0.33)	6.7 (0.56)	–	–
WIWS	† 9.0 (0.00)	9.0 (0.00)	* 0.7 (0.10)	1.1 (0.08)	2.7 (0.26)	2.8 (0.12)	–	–

Table G-6 (continued). Individual-Site Phenotypic Comparison of KK179 to the Conventional Control in the First Year (2010-2011) of Forage Production

	Cutting #4							
	Lodging (0-9 rating)		Crop growth stage (stage number 0-6)		Forage yield (fresh weight ton/acre)		Fall plant height (in)	
Site	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)
CADV	5.8 (0.63)	5.0 (0.41)	2.2 (0.04)	2.3 (0.02)	4.3 (0.37)	3.1 (0.85)	1.8 (0.13)	1.8 (0.12)
CANA	NA	NA	NA	NA	NA	NA	3.1 (0.15)	3.1 (0.18)
IABN	NA	NA	NA	NA	NA	NA	7.5 (0.47)	7.6 (0.56)
IDNP	NA	NA	NA	NA	NA	NA	4.1 (0.25)	4.3 (0.30)
ILCL	NA	NA	NA	NA	NA	NA	4.1 (0.33)	4.1 (0.38)
KSLA	† 9.0 (0.00)	9.0 (0.00)	* 2.0 (0.02)	2.1 (0.03)	4.0 (0.22)	3.6 (0.26)	4.1 (0.24)	4.2 (0.39)
NYNR	NA	NA	NA	NA	NA	NA	4.2 (0.34)	4.1 (0.63)
PACO	NA	NA	NA	NA	NA	NA	9.8 (0.85)	10.2 (0.75)
WATC	NA	NA	NA	NA	NA	NA	7.5 (0.29)	8.3 (0.28)
WIWS	NA	NA	NA	NA	NA	NA	8.8 (0.35)	8.5 (0.41)

Table G-6 (continued). Individual-Site Phenotypic Comparison of KK179 to the Conventional Control in the First Year (2010-2011) of Forage Production

Site	Total forage yield (fresh weight ton/acre)		Spring vigor (1-10 rating)		Spring stand recovery (1-10 rating)		Spring stand count (# stems/foot)	
	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)
CADV	15.6 (0.78)	14.7 (0.81)	7.8 (0.25)	7.5 (0.50)	7.8 (0.75)	7.3 (0.48)	39.8 (6.30)	30.8 (4.82)
CANA	2.5 (0.52)	2.9 (0.47)	5.8 (0.25)	5.3 (0.48)	† 10.0 (0.00)	10.0 (0.00)	–	–
IABN	9.7 (1.23)	8.4 (1.14)	8.5 (0.50)	9.0 (0.41)	8.8 (0.25)	9.5 (0.29)	71.9 (3.38)	73.8 (3.52)
IDNP	18.1 (0.72)	19.1 (1.20)	8.0 (0.41)	8.0 (0.00)	7.8 (0.63)	8.5 (0.65)	86.7 (12.05)	89.8 (12.45)
ILCL	8.2 (0.70)	7.5 (0.50)	7.5 (0.29)	7.3 (0.85)	7.0 (0.71)	7.5 (0.87)	45.6 (1.85)	45.7 (8.22)
KSLA	20.1 (0.35)	20.7 (0.85)	8.0 (0.00)	7.8 (0.25)	7.5 (0.29)	8.0 (0.41)	88.3 (2.32)	84.6 (3.51)
NYNR	10.2 (0.56)	9.8 (0.61)	–	–	–	–	50.5 (7.23)	57.0 (4.43)
PACO	12.5 (0.52)	11.8 (0.67)	7.8 (0.25)	7.3 (0.48)	9.8 (0.25)	10.0 (0.00)	45.3 (1.64)	44.3 (2.50)
WATC	* 18.1 (1.02)	14.9 (1.49)	* 8.5 (0.29)	10.0 (0.00)	7.5 (0.29)	7.3 (0.48)	83.6 (3.02)	87.3 (5.94)
WIWS	10.8 (0.60)	10.8 (0.37)	8.5 (0.29)	9.0 (0.00)	9.8 (0.25)	9.8 (0.25)	54.8 (3.60)	57.8 (2.71)

Note: The experimental design was a randomized complete block with four replications. Means based on n=4 for all characteristics except as follows: for crop growth stage #1 and crop growth stage #2, n=3 for both KK179 and the conventional control at CADV; for crop growth stage #3, n=2 for the conventional control at CANA, n=3 for KK179 at CADV, and n=3 for the conventional control at KSLA; for crop growth stage #4, n=2 for both KK179 and the conventional control at CADV; for forage yield #4, n=3 for the conventional control at CADV; and for total forage yield, n=3 for the conventional control at CADV.
SE = Standard Error
* Indicates a statistically significant difference was detected between KK179 and the conventional control (α=0.05) using ANOVA.
† No statistical comparisons were made due to lack of variability in the data.
– Indicates data were excluded from analysis.
NA Indicates cutting is not applicable for this site.
[1]Regrowth after cutting was not assessed following the last harvest of the season.

Table G-7. Individual-Site Phenotypic Comparison of KK179 to the Conventional Control in the Second Year (2011-2012) of Forage Production

Site	Cutting #1							
	Lodging (0-9 rating)		Crop growth stage (stage number 0-6)		Forage yield (fresh weight ton/acre)		Regrowth after cutting[1] (1-10 rating)	
	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)
CADV	† 9.0 (0.00)	9.0 (0.00)	2.9 (0.04)	2.9 (0.03)	9.5 (1.18)	10.2 (0.39)	9.0 (0.00)	8.8 (0.25)
CANA	7.0 (0.58)	8.0 (0.41)	2.9 (0.19)	2.9 (0.24)	9.4 (0.89)	9.7 (0.30)	9.0 (0.00)	9.0 (0.00)
IABN	6.5 (0.29)	6.0 (0.71)	3.5 (0.06)	3.6 (0.27)	12.1 (0.20)	12.1 (0.27)	8.5 (0.29)	9.3 (0.48)
IDNP	4.5 (0.50)	3.5 (0.29)	2.3 (0.14)	2.3 (0.07)	23.3 (0.92)	23.7 (0.79)	9.0 (0.41)	9.3 (0.25)
ILCL	8.0 (0.00)	8.3 (0.25)	* 2.9 (0.19)	3.2 (0.05)	9.3 (0.23)	9.8 (0.85)	10.0 (0.00)	10.0 (0.00)
KSLA	† 9.0 (0.00)	9.0 (0.00)	1.4 (0.43)	1.5 (0.32)	4.5 (0.30)	5.0 (0.30)	8.0 (0.00)	8.0 (0.00)
NYNR	† 9.0 (0.00)	9.0 (0.00)	–	–	5.8 (0.39)	6.4 (0.28)	8.8 (0.95)	9.5 (0.29)
PACO	–	–	–	–	13.6 (0.56)	12.5 (0.46)	8.0 (0.00)	8.0 (0.41)
WATC	7.0 (0.71)	6.0 (0.71)	3.9 (0.05)	3.9 (0.06)	17.0 (0.66)	17.5 (0.42)	* 9.0 (0.00)	10.0 (0.00)
WIWS	4.0 (0.00)	4.0 (0.00)	2.5 (0.11)	2.3 (0.15)	16.3 (0.52)	15.7 (0.44)	9.0 (0.00)	9.0 (0.00)

Table G-7 (continued). Individual-Site Phenotypic Comparison of KK179 to the Conventional Control in the Second Year (2011-2012) of Forage Production

Site	Cutting #2							
	Lodging (0-9 rating)		Crop growth stage (stage number 0-6)		Forage yield (fresh weight ton/acre)		Regrowth after cutting[1] (1-10 rating)	
	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)
CADV	† 9.0 (0.00)	9.0 (0.00)	* 2.0 (0.20)	2.5 (0.04)	–	–	8.5 (0.29)	8.3 (0.48)
CANA	† 9.0 (0.00)	9.0 (0.00)	2.7 (0.34)	2.8 (0.42)	3.8 (0.28)	4.2 (0.46)	8.0 (0.00)	7.3 (0.75)
IABN	* 5.8 (0.25)	4.3 (0.48)	3.7 (0.23)	3.6 (0.19)	10.9 (0.51)	11.4 (0.45)	8.0 (0.00)	9.0 (0.41)
IDNP	6.0 (1.22)	6.0 (1.41)	3.2 (0.18)	3.1 (0.24)	14.5 (0.41)	15.3 (0.43)	9.0 (0.41)	9.5 (0.29)
ILCL	† 9.0 (0.00)	9.0 (0.00)	4.5 (0.16)	4.7 (0.19)	8.2 (0.14)	7.5 (0.11)	10.0 (0.00)	10.0 (0.00)
KSLA	† 9.0 (0.00)	9.0 (0.00)	* 3.6 (0.34)	4.2 (0.35)	10.6 (0.25)	10.9 (0.57)	8.8 (0.25)	9.0 (0.00)
NYNR	† 9.0 (0.00)	9.0 (0.00)	–	–	3.4 (0.24)	3.5 (0.22)	† 10.0 (0.00)	10.0 (0.00)
PACO	5.8 (0.25)	5.5 (0.29)	–	–	12.4 (0.50)	12.1 (0.41)	9.0 (0.00)	9.0 (0.00)
WATC	9.0 (0.00)	9.0 (0.00)	–	–	13.1 (0.28)	13.2 (0.66)	9.3 (0.25)	9.5 (0.29)
WIWS	5.0 (0.00)	5.3 (0.25)	3.0 (0.09)	3.0 (0.09)	10.5 (0.27)	10.2 (0.23)	9.8 (0.25)	9.0 (0.41)

Table G-7 (continued). Individual-Site Phenotypic Comparison of KK179 to the Conventional Control in the Second Year (2011-2012) of Forage Production

Site	Cutting #3							
	Lodging (0-9 rating)		Crop growth stage (stage number 0-6)		Forage yield (fresh weight ton/acre)		Regrowth after cutting[1] (1-10 rating)	
	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)
CADV	† 9.0 (0.00)	9.0 (0.00)	3.9 (0.05)	4.0 (0.01)	13.4 (0.96)	12.6 (0.45)	9.3 (0.48)	9.5 (0.50)
CANA	† 9.0 (0.00)	9.0 (0.00)	3.0 (0.33)	2.1 (0.52)	1.6 (0.10)	1.8 (0.43)	8.5 (0.50)	8.5 (0.50)
IABN	3.3 (0.25)	4.5 (0.87)	4.0 (0.22)	4.1 (0.19)	7.9 (0.49)	8.5 (0.34)	* 7.0 (0.00)	8.3 (0.48)
IDNP	6.5 (0.87)	7.8 (0.63)	2.6 (0.12)	2.8 (0.19)	10.9 (0.72)	10.9 (0.82)	8.8 (0.48)	9.0 (0.41)
ILCL	† 9.0 (0.00)	9.0 (0.00)	4.5 (0.23)	4.3 (0.16)	5.2 (0.15)	5.0 (0.17)	10.0 (0.00)	10.0 (0.00)
KSLA	5.8 (0.75)	5.3 (0.25)	4.7 (0.15)	4.3 (0.31)	10.9 (0.53)	11.4 (0.14)	6.0 (0.00)	5.8 (0.25)
NYNR	† 9.0 (0.00)	9.0 (0.00)	–	–	3.7 (0.02)	3.6 (0.23)	† 10.0 (0.00)	10.0 (0.00)
PACO	7.3 (0.25)	7.5 (0.29)	4.2 (0.20)	4.0 (0.07)	* 9.1 (0.35)	8.4 (0.12)	* 10.0 (0.00)	9.5 (0.29)
WATC	7.8 (0.63)	7.5 (0.96)	4.0 (0.06)	4.1 (0.04)	12.1 (0.25)	12.1 (0.65)	9.0 (0.00)	9.5 (0.50)
WIWS	5.5 (0.29)	6.3 (0.25)	* 3.2 (0.15)	3.7 (0.33)	8.6 (0.17)	8.4 (0.20)	9.3 (0.25)	9.5 (0.29)

Table G-7 (continued). Individual-Site Phenotypic Comparison of KK179 to the Conventional Control in the Second Year (2011-2012) of Forage Production

Site	Cutting #4							
	Lodging (0-9 rating)		Crop growth stage (stage number 0-6)		Forage yield (fresh weight ton/acre)		Regrowth after cutting[1] (1-10 rating)	
	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)
CADV	† 9.0 (0.00)	9.0 (0.00)	4.0 (0.17)	4.0 (0.05)	6.9 (0.25)	7.4 (0.53)	8.5 (0.29)	9.0 (0.00)
CANA	† 9.0 (0.00)	9.0 (0.00)	2.3 (0.41)	2.8 (0.39)	1.9 (0.23)	1.9 (0.39)	† 9.0 (0.00)	9.0 (0.00)
IABN	8.5 (0.29)	8.5 (0.29)	2.4 (0.07)	2.4 (0.09)	* 5.4 (0.07)	6.0 (0.16)	–	–
IDNP	6.8 (0.85)	8.0 (0.71)	3.0 (0.13)	3.1 (0.18)	9.2 (0.66)	8.6 (0.64)	–	–
ILCL	† 9.0 (0.00)	9.0 (0.00)	3.2 (0.20)	3.6 (0.48)	0.9 (0.08)	0.8 (0.06)	–	–
KSLA	† 9.0 (0.00)	9.0 (0.00)	2.6 (0.22)	2.4 (0.29)	6.7 (0.34)	6.7 (0.14)	6.0 (0.00)	6.0 (0.00)
NYNR	† 9.0 (0.00)	9.0 (0.00)	–	–	3.2 (0.21)	3.3 (0.34)	–	–
PACO	4.3 (0.25)	4.5 (0.29)	2.6 (0.13)	2.6 (0.19)	6.0 (0.19)	6.1 (0.46)	9.0 (0.00)	8.5 (0.29)
WATC	† 9.0 (0.00)	9.0 (0.00)	3.4 (0.03)	3.5 (0.03)	9.3 (0.19)	9.2 (0.26)	9.5 (0.29)	10.0 (0.00)
WIWS	5.3 (0.25)	5.8 (0.25)	2.4 (0.13)	2.5 (0.09)	4.8 (0.16)	5.3 (0.13)	–	–

Table G-7 (continued). Individual-Site Phenotypic Comparison of KK179 to the Conventional Control in the Second Year (2011-2012) of Forage Production

Site	Cutting #5							
	Lodging (0-9 rating)		Crop growth stage (stage number 0-6)		Forage yield (fresh weight ton/acre)		Fall plant height (in)	
	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)
CADV	8.5 (0.29)	8.8 (0.25)	3.3 (0.16)	3.5 (0.09)	6.9 (1.02)	4.9 (0.78)	4.7 (0.80)	4.8 (0.62)
CANA	† 9.0 (0.00)	9.0 (0.00)	0.9 (0.07)	1.0 (0.10)	1.1 (0.13)	1.2 (0.16)	–	–
IABN	–	–	–	–	–	–	3.4 (0.14)	3.7 (0.28)
IDNP	–	–	–	–	–	–	8.7 (0.13)	9.3 (0.31)
ILCL	–	–	–	–	–	–	10.6 (0.61)	11.1 (0.93)
KSLA	† 9.0 (0.00)	9.0 (0.00)	–	–	5.0 (0.21)	5.4 (0.06)	4.6 (0.39)	5.1 (0.25)
NYNR	–	–	–	–	–	–	–	–
PACO	5.8 (0.25)	5.8 (0.48)	1.6 (0.03)	1.7 (0.06)	5.3 (0.26)	5.3 (0.31)	4.9 (0.10)	4.7 (0.13)
WATC	† 9.0 (0.00)	9.0 (0.00)	1.2 (0.06)	1.2 (0.05)	* 4.8 (0.40)	5.4 (0.17)	* 8.8 (0.31)	9.7 (0.13)
WIWS	–	–	–	–	–	–	4.3 (0.10)	4.2 (0.14)

Table G-7 (continued). Individual-Site Phenotypic Comparison of KK179 to the Conventional Control in the Second Year (2011-2012) of Forage Production

Site	Total forage yield (fresh weight ton/acre)		Spring vigor (1-10 rating)		Spring stand recovery (1-10 rating)		Spring stand count (# stems/foot)	
	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)
CADV[2]	36.7 (1.95)	35.8 (0.91)	7.3 (1.20)	6.5 (0.29)	8.0 (1.15)	8.8 (0.25)	11.4 (1.66)	11.1 (0.28)
CANA	17.7 (1.55)	18.7 (1.47)	5.8 (0.25)	5.8 (0.25)	† 10.0 (0.00)	10.0 (0.00)	72.4 (3.92)	67.8 (1.87)
IABN	36.3 (1.09)	38.0 (1.00)	7.5 (0.65)	8.0 (1.08)	* 7.8 (0.25)	9.5 (0.29)	50.2 (2.50)	51.4 (3.27)
IDNP	57.9 (2.42)	58.5 (1.12)	7.5 (0.65)	6.8 (0.75)	7.3 (0.25)	6.5 (0.65)	* 54.8 (4.70)	33.8 (8.22)
ILCL	23.6 (0.35)	23.1 (0.96)	9.5 (0.29)	9.0 (0.00)	8.3 (0.25)	8.0 (0.00)	31.0 (3.23)	32.0 (1.62)
KSLA	37.7 (0.81)	39.4 (0.69)	9.0 (0.00)	7.5 (1.50)	8.3 (0.25)	8.0 (0.00)	105.8 (4.82)	93.8 (6.00)
NYNR	16.0 (0.16)	16.8 (0.68)	5.0 (0.00)	5.0 (0.00)	7.0 (1.35)	8.3 (0.25)	* 59.3 (6.51)	69.7 (1.86)
PACO	46.4 (1.27)	44.5 (1.59)	* 6.5 (0.29)	5.5 (0.29)	8.3 (0.25)	8.3 (0.25)	32.3 (4.00)	38.1 (2.28)
WATC	56.3 (0.85)	57.5 (1.71)	* 9.3 (0.25)	10.0 (0.00)	7.8 (0.48)	8.3 (0.48)	86.2 (3.15)	90.3 (4.18)
WIWS	40.1 (0.47)	39.6 (0.79)	9.8 (0.25)	9.3 (0.25)	8.8 (0.63)	9.0 (0.41)	32.3 (1.27)	31.3 (2.35)

Note: The experimental design was a randomized complete block with four replications. Means based on n=4 for all characteristics except as follows: for crop growth stage #1, n=3 for the conventional control at CANA, IABN, and KSLA and n=2 for KK179 at KSLA; for forage yield #1, n=3 for the conventional control at CADV; for crop growth stage #2, n=3 for KK179 at CADV and at KSLA; for crop growth stage #3, n=2 for KK179 at CADV and n=3 for the conventional control at CADV and CANA; for forage yield #3, n=3 for KK179 at PACO and n=3 for the conventional control at CADV; for crop growth stage #4, n=2 for KK179 at CADV, n=3 for KK179 at CANA, IDNP and KSLA, and n=3 for the conventional control at KSLA; for total forage yield , n=3 for KK179 at PACO and n=3 for the conventional control at CADV; for spring vigor, spring stand recovery, and spring stand count, n=3 for KK179 at CADV.
SE = Standard Error
* Indicates a statistically significant difference was detected between KK179 and the conventional control (α=0.05) using ANOVA.
† No statistical comparisons were made due to lack of variability in the data.
NA Indicates the particular cutting is not applicable for this site.
– Indicates data were excluded from analysis.
[1]Regrowth after cutting was not assessed following the last harvest of the season.
[2]Total forage yield at CADV was calculated from four cuttings, since forage yield for cutting #2 was excluded.

G.14 Combined and Individual Field Site Environmental Interactions Results and Discussion

G.14.1 Combined and Individual Field Site Environmental Interactions Results and Discussion - First Year (2010)

In the assessment for the first year (2010), no differences in the range of plant damage responses to abiotic stressors were observed for 93 comparisons between KK179 and the conventional control. Abiotic stressors evaluated included drought, flood, frost, hail, heat, nutrient deficiency, soil compaction, and wind (Table G-8).

No differences in the range of plant damage responses to disease stressors were observed for 93 comparisons between KK179 and the conventional control. Diseases evaluated included Anthracnose, bacterial wilt, black stem, damping-off, downy mildew, Fusarium wilt, leaf spots, root rot, Sclerotinia crown and stem rot, stem nematode, and Verticillium wilt (Table G-9).

No differences in the range of plant damage responses to arthropod stressors were observed for 96 comparisons between KK179 and the conventional control. Arthropods evaluated included alfalfa caterpillar, alfalfa weevil, aphid, armyworm, blister beetle, cutworm, grasshopper, meadow spittlebug, plant bug, potato leafhopper, spider mite, and thrips (Table G-10).

In an individual-site analysis for the first year (2010), no statistically significant differences were detected between KK179 and the conventional control for alfalfa weevil and potato leafhopper damage among all observations (Table G-11). The lack of differences in both the individual-site analysis and the combined-site -analysis supports the conclusion that the introduction of the trait for reduced G lignin and total lignin is not expected to have an effect on the environment compared to the conventional control (Figure VII-1, Step 2, answer "no").

In the individual-site analysis of arthropod abundance for the first year, no statistically significant differences were detected (α=0.05) between KK179 and the conventional control for 65 of the 69 comparisons of pest- and beneficial-arthropod abundance (Tables G-12 and G-13). No statistically significant differences in abundance were detected between KK179 and the conventional control for the following pest and beneficial arthropods: aphid, alfalfa weevil, alfalfa looper, false chinch bug, green cloverworm, garden webworm, thrips, damsel bug, ladybird beetle, parasitic wasps, and lacewing.

Two statistically significant differences in abundance were detected out of 39 pest-arthropod comparisons (Table G-12). KK179 had significantly higher abundance of Lygus bug than the conventional control in collection #2 at KSLA (1.3 vs. 0.0). KK179 had significantly lower abundance of potato leafhopper in collection #1 at ILCL (152.3 vs. 329.0). The mean abundance values of potato leafhopper in collection #1 at ILCL and of Lygus bug for collection #2 at KSLA were outside the reference ranges (178.8 – 256.8 and 0.0 – 0.3, respectively) However, the differences detected for these taxa were not consistently detected across collection times or sites. Thus, the detected differences were

not indicative of a consistent response associated with the trait and were not considered biologically meaningful in terms of effects on the environment from KK179 compared to the conventional control (See Section VII.B.2.).

Two statistically significant differences in abundance were detected out of 30 beneficial-arthropod comparisons (Table G-13). KK179 had significantly lower abundance of spiders than the conventional control in collection #2 at ILCL and at KSLA (0.0 vs. 1.5 and 0.0 vs. 1.3, respectively). The mean abundance values of KK179 were within the respective reference ranges. Thus, the detected differences were not considered biologically meaningful in terms of effects on the environment from KK179 compared to the conventional control (See Section VII.B.2.).

G.14.2. Combined and Individual Field Site Environmental Interactions Results and Discussion - Second Year (2011)

In an assessment for the second year, no differences in the range of plant damage responses to abiotic stressors were observed for 129 comparisons between KK179 and the conventional control. Abiotic stressors evaluated included drought, frost, hail, heat, heaving, nutrient deficiency, soil compaction, wet soil, wind, and winter injury kill (Table G-14).

No differences in the range of plant damage responses to disease stressors were observed for 129 comparisons between KK179 and the conventional control. Diseases evaluated included Anthracnose, bacterial wilt, black stem, crown rot, downy mildew, Fusarium wilt, leaf spots, root rot, Sclerotinia crown and stem rot, and Verticillium wilt (Table G-15).

No differences in the range of plant damage responses to arthropod stressors were observed for 129 comparisons between KK179 and the conventional control. Arthropods evaluated included alfalfa caterpillar, alfalfa leafminer, alfalfa weevil, aphid, armyworm, bean leaf beetle, blister beetle, cutworm, grasshoppers, green cloverworm, Japanese beetle, Lygus bug, meadow spittlebug, plant bug, potato leafhopper, southern corn rootworm beetle, spider mite, and thrips (Table G-16).

In the individual-site analysis for the second year, three statistically significant differences were detected between KK179 and the conventional control for alfalfa weevil and potato leafhopper damage among all observation times (Table G-17). Alfalfa weevil damage and potato leafhopper damage were higher for KK179 than the conventional control during the observation #1 at KSLA (damage rating of 3.4 vs. 2.7 and 2.2 vs. 1.7, respectively). Potato leafhopper damage was lower for KK179 than the conventional control for observation #4 at KSLA (damage rating of 0.1 vs. 0.7). The differences detected in the individual-site analysis were not detected when data were pooled in a combined-site analysis. Thus, the detected differences were not considered biologically meaningful in terms of effects on the environment from KK179 compared to the conventional control (Figure VII-1, Step 2, answer “no”).

In an individual-site analysis of arthropod abundance for the second year, no significant differences were detected between KK179 and the conventional control for arthropod abundance for 82 of 83 comparisons (Tables G-18 and G-19). No statistically significant differences were detected between KK179 and the conventional control for the following pest- and beneficial-arthropods: aphid, armyworm, false chinch bug, blister beetle, green cloverworm, Lygus bug, meadow spittlebug, potato leafhopper, thrips, soybean looper, spiders, ladybird beetle, lacewing, nabids, and chalcid wasps.

One significant difference was detected out of 50 pest-arthropod comparisons between KK179 and the conventional control (Table G-18). KK179 had a lower abundance of alfalfa weevils compared to the conventional control for collection #1 at ILCL (0.3 vs. 7.0). The mean abundance value of KK179 was within the reference range. Thus, the detected difference was not considered biologically meaningful in terms of effects on the environment from KK179 compared to the conventional control (See Section VII.B.2.).

No statistically significant differences in abundance were detected between KK179 and the conventional control out of 33 beneficial-arthropod comparisons (Table G-19). The lack of differences supports the conclusion that the introduction of the trait for reduced G lignin and total lignin is not expected to affect the environment compared to the conventional control.

Table G-8. Combined-Site Qualitative Assessment: Abiotic Stressor Evaluations Using an Observational Severity Scale for KK179 and the Conventional Control in the First Year

Abiotic Stressor	Number of observations across all sites in 2010	Number of observations with no differences between KK179 and the conventional control across all sites in 2010
Total	**93**	**93**
Drought	16	16
Flood[1]	15	15
Frost damage	1	1
Hail damage	7	7
Heat	22	22
Nutrient deficiency	11	11
Soil compaction	5	5
Wind damage	16	16

Note: The experimental design was a randomized complete block with four replications. Observations were made during each crop growth cycle, prior to harvest. Data were not subjected to statistical analysis.
No differences were observed between KK179 and the conventional control during any observation for damage caused by any of the assessed abiotic stressors.
[1]Includes wet soil and excess moisture.

Table G-9. Combined-Site Qualitative Assessment: Disease Damage Evaluations Using an Observational Severity Scale for KK179 and the Conventional Control in the First Year (2010)

Disease Stressor	Number of observations across all sites in 2010	Number of observations with no differences between KK179 and the conventional control across all sites in 2010
Total	**93**	**93**
Anthracnose	6	6
Bacterial wilt	7	7
Black stem	8	8
Damping-off	3	3
Downy mildew	13	13
Fusarium wilt	6	6
Leaf spots	26	26
Root rot[1]	6	6
Sclerotinia crown and stem rot	4	4
Stem nematode	4	4
Verticillium wilt	10	10

Note: The experimental design was a randomized complete block with four replications. Observations were made during each crop growth cycle, prior to harvest. Data were not subjected to statistical analysis.
No differences were observed between KK179 and the conventional control during any observation for damage caused by any of the assessed disease stressors.
[1]Includes *Phytophthora.*

Table G-10. Combined-Site Qualitative Assessment: Insect Stressor Evaluations Using an Observational Severity Scale for KK179 and the Conventional Control in the First Year (2010)

Insect Stressor	Number of observations across all sites in 2010	Number of observations with no differences between KK179 and the conventional control across all sites in 2010
Total	**96**	**96**
Alfalfa caterpillar	1	1
Alfalfa weevil	5	5
Aphid	29	29
Armyworm	3	3
Blister beetle	4	4
Cutworm	7	7
Grasshopper	10	10
Meadow spittlebug	4	4
Plant bug	8	8
Potato leafhopper	10	10
Spider mite	2	2
Thrips	13	13

Note: The experimental design was a randomized complete block with four replications. Observations were made during each crop growth cycle, prior to harvest. Data were not subjected to statistical analysis.
No differences were observed between KK179 and the conventional control during any observation for damage caused by any of the assessed insect stressors.

Table G-11. Individual-Site Analysis: Quantitative Assessment of Potato Leafhopper and Alfalfa Weevil Damage to KK179 Compared to the Conventional Control in the First Year (2010)

Site	Damage (0-10 rating scale)			
	Alfalfa weevil (*Hypera postica*)		Potato leafhopper (*Empoasca fabae*)	
	KK179 Mean (S.E.)	Control Mean (S.E.)	KK179 Mean (S.E.)	Control Mean (S.E.)
Observation #1				
ILCL	0.3 (0.03)	0.5 (0.16)	0.7 (0.04)	0.7 (0.14)
KSLA	0.1 (0.06)	0.2 (0.05)	0.5 (0.02)	0.7 (0.08)
NYNR	† 0.0 (0.00)	0.0 (0.00)	† 0.0 (0.00)	0.0 (0.00)
Observation #2				
ILCL	0.5 (0.04)	0.7 (0.13)	0.7 (0.15)	0.8 (0.10)
KSLA	0.0 (0.03)	0.0 (0.00)	† 0.0 (0.00)	0.0 (0.00)
NYNR	† 0.0 (0.00)	0.0 (0.00)	† 0.0 (0.00)	0.0 (0.00)
Observation #3				
ILCL	0.1 (0.05)	0.2 (0.06)	0.1 (0.08)	0.1 (0.03)
KSLA	† 0.0 (0.00)	0.0 (0.00)	† 0.0 (0.00)	0.0 (0.00)
NYNR	† 0.0 (0.00)	0.0 (0.00)	† 0.0 (0.00)	0.0 (0.00)
Observation #4				
KSLA	† 0.0 (0.00)	0.0 (0.00)	† 0.0 (0.00)	0.0 (0.00)

Note: The experimental design was a randomized complete block with four replications. Means based on n=4.
S.E. = Standard Error
No statistically significant differences were detected between KK179 and the conventional control (α=0.05) using ANOVA.
† No statistical comparisons were made due to lack of variability in the data.

Table G-12. Abundance of Pest Arthropods in Samples Collected from KK179, the Conventional Control, and the Conventional Commercial Reference Varieties in the First Year (2010)

Site	Pest Arthropod								
	Aphid (Aphididae)			Alfalfa weevil (*Hypera postica*)			Alfalfa looper (*Autographa californica*)		
	KK179 Mean (SE)	Control Mean (SE)	Reference Range[1]	KK179 Mean (SE)	Control Mean (SE)	Reference Range[1]	KK179 Mean (SE)	Control Mean (SE)	Reference Range[1]
Collection #1									
ILCL	–	–	–	–	–	–	–	–	–
KSLA	22.3 (3.20)	26.0 (4.56)	17.5 – 27.3	0.8 (0.48)	0.0 (0.00)	0.0 – 0.5	–	–	–
NYNR	22.8 (4.77)	15.8 (3.99)	17.0 – 24.5	–	–	–	–	–	–
Collection #2									
ILCL	12.8 (3.77)	13.8 (3.71)	13.8 – 22.8	–	–	–	–	–	–
KSLA	167.8 (35.73)	181.3 (25.38)	114.0 – 294.5	–	–	–	–	–	–
NYNR	156.5 (19.75)	118.3 (6.16)	116.8 – 159.5	–	–	–	–	–	–
Collection #3									
ILCL	44.5 (17.33)	41.5 (8.21)	28.0 – 44.3	–	–	–	–	–	–
KSLA	510.3 (62.90)	509.0 (172.02)	404.0 – 813.5	–	–	–	0.8 (0.25)	0.5 (0.29)	0.5 – 0.8
NYNR	8.3 (0.85)	12.3 (2.66)	7.0 – 11.5	–	–	–	–	–	–
Collection #4									
KSLA	5.8 (1.89)	9.0 (1.78)	5.8 – 9.0	–	–	–	–	–	–

Table G-12 (continued). Abundance of Pest Arthropods in Samples Collected from KK179, the Conventional Control, and the Conventional Commercial Reference Varieties in the First Year (2010)

Site	Pest Arthropod								
	False chinch bug (*Nysius raphanus*)			Green cloverworm (*Hypena scabra*)			Garden webworm (*Achyra rantalis*)		
	KK179 Mean (SE)	Control Mean (SE)	Reference Range	KK179 Mean (SE)	Control Mean (SE)	Reference Range	KK179 Mean (SE)	Control Mean (SE)	Reference Range
Collection #1									
ILCL	4.5 (1.32)	10.0 (2.86)	7.0 – 11.3	1.3 (0.25)	1.8 (1.11)	1.3 – 4.3	–	–	–
KSLA	9.0 (1.96)	7.5 (0.50)	6.3 – 13.8	–	–	–	–	–	–
NYNR	–	–	–	–	–	–	–	–	–
Collection #2									
ILCL	7.3 (1.11)	9.5 (1.94)	10.0 – 13.3	–	–	–	2.3 (0.63)	2.8 (1.03)	1.5 – 3.8
KSLA	1.0 (0.41)	1.5 (1.50)	0.8 – 1.3	–	–	–	–	–	–
NYNR	2.0 (0.71)	1.8 (0.75)	0.8 – 1.5	–	–	–	–	–	–
Collection #3									
ILCL	6.8 (1.93)	7.5 (2.02)	3.8 – 10.3	–	–	–	–	–	–
KSLA	10.5 (3.01)	10.3 (0.85)	9.0 – 10.5	–	–	–	3.3 (0.75)	3.8 (0.75)	1.8 – 3.0
NYNR	–	–	–	–	–	–	–	–	–
Collection #4									
KSLA	6.0 (1.58)	3.0 (1.08)	3.8 – 6.8	–	–	–	–	–	–

Table G-12 (continued). Abundance of Pest Arthropods in Samples Collected from KK179, the Conventional Control, and the Conventional Commercial Reference Varieties in the First Year (2010)

Site	Pest Arthropod								
	Lygus bug (Miridae)			Potato leafhopper (*Empoasca fabae*)			Thrips (Thysanoptera)		
	KK179 Mean (SE)	Control Mean (SE)	Reference Range	KK179 Mean (SE)	Control Mean (SE)	Reference Range	KK179 Mean (SE)	Control Mean (SE)	Reference Range
Collection #1									
ILCL	7.8 (1.49)	8.8 (3.07)	6.8 – 11.0	*152.3 (30.90)	329.0 (90.25)	178.8 – 256.8	–	–	–
KSLA	0.3 (0.25)	1.5 (0.96)	0.7 – 1.3	6.5 (1.66)	11.0 (1.78)	6.7 – 13.3	–	–	–
NYNR	1.5 (0.50)	1.0 (0.71)	1.5 – 2.3	12.0 (3.24)	18.5 (3.07)	12.3 – 14.3	–	–	–
Collection #2									
ILCL	–	–	–	35.8 (7.79)	29.0 (11.71)	19.5 – 27.8	–	–	–
KSLA	* 1.3 (0.48)	0.0 (0.00)	0.0 – 0.3	–	–	–	8.5 (2.72)	15.8 (5.85)	6.8 – 12.5
NYNR	11.8 (0.95)	11.8 (3.71)	5.3 – 11.0	–	–	–	–	–	–
Collection #3									
ILCL				–	–	–	9.0 (1.68)	10.3 (3.97)	6.5 – 8.8
KSLA	0.3 (0.25)	0.8 (0.48)	0.0 – 0.5	–	–	–	–	–	–
NYNR	1.8 (0.63)	4.3 (2.36)	1.8 – 4.8	25.5 (1.04)	19.8 (1.49)	14.8 – 21.8	12.0 (3.56)	16.3 (2.50)	11.5 – 26.0
Collection #4									
KSLA	–	–	–	1.5 (0.29)	1.5 (0.29)	0.3 – 1.3	27.0 (3.76)	24.8 (2.78)	16.3 – 26.8

Note: The experimental design was a randomized complete block with four replications. Means based on n=4.
SE = Standard Error
* Indicates a statistically significant difference was detected between KK179 and the conventional control (α=0.05) using ANOVA.
– Indicates arthropod was not evaluated.
[1]Reference range is the minimum and maximum mean values among the conventional commercial reference varieties.

Table G-13. Abundance of Beneficial Arthropods in Samples Collected from KK179, the Conventional Control, and the Conventional Commercial Reference Varieties in the First Year (2010)

	Beneficial Arthropod								
	Spiders (Araneae)			Damsel bug (*Nabis* spp.)			Ladybird beetle (Coccinellidae)		
Site	KK179 Mean (SE)	Control Mean (SE)	Reference Range[1]	KK179 Mean (SE)	Control Mean (SE)	Reference Range[1]	KK179 Mean (SE)	Control Mean (SE)	Reference Range[1]
Collection #1									
ILCL	1.5 (0.29)	1.5 (0.50)	0.8 – 1.5	1.8 (0.85)	1.0 (0.41)	0.8 – 2.0	2.0 (0.91)	2.3 (0.85)	2.3 – 3.0
KSLA	–	–	–	1.0 (0.58)	0.8 (0.25)	0.5 – 2.0	1.0 (0.71)	0.3 (0.25)	0.3 – 2.8
NYNR	–	–	–	0.0 (0.00)	0.0 (0.00)	0.0 – 0.3	0.0 (0.00)	0.3 (0.25)	0.0 – 0.5
Collection #2									
ILCL	* 0.0 (0.00)	1.5 (0.65)	0.0 – 1.8	0.5 (0.29)	0.5 (0.29)	0.3 – 2.0	1.5 (0.65)	1.8 (0.75)	0.5 – 3.0
KSLA	* 0.0 (0.00)	1.3 (0.48)	0.0 – 0.5	–	–	–	0.3 (0.25)	0.0 (0.00)	0.0 – 0.3
NYNR	–	–	–	1.5 (0.65)	0.5 (0.50)	0.3 – 1.5	1.8 (0.85)	1.3 (0.48)	0.5 – 3.0
Collection #3									
ILCL	0.0 (0.00)	0.5 (0.29)	0.0 – 0.5	0.0 (0.00)	0.5 (0.29)	0.0 – 0.5	–	–	–
KSLA	2.5 (0.65)	1.5 (0.87)	1.3 – 2.5	–	–	–	5.5 (1.04)	2.8 (0.63)	2.5 – 4.8
NYNR	–	–	–	0.5 (0.29)	0.8 (0.48)	0.3 – 1.0	0.3 (0.25)	0.5 (0.29)	0.8 – 1.8
Collection #4									
KSLA	1.3 (0.48)	1.0 (0.41)	0.8 – 2.3	0.5 (0.29)	0.3 (0.25)	0.0 – 0.5	–	–	–

Table G-13 (continued). Abundance of Beneficial Arthropods in Samples Collected from KK179, the Conventional Control, and the Conventional Commercial Reference Varieties in the First Year (2010)

Site	Beneficial Arthropod					
	Parasitic wasps (Hymenoptera)[2]			Lacewing (Chrysopidae)		
	KK179 Mean (SE)	Control Mean (SE)	Reference Range	KK179 Mean (SE)	Control Mean (SE)	Reference Range
Collection #1						
ILCL	–	–	–	–	–	–
KSLA	5.0 (1.47)	9.8 (1.18)	5.0 – 12.3	–	–	–
NYNR	0.0 (0.00)	0.5 (0.50)	0.0 – 0.5	–	–	–
Collection #2						
ILCL	–	–	–	–	–	–
KSLA	0.8 (0.25)	2.0 (0.91)	0.8 – 1.5	–	–	–
NYNR	1.0 (1.00)	1.0 (0.71)	0.5 – 1.5	–	–	–
Collection #3						
ILCL	–	–	–	0.0 (0.00)	0.0 (0.00)	0.0 – 0.5
KSLA	3.0 (1.08)	1.8 (0.85)	1.8 – 3.0	–	–	–
NYNR	2.8 (0.75)	1.5 (0.65)	0.5 – 3.8	–	–	–
Collection #4						
KSLA	0.5 (0.50)	1.5 (0.65)	1.0 – 1.8	–	–	–

Note: The experimental design was a randomized complete block with four replications. Means based on n=4.
SE = Standard Error
* Indicates a statistically significant difference was detected between KK179 and the conventional control (α=0.05) using ANOVA.
– Indicates arthropod was not evaluated.
[1]Reference range is the minimum and maximum mean values among the conventional commercial reference varieties.
[2]Parasitic wasps include braconids, ichneumons, and micro-parasitic Hymenoptera.

Table G-14. Combined-Site Qualitative Assessment: Abiotic Stressor Evaluations Using an Observational Severity Scale for KK179 and the Conventional Control in the Second Year (2011)

Abiotic Stressor	Number of observations across all sites in 2011	Number of observations with no differences between KK179 and the conventional control across all sites in 2011
Total	**129**	**129**
Drought	22	22
Frost damage	3	3
Hail damage	8	8
Heat	19	19
Heaving	1	1
Nutrient deficiency	10	10
Soil compaction	6	6
Wet soil[1]	22	22
Wind damage	30	30
Winter injury kill	8	8

Note: The experimental design was a randomized complete block with four replications. Observations were made during each crop growth cycle, prior to harvest. Data were not subjected to statistical analysis.
No differences were observed between KK179 and the conventional control during any observation for damage caused by any of the assessed abiotic stressors.
[1]Includes wet soil and excess moisture.

Table G-15. Combined-Site Qualitative Assessment: Disease Damage Evaluations Using an Observational Severity Scale for KK179 and the Conventional Control in the Second Year (2011)

Disease Stressor	Number of observations across all sites in 2011	Number of observations with no differences between KK179 and the conventional control across all sites in 2011
Total	**129**	**129**
Anthracnose	7	7
Bacterial wilt	10	10
Black stem[1]	12	12
Crown Rot	3	3
Downy mildew	28	28
Fusarium wilt	4	4
Leaf spots[2]	36	36
Root rot[3]	6	6
Sclerotinia crown and stem rot	4	4
Verticillium wilt	19	19

Note: The experimental design was a randomized complete block with four replications. Observations were made during each crop growth cycle, prior to harvest. Data were not subjected to statistical analysis. No differences were observed between KK179 and the conventional control during any observation for damage caused by any of the assessed disease stressors.
[1]Includes summer black stem.
[2]Includes common leaf spot.
[3]Includes *Phytophthora.*

Table G-16. Combined-Site Qualitative Assessment: Insect Stressor Evaluations Using an Observational Severity Scale for KK179 and the Conventional Control in the Second Year (2011)

Insect Stressor	Number of observations across all sites in 2011	Number of observations with no differences between KK179 and the conventional control across all sites in 2011
Total	**129**	**129**
Alfalfa caterpillar	5	5
Alfalfa leafminer	5	5
Alfalfa weevil	10	10
Aphid[1]	38	38
Armyworm	3	3
Bean leaf beetle	1	1
Blister beetle	4	4
Cutworm	2	2
Grasshopper	16	16
Green cloverworm	1	1
Japanese beetle	1	1
Lygus bug	4	4
Meadow spittlebug	5	5
Plant bug	4	4
Potato leafhopper	11	11
Southern corn rootworm beetle	1	1
Spider mite	1	1
Thrips	17	17

Note: The experimental design was a randomized complete block with four replications. Observations were made during each crop growth cycle, prior to harvest. Data were not subjected to statistical analysis. No differences were observed between KK179 and the conventional control during any observation for damage caused by any of the assessed insect stressors.
[1]Includes pea aphid.

Table G-17. Individual-Site Analysis: Quantitative Assessment of Potato Leafhopper and Alfalfa Weevil Damage to KK179 Compared to the Conventional Control in the Second Year (2011)

Site	Damage (0-10 rating scale)			
	Alfalfa weevil (*Hypera postica*)		Potato leafhopper (*Empoasca fabae)*	
	KK179 Mean (SE)	Control Mean (SE)	KK179 Mean (SE)	Control Mean (SE)
Observation #1				
ILCL	0.2 (0.08)	0.1 (0.06)	0.3 (0.10)	0.1 (0.13)
KSLA	* 3.4 (0.09)	2.7 (0.17)	* 2.2 (0.25)	1.7 (0.10)
NYNR	† 0.0 (0.00)	0.0 (0.00)	† 0.0 (0.00)	0.0 (0.00)
Observation #2				
ILCL	0.6 (0.10)	0.7 (0.10)	0.4 (0.07)	0.3 (0.06)
KSLA	† 0.0 (0.00)	0.0 (0.00)	† 0.0 (0.00)	0.0 (0.00)
NYNR	† 0.0 (0.00)	0.0 (0.00)	† 0.0 (0.00)	0.0 (0.00)
Observation #3				
ILCL	0.6 (0.10)	0.5 (0.18)	0.4 (0.03)	0.3 (0.12)
KSLA	† 0.0 (0.00)	0.0 (0.00)	† 0.0 (0.00)	0.0 (0.00)
NYNR	† 0.0 (0.00)	0.0 (0.00)	† 0.0 (0.00)	0.0 (0.00)
Observation #4				
ILCL	0.1 (0.05)	0.1 (0.05)	0.4 (0.05)	0.3 (0.03)
KSLA	† 0.0 (0.00)	0.0 (0.00)	* 0.1 (0.13)	0.7 (0.36)
NYNR	† 0.0 (0.00)	0.0 (0.00)	1.5 (0.65)	1.5 (0.65)
Observation #5				
KSLA	† 0.0 (0.00)	0.0 (0.00)	† 0.0 (0.00)	0.0 (0.00)

Note: The experimental design was a randomized complete block with four replications. Means based on n=4.
SE = Standard Error
* Indicates a statistically significant difference was detected between KK179 and the conventional control (α=0.05) using ANOVA.
† No statistical comparisons were made due to lack of variability in the data.

Table G-18. Abundance of Pest Arthropods in Samples Collected from KK179, the Conventional Control, and the Conventional Commercial Reference Varieties in the Second Year (2011)

Site	Pest Arthropod								
	Aphid (Aphididae)			Alfalfa weevil (*Hypera postica*)			Armyworm (*Spodoptera* spp.)		
	KK179 Mean (SE)	Control Mean (SE)	Reference Range[1]	KK179 Mean (SE)	Control Mean (SE)	Reference Range[1]	KK179 Mean (SE)	Control Mean (SE)	Reference Range[1]
Collection #1									
ILCL	52.8 (2.14)	53.8 (16.04)	60.0 - 100.8	* 0.3 (0.25)	7.0 (5.05)	0.0 – 1.0	–	–	–
KSLA	81.5 (11.38)	75.3 (10.95)	64.3 – 97.0	25.0 (7.69)	29.8 (5.12)	22.0 – 43.3	–	–	–
NYNR	185.8 (19.26)	181.5 (38.49)	220.0 – 252.0	44.3 (10.26)	48.5 (6.74)	35.5 – 64.3	–	–	–
Collection #2									
ILCL	–	–	–	–	–	–	–	–	–
KSLA	173.0 (24.19)	177.0 (25.26)	158.3 – 281.3	–	–	–	–	–	–
NYNR	52.5 (5.07)	52.3 (7.09)	41.0 – 58.0	–	–	–	–	–	–
Collection #3									
ILCL	73.5 (14.39)	54.3 (7.38)	76.3 – 87.8	–	–	–	–	–	–
KSLA	186.5 (25.31)	189.3 (31.98)	134.3 – 236.0	–	–	–	–	–	–
NYNR	114.0 (17.97)	96.3 (8.40)	62.3 – 112.0	–	–	–	–	–	–
Collection #4									
ILCL	141.5 (29.22)	112.8 (18.14)	99.3 – 134.3	–	–	–	–	–	–
KSLA	167.8 (35.46)	232.8 (24.54)	189.3 – 276.5	–	–	–	–	–	–
NYNR	3.3 (1.60)	4.8 (1.11)	4.8 – 6.8	–	–	–	0.3 (0.25)	0.0 (0.00)	0.0 – 0.8
Collection #5									
KSLA	11.0 (2.35)	8.3 (1.31)	6.5 – 8.0	–	–	–	5.3 (2.21)	3.8 (1.55)	3.5 – 6.5

Table G-18 (continued). Abundance of Pest Arthropods in Samples Collected from KK179, the Conventional Control, and the Conventional Commercial Reference Varieties in the Second Year (2011)

Site	Pest Arthropod								
	False chinch bug (*Nysius raphanus*)			Blister beetle (Meloidae)			Green cloverworm (*Plathypena scabra*)		
	KK179 Mean (SE)	Control Mean (SE)	Reference Range	KK179 Mean (SE)	Control Mean (SE)	Reference Range	KK179 Mean (SE)	Control Mean (SE)	Reference Range
Collection #1									
ILCL	–	–	–	–	–	–	–	–	–
KSLA	1.5 (0.50)	2.3 (1.03)	2.0 – 4.8	–	–	–	–	–	–
NYNR	–	–	–	–	–	–	–	–	–
Collection #2									
ILCL	–	–	–	–	–	–	–	–	–
KSLA	21.3 (5.22)	20.3 (2.69)	19.0 – 29.5	0.3 (0.25)	0.5 (0.50)	0.0 – 1.0			
NYNR	–	–	–	–	–	–	–	–	–
Collection #3									
ILCL	–	–	–	–	–	–	–	–	–
KSLA	18.5 (7.92)	17.3 (2.29)	7.0 – 20.0	–	–	–	–	–	–
NYNR	–	–	–	–	–	–	1.3 (0.63)	1.8 (0.75)	0.0 – 1.3
Collection #4									
ILCL	–	–	–	–	–	–	–	–	–
KSLA	3.0 (0.41)	5.8 (2.10)	6.0 – 9.0	–	–	–	–	–	–
NYNR	–	–	–	–	–	–	–	–	–
Collection #5									
KSLA	4.0 (1.73)	3.3 (1.11)	4.0 – 5.8	–	–	–	–	–	–

Table G-18 (continued). Abundance of Pest Arthropods in Samples Collected from KK179, the Conventional Control, and the Conventional Commercial Reference Varieties in the Second Year (2011)

Site	Pest Arthropod								
	Lygus bug (*Lygus hesperus*)			Meadow spittlebug (*Philaenus spumarius*)			Potato leafhopper (*Empoasca fabae*)		
	KK179 Mean (SE)	Control Mean (SE)	Reference Range	KK179 Mean (SE)	Control Mean (SE)	Reference Range	KK179 Mean (SE)	Control Mean (SE)	Reference Range
Collection #1									
ILCL	3.0 (1.08)	2.5 (1.50)	1.0 – 6.3	–	–	–	–	–	–
KSLA	–	–	–	–	–	–	–	–	–
NYNR	9.8 (0.85)	9.0 (4.83)	6.3 – 11.3	2.0 (1.00)	0.8 (0.75)	0.3 – 2.5	3.0 (1.08)	5.0 (0.71)	2.3 – 7.5
Collection #2									
ILCL	14.5 (1.32)	16.8 (5.53)	9.5 – 16.5	–	–	–	74.5 (11.53)	69.0 (3.19)	48.3 – 95.8
KSLA	–	–	–	–	–	–	–	–	–
NYNR	8.3 (2.93)	11.8 (1.75)	8.3 – 14.0	–	–	–	49.3 (14.87)	65.0 (14.96)	37.3 – 52.8
Collection #3									
ILCL	6.8 (0.85)	12.0 (3.72)	5.8 – 12.0	–	–	–	109.8 (21.26)	129.5 (32.07)	85.3 – 100.3
KSLA	–	–	–	–	–	–	3.8 (1.49)	2.5 (0.65)	0.8 – 2.3
NYNR	21.5 (5.20)	19.8 (3.12)	13.3 – 16.3	–	–	–	215.5 (83.92)	169.3 (31.20)	116.8 – 137.0
Collection #4									
ILCL	–	–	–	–	–	–	22.0 (6.65)	14.3 (2.02)	9.8 – 17.0
KSLA	–	–	–	–	–	–	–	–	–
NYNR	2.0 (1.68)	1.8 (0.25)	2.3 – 3.8	–	–	–	6.5 (1.04)	3.5 (0.87)	1.0 – 4.0
Collection #5									
KSLA	–	–	–	–	–	–	–	–	–

Table G-18 (continued). Abundance of Pest Arthropods in Samples Collected from KK179, the Conventional Control, and the Conventional Commercial Reference Varieties in the Second Year (2011)

	Pest Arthropod					
	Thrips (Thysanoptera)			Soybean looper (*Pseudoplusia includens*)		
Site	KK179 Mean (SE)	Control Mean (SE)	Reference Range	KK179 Mean (SE)	Control Mean (SE)	Reference Range
Collection #1						
ILCL	32.0 (1.08)	33.0 (12.73)	27.8 – 48.3	–	–	–
KSLA	25.3 (2.50)	37.5 (9.95)	37.8 – 46.8	–	–	–
NYNR	–	–	–			
Collection #2						
ILCL	181.5 (33.26)	177.0 (10.23)	87.5 – 166.5	–	–	–
KSLA	739.3 (174.43)	976.3 (118.06)	796.5 – 1190.5	–	–	–
NYNR	–	–	–	–	–	–
Collection #3						
ILCL	–	–	–	–	–	–
KSLA	138.5 (39.86)	172.8 (43.35)	132.3 – 213.8	–	–	–
NYNR	–	–	–	–	–	–
Collection #4						
ILCL	52.5 (4.56)	49.3 (5.62)	36.3 – 58.0	–	–	–
KSLA	76.3 (7.04)	137.3 (18.20)	115.3 – 160.3	2.5 (0.96)	2.5 (1.04)	2.3 – 3.8
NYNR	12.0 (2.38)	10.3 (2.46)	8.0 – 14.0	–	–	–
Collection #5						
KSLA	174.0 (25.44)	208.0 (18.52)	157.3 – 223.0	–	–	–

Note: The experimental design was a randomized complete block with four replications. Means based on n=4.
SE = Standard Error
* Indicates a statistically significant difference was detected between KK179 and the conventional control (α=0.05) using ANOVA.
– Indicates arthropod was not evaluated.
[1]Reference range is the minimum and maximum mean values among the conventional commercial reference varieties.

Table G-19. Abundance of Beneficial Arthropods in Samples Collected from KK179, the Conventional Control, and the Conventional Commercial Reference Varieties in the Second Year (2011)

Site	Beneficial Arthropod								
	Spiders (Araneae)			Ladybird beetle (Coccinellidae)			Lacewing (Chrysopidae)		
	KK179 Mean (SE)	Control Mean (SE)	Reference Range[1]	KK179 Mean (SE)	Control Mean (SE)	Reference Range[1]	KK179 Mean (SE)	Control Mean (SE)	Reference Range[1]
Collection #1									
ILCL	0.0 (0.00)	0.3 (0.25)	0.0 – 0.5	1.5 (0.29)	3.0 (1.73)	1.8 – 2.5	0.3 (0.25)	0.0 (0.00)	0.0 – 1.5
KSLA	0.5 (0.29)	0.3 (0.25)	0.3 – 0.5	1.0 (0.71)	1.0 (0.71)	0.8 – 2.3	–	–	–
NYNR	–	–	–	–	–	–	–	–	–
Collection #2									
ILCL	–	–	–	–	–	–	–	–	–
KSLA	0.8 (0.25)	1.0 (0.71)	0.8 – 1.5	2.3 (1.31)	1.5 (0.65)	1.5 – 2.0	–	–	–
NYNR	0.0 (0.00)	0.3 (0.25)	0.0 – 0.3	–	–	–	–	–	–
Collection #3									
ILCL	0.8 (0.25)	1.8 (0.25)	1.0 – 2.3	–	–	–	0.3 (0.25)	0.3 (0.25)	0.3 – 0.5
KSLA	2.8 (0.75)	4.5 (1.44)	3.3 – 4.0	1.3 (0.48)	0.8 (0.25)	0.5 – 2.8	0.5 (0.29)	0.5 (0.50)	0.5 – 1.0
NYNR	1.3 (0.25)	0.8 (0.25)	1.0 – 1.8	7.5 (1.66)	6.0 (1.08)	3.3 – 5.5	1.0 (0.41)	0.8 (0.25)	0.5 – 2.0
Collection #4									
ILCL	4.0 (1.47)	1.3 (0.95)	1.3 – 2.8	0.3 (0.25)	0.5 (0.29)	0.0 – 1.3	–	–	–
KSLA	2.0 (0.58)	4.3 (0.48)	2.8 – 4.8	–	–	–	0.5 (0.29)	0.8 (0.25)	0.0 – 1.0
NYNR	0.0 (0.00)	0.3 (0.25)	0.3 – 0.8	0.0 (0.00)	0.0 (0.00)	0.0 – 0.3	–	–	–
Collection #5									
KSLA	3.0 (0.71)	4.0 (1.08)	3.3 – 6.3	0.3 (0.25)	0.3 (0.25)	0.0 – 0.5	1.5 (0.29)	0.8 (0.48)	0.5 – 1.3

Table G-19 (continued). Abundance of Beneficial Arthropods in Samples Collected from KK179, the Conventional Control, and the Conventional Commercial Reference Varieties in the Second Year (2011)

	Beneficial Arthropod					
	Nabids (Nabidae)			Chalcid wasps (Chalcidoidea)		
Site	KK179 Mean (SE)	Control Mean (SE)	Reference Range	KK179 Mean (SE)	Control Mean (SE)	Reference Range
Collection #1						
ILCL	–	–	–	–	–	–
KSLA	–	–	–	–	–	–
NYNR	–	–	–	–	–	–
Collection #2						
ILCL	–	–	–	–	–	–
KSLA	1.0 (0.71)	2.3 (0.48)	0.8 – 1.8	–	–	–
NYNR	0.5 (0.29)	0.5 (0.29)	0.0 – 0.8	–	–	–
Collection #3						
ILCL	0.8 (0.48)	0.3 (0.25)	0.0 – 0.5	–	–	–
KSLA	1.8 (1.44)	0.3 (0.25)	0.0 – 0.5	–	–	–
NYNR	0.8 (0.48)	1.3 (0.25)	0.0 – 1.5	–	–	–
Collection #4						
ILCL	–	–	–	–	–	–
KSLA	0.0 (0.00)	0.5 (0.29)	0.0 – 0.8	–	–	–
NYNR	–	–	–	–	–	–
Collection #5						
KSLA	0.8 (0.48)	0.8 (0.48)	0.8 – 1.5	9.5 (2.99)	4.5 (0.65)	3.8 – 7.8

Note: The experimental design was a randomized complete block with four replications. Means based on n=4.
SE = Standard Error
* Indicates a statistically significant difference was detected between KK179 and the conventional control (α=0.05) using ANOVA.
– Indicates arthropod was not evaluated.
[1]Reference range is the minimum and maximum mean values among the conventional commercial reference varieties.

References for Appendix G

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SAS. 2008. SAS/STAT software version 9.2. SAS Institute, Inc., Cary, North Carolina.

Appendix H: Materials and Methods from Phenotypic and Agronomic Assessment of KK179 under Field Conditions during Seed Production

H.1. Materials

Agronomic and phenotypic characteristics were assessed for KK179, the conventional control (C_0-Syn1), and seven conventional commercial reference varieties grown under similar agronomic conditions of seed production (Table H-1).

H.2. Characterization of the Materials

The presence or absence of KK179 in the starting seed of KK179, the conventional control, and three of the conventional commercial reference varieties, which were produced in the same production with KK179 and the conventional control, was verified by event-specific PCR analyses.

H.3. Field Sites and Plot Design

A seed production trial was established in 2010 at a single location in Canyon County, ID. Seed of KK179, the conventional control, seven conventional commercial reference varieties, and one other material was planted in a randomized complete block design with six replications. Each plot consisted of two 20 foot-long rows spaced 22 inches apart.

H.4. Planting and Field Operations

Planting information, soil description, and cropping history of the study area are listed in Table H-2. Prior to planting, the Principal Investigator at the site prepared a proper seed bed according to local agronomic practices, including tillage, fertilization and pH adjustment, and pest management. After planting, all plots were thinned to a uniform density of 200 plants per plot. During the growing season, the field site was scouted for agronomic conditions and pest populations, including pest arthropods, diseases, and weeds. Fertilizer, irrigation, agricultural chemicals, and other management treatments were applied as necessary. All maintenance operations were performed uniformly across the entire plot area.

H.5. Study Management

The field trial was uniformly managed under the local agronomic practices for seed production. Plots were not harvested for forage. Bees were introduced into the field in two separate releases in July. Irrigation was restricted following bee release. All plots were swathed September 13, 2010, and seed was harvested on September 17, 2010.

H.6. Phenotypic Observations

KK179 was compared to the conventional control for seedling establishment, seedling vigor, lodging, seed weight, seeds per pod, split pods, and seed yield. A description of each phenotypic characteristic assessed is listed in Table VII-1.

H.7. Data Assessment

Experienced scientists familiar with the experimental design and evaluation criteria were involved in all components of data collection, summarization, and analysis. Study personnel assessed that measurements were taken properly, data were consistent with expectations based on experience with the crop, and the experiment was carefully monitored. Prior to analysis, the overall dataset was evaluated for evidence of biologically-relevant changes and for possible evidence of an unexpected plant response. Any unexpected observations or issues during the study that would impact the study objectives were noted. Data were then subjected to statistical analysis as indicated below.

H.8. Statistical Analysis

An analysis of variance was conducted according to a randomized complete block design using SAS® (SAS, 2008) to compare KK179 and the conventional control for seedling establishment, seedling vigor, lodging, seed weight, seed per pod, split pods, and seed yield. The level of statistical significance was predetermined to be 5% (α=0.05). KK179 and the conventional control were not statistically compared to the conventional commercial reference varieties. Minimum and maximum mean values were calculated for each characteristic from the seven conventional commercial reference varieties.

Table H-1. Starting Seed for Phenotypic and Agronomic Assessment during Seed Production

Material Name	Genotype / Phenotype	Monsanto Lot Number
KK179	Reduced Lignin; Syn1 population	11265471
C_0-Syn1	Conventional; Syn1 population	11265474
WL 319HQ	Conventional	11265484
DKA50-18	Conventional	11265481
Vernal	Conventional	11265487
Dow/Dairyland Hybriforce 400	Conventional	11265485
Ranger	Conventional	11265488
Pioneer 54V09	Conventional	11265480
Pioneer 54H11	Conventional	11265478

Note: KK179 was grown under USDA-APHIS Permit 09-308-108rm. The field trial included one additional material that is outside of the scope of objectives of this evaluation of KK179.

Table H-2. Field and Planting Information

Plot area (ft^2)	Rows (#/plot)	Planting date	Planting density[1] (lb/A)	Soil Series	OM[2] (%)	2009 Crop
73.3	2	4/20/2010	2.6	Sandy Loam	1.5	Corn

[1]All plots were thinned to a uniform density of 200 plants per plot after seedling establishment and seedling vigor data were collected.
[2]OM =Organic Matter

References for Appendix H

SAS. 2008. SAS/STAT software version 9.2. SAS Institute, Inc., Cary, North Carolina.

Appendix I: Materials and Methods for Pollen Morphology and Viability Assessment

I.1. Plant Production

KK179, the conventional control (C_0-Syn1), and four conventional commercial reference varieties were grown under similar agronomic conditions in a greenhouse (Table I-1). Approximately 1-2 weeks after planting, seedlings of KK179 were sampled and tested using event-specific PCR analyses. Plants not containing KK179 were removed from the trial. The greenhouse trial was arranged in a randomized complete block design with four replications, with 10 plants of each material per replication.

I.2. Flower Collection

A minimum of eight racemes were collected from each entry in each replication. Each set of racemes was placed in a labeled container and stored on wet ice for transport and storage in the microscope laboratory. A minimum of 15 mature flowers were removed from the collected racemes of each entry within a replication.

I.3. Pollen Sample Preparation

From the collected flowers, five individual sub-samples were prepared. Each sub-sample consisted of the pollen from three to five flowers. For each sub-sample, the pollen was then stained with Alexander's stain diluted 1:5 (Alexander, 1980).

I.4. Data Collection

Pollen characteristics were assessed by viewing samples under an Olympus BX51 light microscope equipped with an Olympus DP70 digital color camera. The microscope and camera were connected to a computer running Microsoft Windows 2000 Professional (© 1981-1999, Microsoft Corp.) and installed with associated DP Controller v1.2.1.108 and DP Manager v1.2.1.107 camera software (© 2001-2003, Olympus Optical Co., Ltd.) and Image-Pro Plus v6.2.1.491 imaging software (© 1993-2007, Media Cybernetics, Inc.).

I.4.1. Pollen Viability

When exposed to the staining solution, viable pollen grains stained red to purple due to the presence of living cytoplasmic content. Non-viable pollen grains stained light blue to green or colorless and may have appeared round to collapsed in shape, depending on the degree of hydration. For each pollen sample, the number of viable and non-viable pollen grains was counted from a random field of view under the microscope. A minimum of 75 pollen grains were counted for each sample. Dense clusters of pollen or pollen grains adhering to flower parts were not counted because they may not have absorbed the staining solution uniformly.

I.4.2. Pollen Diameter

Pollen grain diameter was measured along two perpendicular axes for 10 representative pollen grains per replication. Mean pollen diameter for each replicate was calculated from the 20 total measurements as shown in Table VII-14.

I.4.3. General Pollen Morphology

General pollen morphology of KK179, the conventional control, and the conventional commercial reference varieties was observed as shown in Figure I-1.

I.5. Statistical Analysis

An analysis of variance was conducted according to a randomized complete block design using SAS® (SAS, 2008). The level of statistical significance was predetermined to be 5% (α=0.05). KK179 was compared to the conventional control for percent viable pollen and pollen grain diameter. KK179 and the conventional control were not statistically compared to the conventional commercial reference varieties. Minimum and maximum mean values were calculated for each characteristic from the four conventional commercial reference varieties. General pollen morphology was qualitative; therefore, no statistical analysis was conducted on these observations.

Table I-1. Starting Seed for Pollen Morphology and Viability Assessment

Material	Genotype / Phenotype	Monsanto Lot Number
KK179	Reduced Lignin; Syn1 population	11265471
C_0-Syn1	Conventional; Syn1 population	11265474
Pioneer 54H11	Conventional	11265478
WL 319HQ	Conventional	11265484
DKA50-18	Conventional	11265481
Dow/Dairyland HybriForce 400	Conventional	11265485

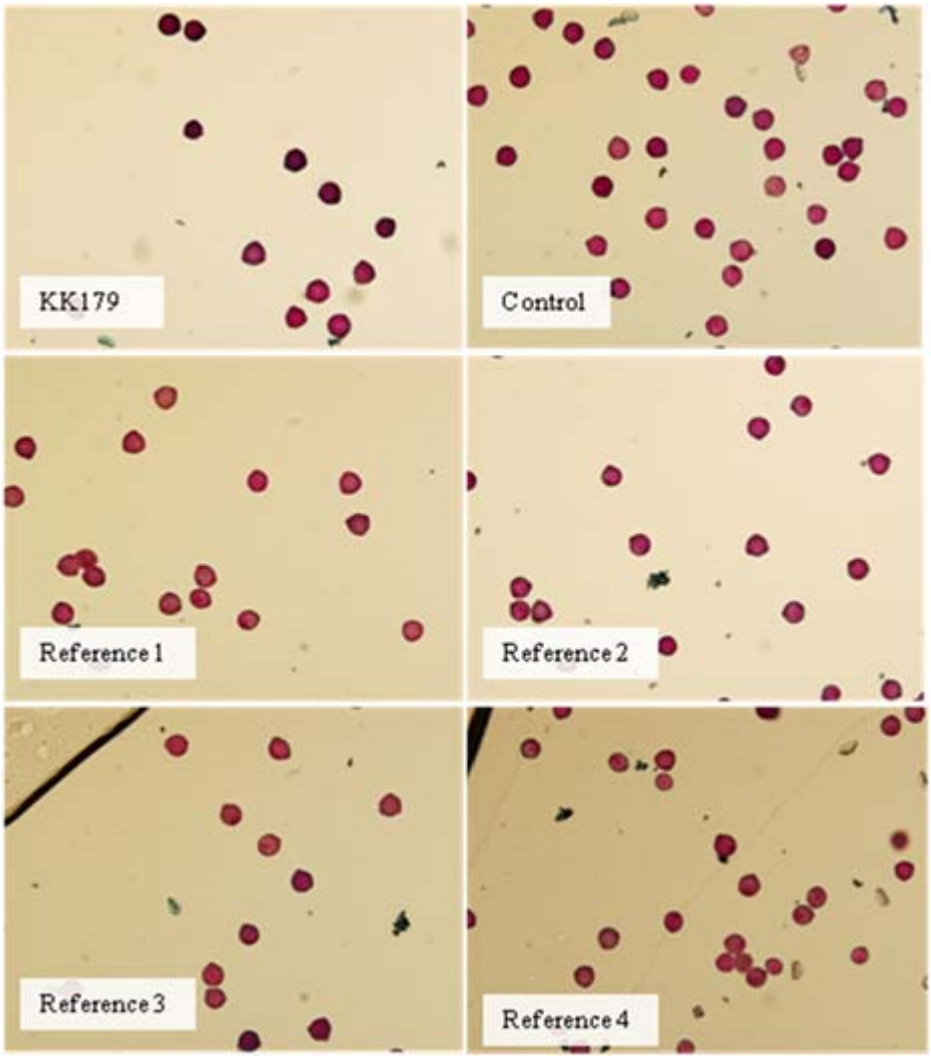

Figure I-1. General Morphology of Pollen from KK179, the Control, and Conventional Commerical Reference Varieties under 200X Magnification

The alfalfa pollen samples were stained with Alexander's stain diluted 1:5. Viable pollen grains stained red to purple, while non-viable pollen grains stained blue to green and may appear round to collapsed depending on the degree of hydration. No visual differences were observed between KK179 and the conventional control or conventional commercial reference alfalfa varieties.

References for Appendix I

Alexander, M.P. 1980. A versatile stain for pollen fungi, yeast and bacteria. Stain Technology 55: 13-18.

SAS. 2008. SAS/STAT software version 9.2. SAS Institute, Inc., Cary, North Carolina.

Appendix J: Materials and Methods for Flower Morphology Assessment

J.1. Plant Production

Transplants of KK179, the conventional control (C_0-Syn1), and four conventional commercial reference varieties were grown under similar agronomic conditions in a field trial in 2010 in Walworth County, WI (WIDL). Prior to transplantation, seedlings of KK179 were sampled and tested using event-specific PCR analyses. Plants not containing KK179 were not used in the field trial. The trial was arranged in a randomized complete block design with four replications. Each plot consisted of seven 3.5-foot rows, with seven plants per row, for a total of 49 plants per plot.

J.2. Flower Collection

Racemes (*i.e.*, a type of inflorescence) were collected from a single plot at a time, in plot sequence, at approximately the 10% bloom stage. From each plot, a minimum of 10 racemes were collected from among the inner 25 plants and were non-systematically selected from the top, middle, or bottom of a minimum of 10 separate plants. Racemes were placed in a single clean container that was pre-labeled with entry number, entry name, and plot number from which the sample originated. Each raceme had approximately 5-40 flowers, with not less than half of the individual flowers fully opened. The containers were kept on wet ice from collection until the photographs and measurements were taken.

J.3. Flower Sample Preparation

Ten racemes were taken out from the labeled plastic container from each plot using a forceps and placed on a white paper for detailed evaluation. These 10 racemes of each plot were arranged in a group (2 rows × 5 plants) and were photographed, with the plot number, location, date, and a scale bar identified in each photograph.

From each raceme, one individual non-senesced flower was detached from the rachis (*i.e.*, main stem of the raceme) with a forceps, for a total of 10 flowers per plot. Each flower was tripped using a forceps by pushing the fused keel petals and then evaluated. A single flower from each plot was then non-systematically selected and photographed.

J.4. Data Collection

KK179 was compared to the conventional control for flowers per raceme, standard petal length, keel petal length, calyx tube diameter, sexual column length, wing petal length, flower color class (USDA-ARS, 1972), gross raceme morphology, and gross flower morphology (Table VII-15). Flower characteristics were assessed by viewing samples with an Olympus® SZ60 stereo-zoom microscope equipped with a Nikon© D5000 digital

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color camera. Descriptions of each characteristic evaluated are listed in Table VII-1 and are shown in Figure J-1.

J.5. General Flower Morphology

Gross raceme morphology and gross flower morphology were observed from digital images of whole racemes and individual flowers of KK179, the conventional control, and the conventional commercial reference varieties (Figures J-2 and J-3).

J.6. Statistical Analysis

An analysis of variance was conducted according to a randomized complete block design using SAS (SAS, 2008) to compare KK179 and the conventional control for the characteristics: flowers per raceme, standard petal length, keel petal length, calyx tube diameter, sexual column length, and wing petal length. The level of statistical significance was predetermined to be 5% (α=0.05). KK179 and the conventional control were not statistically compared to the conventional commercial reference varieties. Minimum and maximum mean values were calculated for each characteristic from the four conventional commercial reference varieties. Analysis of variance and the calculation for quantitative descriptive statistics was not appropriate for flower color class, gross raceme morphology, and gross flower morphology due to the nominal nature of the responses. For these characteristics, the rating observed most often is reported for KK179 and the conventional control, and the reference range consists of the minimum and maximum observed ratings from among the conventional commercial reference varieties.

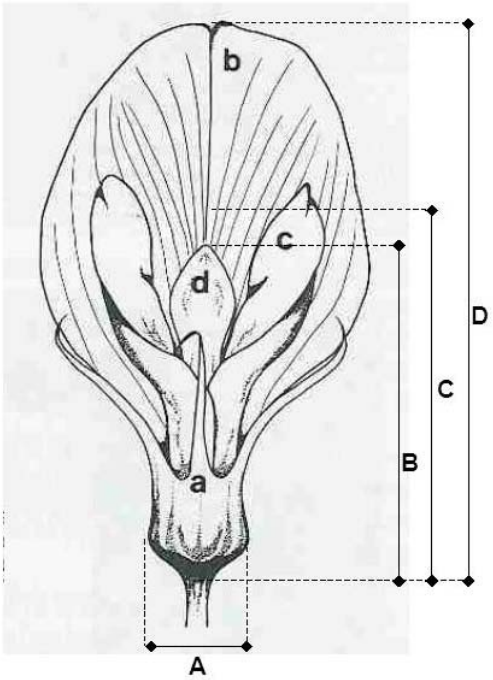

Figure J-1 Diagram of an Untripped (Unopened) Alfalfa Flower

a-sepals, **b**-standard petal, **c**-wing petals, and **d**-fused keel petals, which conceal the sexual column.

Intact flowers were tripped to reveal the sexual column and measurements were recorded for: **A**- diameter of calyx, **B**- length of keel petals, **C**-length of sexual column after release from keel petals (sexual column is not shown in the diagram) and, **D**- length of standard petal.

The figure was produced by Dr. Larry Teuber of the University of California-Davis.

Table J-1. Starting Plants for Flower Morphology Assessment

Material Name	Genotype / Phenotype	Monsanto Seed Lot Number	Monsanto Transplant Lot Number[1]
KK179	Reduced Lignin; Syn1 population	11265471	11266289
C_0-Syn1	Conventional; Syn1 population	11265474	11266292
Pioneer 54V54	Conventional	11265475	11266293
Croplan LegenDairy 5.0	Conventional	11265476	11266294
Dow/Dairyland HybriForce 400	Conventional	11265485	11266303
Vernal	Conventional	11265487	11266305

Note: The field production source of flowers evaluated also included two additional materials that are outside of the scope of the objectives of this evaluation of KK179.
[1]Transplants were produced in a greenhouse and were analyzed by PCR to verify identity.

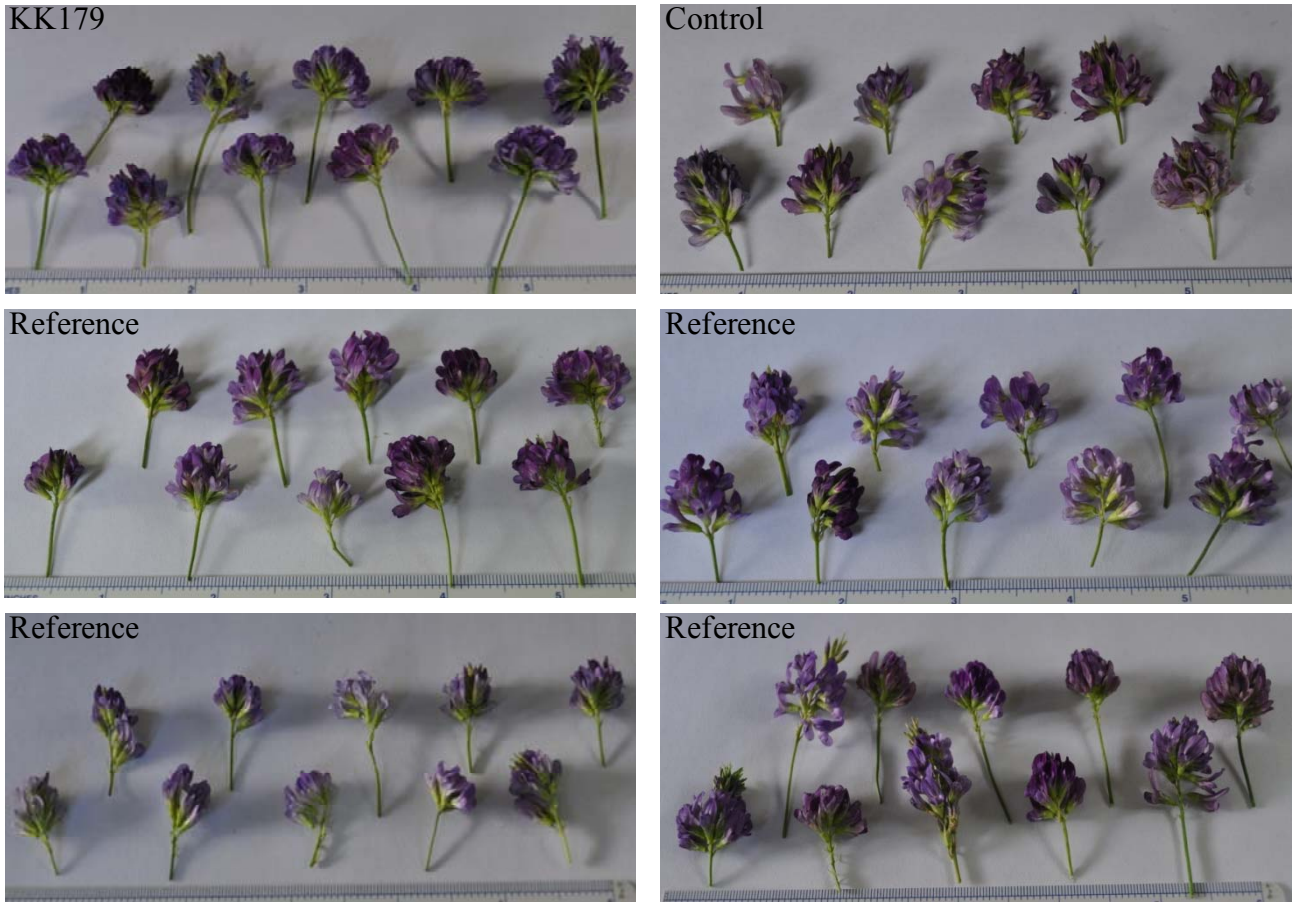

Figure J-2. General Morphology of Whole Racemes from KK179, the Conventional Control, and the Conventional Commercial Reference Varieties

No visual differences were observed between KK179 and the conventional control or the conventional commercial reference varieties.

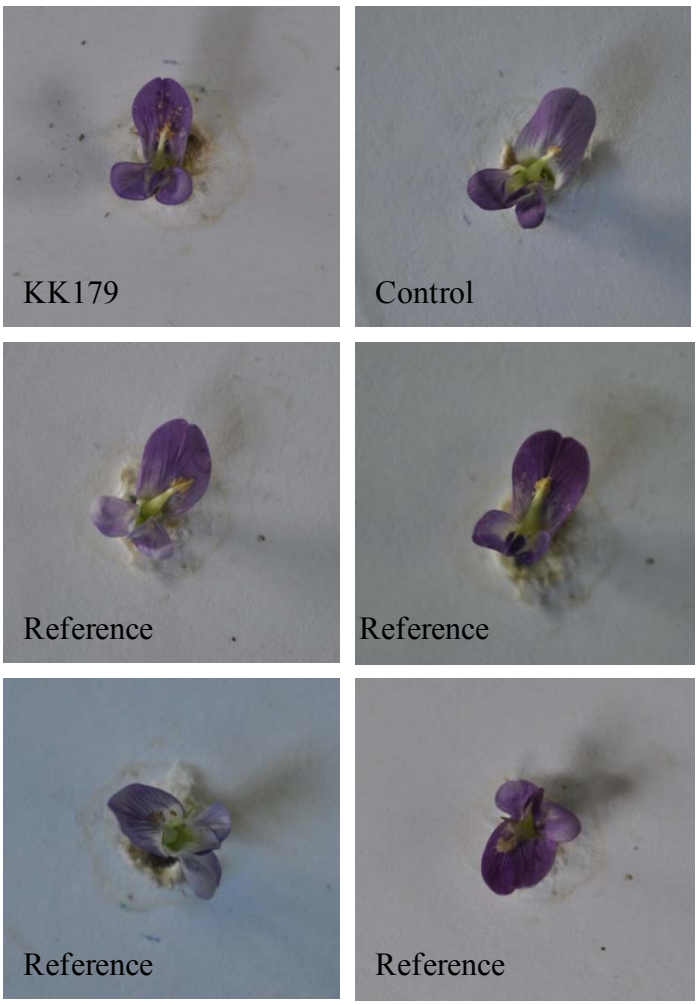

Figure J-3. General Morphology of Tripped Flowers from KK179, the Conventional Control, and the Conventional Commercial Reference Varieties

No visual differences were observed between KK179 and the conventional control or the conventional commercial reference varieties.

References for Appendix J

SAS. 2008. SAS/STAT software version 9.2. SAS Institute, Inc., Cary, North Carolina.

USDA-ARS. 1972. A system for visually classifying alfalfa flower color. Agriculture Handbook No. 424. U.S. Department of Agriculture, Agricultural Research Service, Washington, D.C. http://www.naaic.org/Resources/colorguide/flowercolor.html [Accessed April 4, 2012].

Appendix K: Materials and Methods for Symbiont Assessment

K.1. Background

Members of the bacterial families Rhizobiaceae and Bradyrhizobiaceae form a highly complex and specific symbiotic relationship with leguminous plants and fix a substantial portion of the world's supply of organic nitrogen (Gage, 2004). The nitrogen-fixing plant-microbe symbiosis results in the formation of root nodules, which provide an environment in which differentiated bacteria called bacteroids are capable of reducing or "fixing" atmospheric nitrogen. The product of nitrogen fixation, ammonia, can then be utilized by the plant. In alfalfa, atmospheric nitrogen is fixed into organic nitrogen through a symbiotic association with the bacterium *Sinorhizobium meliloti* which can contribute up to 64% of alfalfa's nitrogen requirement (Lanyon and Griffith, 1988).

The relative effectiveness of the symbiotic relationship between a leguminous plant and its rhizobial symbiont can be assessed by various methods. Measurement of nodule dry weight along with plant growth and nitrogen status are commonly used to assess differences in the symbiotic relationship between a legume and its associated rhizobia (Israel et al., 1986).

K.2. Materials

KK179, the conventional control (C_0-Syn1), and six conventional commercial reference varieties were used in a symbiont interaction assessment (Table K-1). Nodules, root tissue, and shoot tissue collected from KK179, the conventional control, and the conventional commercial reference varieties were evaluated.

K.3. Characterization of the Materials

The presence or absence of KK179 in the starting seed of KK179 and the conventional control was verified by PCR analyses. Approximately 1-2 weeks after planting, seedlings of KK179 were sampled and tested using event-specific PCR analyses. Plants not containing KK179 were removed from the trial.

K.4. Greenhouse Phase and Experimental Design

Starting seed of KK179, the conventional control, and six conventional commercial reference varieties were planted in 6-inch pots containing a custom blend of nitrogen deficient, peat-based potting medium (½ bag each of Premier Horticulture Promix PGX and Promix BX, ¼ cup lime and 2 cups sand). Plants were grown in a greenhouse where temperatures ranged from approximately 20 °C to 35 °C. Prior to planting, pots were saturated with water and allowed to drain for a minimum of four hours. Following this, approximately 500 ml of nitrogen-free nutrient solution was added to each pot. Ten replicate pots were planted with 10 seeds per pot for each of KK179, the conventional control, and the six conventional commercial reference varieties. At planting, each seed was inoculated with a dry, clay-based inoculant containing a minimum of 7×10^7 cells of *S. meliloti* (Dormal, Becker Underwood, Ames, IA). Pots were arranged in ten replicated

blocks containing one pot each of KK179, the conventional control, and the six conventional commercial reference varieties using a randomized complete block design.

Replicates 1, 2, 3, and 4 were planted on September 6, 2011; replicates 5, 6, and 7 were planted on September 7, 2011; and replicates 8, 9 and 10 were planted September 8, 2011. A nitrogen-free nutrient solution (target of 250 ml) was added weekly after plant emergence. In addition, the plant developmental growth stage was evaluated and documented once per week.

Plants from all replicates were thinned to three seedlings per pot on September 27, 2011. Replicates 1, 2, 3, and 4 plants were harvested on October 25, 2011; replicates 5, 6, and 7 were harvested on October 26, 2011; and replicates 8, 9, and 10 were harvested on October 27, 2011. Ten pots containing three plants each were harvested for each of KK179, the conventional control, and the six conventional commercial reference varieties with the following exceptions: four pots had an extra plant emerge after thinning, which was not noticed until harvest, and one pot contained plants that were dead at harvest time. These pots were excluded from the statistical analysis.

K.5. Plant Harvesting/Data Collection

Six weeks after emergence, plants were excised at the surface of the potting medium and shoot and root plus nodule material were removed from the pots. The shoot material was cut into smaller pieces and placed in labeled bags. The plant roots with nodules were separated from the potting medium by washing with water. Excess moisture was removed using absorbent paper towels, and the roots plus nodules were placed in labeled bags. The same day that plants were harvested, nodules were removed by hand from the roots of each plant and weighed to determine the fresh weight (fwt) of the nodules. Nodules were not enumerated as the alfalfa nodules were branched and clustered, making it impossible to record an accurate count. This was due to the fact that alfalfa nodules are indeterminate and continually form new nodule tissue (Gage, 2004). The remaining root and shoot fresh weight was recorded for each plant. Nodules as well as root and shoot material were placed in a drying oven on the same day as collected. The plant material was dried for at least 72 hours at approximately 65 °C to determine dry weight (dwt).

After drying, the shoot tissue was ground with a Harbil 5G high-speed paint shaker and stored at -20 °C prior to shipping to Agvise Laboratories for total nitrogen analysis. Shoot percent total nitrogen was determined by combustion using a nitrogen analyzer according to a study-specific work procedure. Shoot total nitrogen (g) was calculated using the shoot dwt and shoot percent of total nitrogen values. Any remaining seeds and plants were devitalized per USDA-APHIS requirements.

K.6. Statistical Analysis

The data analyzed consisted of five measurements taken at the six-week sampling period: nodule dwt (g), shoot dwt (g), root dwt (g), shoot percent total nitrogen, and shoot total nitrogen (g) (Table VII-16).

An analysis of variance was conducted using a randomized complete block design with ten replications for each of KK179, the conventional control, and the six conventional commercial reference varieties. Data were analyzed using SAS (SAS, 2008) with the level of statistical significance predetermined to be 5% (α=0.05). The means of KK179 and the conventional control were compared to each other. Minimum and maximum values (reference range) were determined for the six conventional commercial reference varieties. No statistical comparisons were made between KK179 and the conventional commercial reference varieties.

Table K-1. Starting Seed of KK179, Conventional Control, and Conventional Commercial Reference Varieties Used in the Symbiont Assessment

Starting Seed Name	Genotype / Phenotype	Monsanto Lot Number
KK179	Reduced Lignin; Syn1 population	11265471
C_0-Syn1	Conventional; Syn1 population	11265474
DKA50-18	Conventional	11265481
FG78T803	Conventional	11293630
FG98M817	Conventional	11293629
Ranger	Conventional	11265488
Vernal	Conventional	11265487
WL 319HQ	Conventional	11265484

References for Appendix K

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Israel, D.W., J.N. Mathis, W.M. Barbour and G.H. Elkan. 1986. Symbiotic effectiveness and host-strain interactions of *Rhizobium fredii* USDA 191 on different soybean cultivars. Applied and Environmental Microbiology 51: 898-903.

Lanyon, L.E. and W.K. Griffith. 1988. Nutrition and fertilizer use. Pages 333-372 in Alfalfa and Alfalfa Improvement. A.A. Hanson, D.K. Barnes, and R.R. Hill, Jr. (eds.). American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc., Madison, Wisconsin.

SAS. 2008. SAS/STAT software version 9.2. SAS Institute, Inc., Cary, North Carolina.