FDA U.S. FOOD & DRUG ADMINISTRATION
CENTER FOR VETERINARY MEDICINE

Biotechnology Notification File No. 000184
CVM Note to the File

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From: Jing Ning, Ph.D.

To: Administrative Record, BNF No. 000184

Subject: Event Altered Lignin Alfalfa

Keywords: Alfalfa, *Medicago sativa* L., altered lignin profile, Transcription Activator-Like Effector Nuclease (TALEN), [redacted] gene, genome editing, gene knock out, Cibus, Inc., Calyxt, Inc.

Purpose

This document summarizes the Food and Drug Administration (FDA) Center for Veterinary Medicine's (CVM, we) evaluation of biotechnology notification file (BNF) number 000184. Cibus, Inc. (Cibus)[1] submitted a safety and nutritional assessment for an altered lignin profile alfalfa variety (hereafter referred to as altered lignin alfalfa[2]) and additional information afterwards. CVM evaluated the information in Cibus' submissions to ensure that regulatory and safety issues regarding animal food derived from altered lignin alfalfa have been resolved prior to commercial distribution. FDA's Human Foods Program summarizes its evaluation of uses of altered lignin alfalfa in human food in a separate document.

In CVM's evaluation, we considered all of the information provided by Cibus as well as publicly available information and information in the agency's files. Here we discuss the outcome of the consultation for animal food use, but do not intend to restate the information provided in the final consultation in its entirety.

Intended Effects

The intended effect of the genetic modification in altered lignin alfalfa is to reduce the content of coniferyl and sinapyl alcohols, two of the monolignols used in lignin synthesis[3]. Cibus accomplished this by deletion of nucleotide sequences within the homologues of the [redacted] gene, which results in the inactivation of [redacted].

[1] The FDA was notified on August 9, 2023 of a merger between Calyxt, Inc. and Cibus, Inc.
[2] Cibus refers to the altered lignin alfalfa variety as "IQ alfalfa" in the BNF submission.

Regulatory Considerations

The purposes of this evaluation are (1) to assess whether Cibus has introduced into animal food a substance requiring premarket approval as a food additive and (2) to determine whether use of the new plant variety in animal food raises other regulatory issues with respect to the Federal Food, Drug, and Cosmetic Act (FD&C Act).

Genetic Modification and Characterization

Introduced DNA and Transformation Method

Transient expression of three plasmids in alfalfa protoplasts was accomplished using polyethylene glycol mediated transformation. Each of two Transcription Activator-Like Effector Nuclease (TALEN)[4] plasmids carried the coding sequence for DNA-binding motifs that specifically recognize target sequences (TAL effector) and a monomer of the restriction endonuclease, FOK1. Expression of the plasmids in protoplasts leads to double stranded breaks in the [redacted] alleles, which are repaired through cell mediated DNA strand break repair. The third plasmid was designed to enhance genome editing. The protoplast cells were then grown in media that induced calli formation and whole plants were regenerated from calli. Cibus states that plasmids were segregated out during cell division because they do not contain sequences (such as selection markers) that enhance maintenance of plasmids in plant cells. The [redacted] alleles were polymerase chain reaction (PCR) amplified using genomic DNA isolated from the [redacted] plants. The amplicon sequences were determined by Illumina sequencing. The [redacted] plants that contained deletions in [redacted] were selected for further development.

Cibus confirmed using PCR analysis that nucleotide sequences that are present in the three plasmids were not present in [redacted] plants selected for further analyses. Cibus also performed whole genome sequencing and collected sufficient data to achieve at least 45.1-fold read coverage. Bioinformatics analyses were used to confirm that there were no transformation plasmid sequences in altered lignin alfalfa genome. Cibus concludes that the genotype of the [redacted] alleles in the [redacted] plants consisted of [redacted], respectively. The deletions in the [redacted] lead to out of frame reads that result in early termination of the transcripts.[5]

Cibus conducted *in silico* analysis to determine whether any of the TALEN could bind to unintended nucleotide sequences in the reference genome and lead to off-target changes in altered lignin alfalfa genome. Cibus concludes that the TALEN pairs used to generate

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[4] A TALEN is an artificial fusion protein that comprises of two functional domains, a DNA-binding domain and a non-specific DNA-cleaving nuclease domain. TALENs function in pairs and they form a dimer to create double-strand break between the two binding sites.

[5] [redacted] Cibus notes that plants have mechanisms like nonsense-mediated mRNA decay, nonstop mRNA decay, and no-go decay that help prevent translation of altered transcripts.

altered lignin alfalfa were highly specific and, thus, there were no TALEN mediated off-target mutations present in altered lignin alfalfa.

Stability and Inheritance

Cibus states that the stability of the intended edits in altered lignin alfalfa was confirmed by PCR amplification of the [redacted] alleles, followed by sequencing of the amplicons from plants belonging to several [redacted] populations. Cibus also states that segregation ratios for the [redacted] alleles were determined for crosses between [redacted]. Cibus states that the results of Chi-square analysis of the segregation data from multiple generations of breeding show that the [redacted] alleles are inherited according to the expected Mendelian principles.

Protein Safety

[redacted]. Cibus highlights that all edits of [redacted] result in deletion of the dimerization and methyltransferase domains. Cibus concludes that in the unlikely event that a protein is produced by the modified alleles, it is expected that the protein would be non-functional. The wild type gene allele would be anticipated to produce the full-length protein, which is 365 amino acids in length. Furthermore, Cibus used bioinformatic analyses to identify potential new open reading frames related to the edited sequences that could lead to production of putative peptides and to assess whether these putative peptides could be toxins. Cibus compared these putative peptide sequences to those in the National Center for Bioinformatics Information nonredundant protein sequence database filtered using the keywords “tox, toxic, and toxin”. Cibus concludes that the modified [redacted] alleles do not contain putative peptide sequences that have relevant similarity to known toxins.

Animal Food Use

Cibus states that altered lignin alfalfa is expected to be grown for the same uses as currently commercialized Alfalfa. Alfalfa (*Medicago sativa* L.) is grown as a perennial forage crop that is harvested several times throughout the growing season. It is one of the principal forage crops cultivated in the United States for animal food. Alfalfa forage products, including hay, grazing, and silage, are valued for their high protein content and highly digestible fiber[6] for ruminants and horses.

Composition

Scope of Analysis

Cibus analyzed the nutrient composition of forage samples from altered lignin alfalfa, conventional unedited alfalfa[7] (control), and three unedited commercial alfalfa varieties (reference varieties) that were grown and harvested under similar conditions. The selected components were based on the Organisation for Economic Co-operation and Development (OECD) alfalfa composition consensus document[8], lignin biosynthetic pathway and phenylpropanoid metabolism in alfalfa.

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[6] Li, X. H., & Brummer, E. C. 2012. Applied Genetics and Genomics in Alfalfa Breeding. Agronomy. 2(1), 40-61

[7] The conventional unedited alfalfa control has similar genetic and agronomic background to altered lignin alfalfa, except for the modified [redacted] alleles.

[8] Organisation of Economic Co-operation and Development. 2005. Consensus Document on Compositional Considerations for New Varieties of Alfalfa and Other Temperate Forage Legumes: Key

Study Design

Cibus conducted field trials at three locations with four replicate plots at each location in the United States. The five alfalfa varieties (altered lignin alfalfa, control, and three reference varieties) were included in each replicate. Representative forage samples were harvested at the bud stage of growth (<10% bloom) from each entry and bulked for analysis. Bulked samples were dried and ground prior to sending for analysis.

Cibus presents mean values and ranges of each component for altered lignin alfalfa and the control. In addition, the across-locations means for components were compared to the range of values obtained for three reference varieties that were grown at the three sites and to ranges published in the OECD alfalfa composition consensus document or scientific literature. Results were all expressed on a dry matter basis.

Results of Analyses

Cibus analyzed proximates (moisture, crude protein, total fat, carbohydrates by calculation, and ash). Cibus reports that the values for proximates were within the range of the control and all of the values fell within the ranges for these components as reported in the OECD consensus document or scientific literature. Cibus also analyzed fiber components [neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), syringyl (S) lignin, and guaiacyl (G) lignin][9]. The mean values for NDF and ADF were numerically lower for altered lignin alfalfa when compared to the mean value for control. However, the range of values for altered lignin alfalfa and control fell within the range of values for these components that were reported in either the reference varieties or OECD consensus document. Cibus also measured the concentrations of 18 amino acids and nine minerals. The mean values for these components in altered lignin alfalfa and control fell within the ranges for these components as reported in the reference varieties and OECD consensus document. Cibus also highlights that mean values for five secondary metabolites (sinapine, coumesterol, *p*-coumaric acid, ferulic acid, and total polyphenols) were similar for altered lignin alfalfa and control and these values fell within the ranges for the reference varieties. Cibus concludes that, except for the intended alteration in lignin profile, altered lignin alfalfa is compositionally equivalent to the conventional unedited control and other reference varieties of alfalfa.

Cibus argues that the biosynthetic pathways of coniferyl and sinapyl alcohols are not entirely eliminated and predicts that there would not be a large buildup of intermediates that would affect other metabolic pathways. Cibus also notes that the isoflavone and saponin biosynthetic pathways are distinct and separate from the lignin biosynthetic pathway, with no common intermediates. Thus, Cibus concludes that levels of isoflavones and saponins in altered lignin alfalfa would not be affected by the reduction in [redacted] activity in altered lignin alfalfa.

Feed Nutrients, Anti-Nutrients and Secondary Plant Metabolites. Organisation for Economic Co-operation and Development, ENV/JM/MONO (2005)13. OECD, Paris.

[9] The results of statistical analyses were not provided.

The level of ADL was measured in altered lignin alfalfa, control, and reference varieties.[10] Cibus states that the mean value for ADL in altered lignin alfalfa was different from than in the control (6.51 versus 7.18% of dry weight). The mean values for ADL in altered lignin alfalfa fell within the range of values reported in OECD consensus document, while the lowest value for altered lignin alfalfa was slightly lower than the range of values obtained for the reference varieties grown at the same locations. The levels of S and G lignin were also measured. Cibus reports different levels of S lignin (2.08 versus 2.55% of dry weight) and G lignin (3.74 versus 4.55% of dry weight) in altered lignin alfalfa when compared to the control. However, the mean values for S and G lignin in altered lignin alfalfa fell within the range of values for the reference varieties.

Summary of Compositional Analyses

Cibus highlights that the gene edited [redacted] alleles in altered lignin alfalfa do not meaningfully impact nutrient composition other than the intended alteration in the amount of G, S lignin monomeric subunits and ADL. Cibus further concludes that altered lignin alfalfa is comparable in nutritional value to alfalfa varieties that are currently used in animal food in the United States.

Conclusion

CVM evaluated Cibus' submissions to determine whether altered lignin alfalfa raises any safety or regulatory issues with respect to its uses in animal food. Based on the information provided by Cibus and other information available to the agency, CVM did not identify any safety or regulatory issues under the FD&C Act that would require further evaluation at this time.

Cibus concludes that altered lignin alfalfa and the animal foods derived from it are as safe as and are not materially different in composition or any other relevant parameter from conventional alfalfa varieties grown, marketed, and consumed in the United States. At this time, based on Cibus' data and information, CVM considers Cibus' consultation on altered lignin alfalfa for use in animal food to be complete.

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[10] Cibus states that samples were analyzed at USDA-Dairy Forage Research Laboratory using near infrared red spectrometry analysis.