Appendix P. FDA Submission

blank page

Biotechnology Consultation Agency Response Letter BNF No. 000084

December 10, 2004

Glen Rogan
Regulatory Affairs Manager
Monsanto Company
800 N. Lindbergh Blvd.
St. Louis, MO 63167

Dear Mr. Rogan:

This is in regard to the consultation that Monsanto Company (Monsanto) and Forage Genetics Incorporated (Forage Genetics) initiated with the Food and Drug Administration (FDA) (Center for Veterinary Medicine and Center for Food Safety and Applied Nutrition) on their genetically engineered alfalfa event J101 and event J163. According to Monsanto and Forage Genetics, these new events are both engineered to express the same new protein, CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS). The CP4 EPSPS protein confers tolerance to glyphosate herbicide. All materials relevant to this notification have been placed in a file designated BNF 0084. This file will be maintained in the Office of Food Additive Safety.

As part of bringing the consultation regarding these products to closure, Monsanto and Forage Genetics jointly submitted a summary of their safety and nutritional assessment of the genetically engineered alfalfa varieties, dated October 6, 2003. Monsanto and Forage Genetics submitted additional information in a submission dated June 15, 2004. These communications informed the FDA of the steps taken by Monsanto and Forage Genetics to ensure that these products comply with the legal and regulatory requirements that fall within FDA's jurisdiction. Based on the safety and nutritional assessment Monsanto and Forage Genetics have conducted, it is our understanding that Monsanto and Forage Genetics have concluded that food and feed derived from the new alfalfa varieties are not materially different in composition, safety, and other relevant parameters from food and feed derived from alfalfa varieties currently on the market, and that the genetically engineered alfalfa varieties do not raise issues that would require premarket review or approval by the FDA.

It is the responsibility of Monsanto and Forage Genetics to obtain all appropriate clearances, including those from the Environmental Protection Agency and the United States Department of Agriculture, before marketing food or feed derived from either alfalfa event J101 or event J163.

Based on the information Monsanto and Forage Genetics have presented to FDA, we have no further questions concerning food or feed derived from alfalfa event J101 or event J163 at this time. However, as you are aware, it is Monsanto's and Forage Genetics' continued responsibility to ensure that foods marketed by the firms are safe, wholesome, and in compliance with all applicable legal and regulatory requirements.

Sincerely yours,

Laura M. Tarantino, Ph.D.
Director
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

CFSAN/Office of Food Additive Safety December 8, 2004

Biotechnology Consultation

Note to the File

BNF No. 000084

Date: December 8, 2004

Subject: Glyphosate-tolerant (Roundup Ready®) Alfalfa Event J101 and Event J163

Keywords: alfalfa, *Medicago sativa* L., glyphosate (N-phosphonomethylglycine), glyphosate-tolerant, herbicide-tolerant, CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS), *Agrobacterium* sp. strain CP4, Roundup Ready®

1.0 Background

In a submission dated October 6, 2003, Monsanto Company (Monsanto) and Forage Genetics Incorporated (Forage Genetics) (jointly the notifiers) provided summary data and information supporting their safety assessment of two glyphosate-tolerant alfalfa (*Medicago sativa* L.) lines. One line contains the transformation event designated J101, and the second line contains the event designated J163. The notifiers intend to combine event J101 and event J163 through conventional breeding to produce commercial glyphosate-tolerant alfalfa seed. Monsanto and Forage Genetics submitted additional information in a submission dated June 15, 2004. Monsanto has successfully completed prior consultations with the agency on other glyphosate-tolerant plants expressing CP4 EPSPS protein.

2.0 Intended Effect

The intended effect of this genetic modification is to confer tolerance to the herbicide glyphosate, which is the active ingredient in Roundup®. The notifiers achieved this by transforming the parent alfalfa R2336 with a 5-enolpyruvylshikimate-3-phosphate synthase gene (*cp4 epsps*) from *Agrobacterium* sp. CP4 strain. The 5-enolpyruvylshikimate-3-phosphate synthase protein (CP4 EPSPS) encoded by this gene provides tolerance to glyphosate herbicide.

3.0 Method of Development

3.1 Genetic Modifications

Glyphosate-tolerant alfalfa events J101 and J163 were generated by *Agrobacterium*-mediated transformation of *Medicago sativa* L. line R2336 callus with the binary T-DNA vector PV-MSHT4. Transformant cells were identified through selection for glyphosate tolerance. The T-DNA segment of the PV-MSHT4 vector contains a single *cp4 epsps* expression cassette with the following elements intended for transfer to the recipient plant line during transformation.

Name	Description
RB	Right border
P-eFMV	35S promoter of the Figwort Mosaic Virus with duplicated enhancer region
HSP70-Leader	The petunia heat shock protein 70 5' untranslated leader sequence
CTP2	Chloroplast transit peptide coding sequence derived from the *Arabidopsis thaliana epsps* gene
cp4 epsps	*cp4 epsps* gene derived from *Agrobacterium* sp. strain CP4
E9 3'	Terminator sequence from the pea ribulose-1,5-bisphosphate carboxylase, small subunit E9 gene
LB	Left border

The transformation vector PV-MSHT4 contains the *ori-322* origin of DNA replication, which allows the replication of the vector in the intermediate host *E. coli*, and the streptomycin adenyltransferase (*aad*) gene, which encodes a protein conferring resistance to the antibiotics streptomycin and spectinomycin. The *ori-322* and *aad* genes are located on the vector backbone, outside of the T-DNA border sequences; therefore, the genes are not intended for transfer to the recipient plant line during transformation.

3.2 Insert Characterization and Stability

The notifiers characterized events J101 and J163 using restriction endonuclease digestion and Southern blot analysis. According to the notifiers, the results of their characterization support the conclusion that, for events J101 and J163, a single, intact copy of the *cp4 epsps* gene cassette (of the PV-MSHT4 vector T-DNA) was incorporated into *Medicago sativa* L. line R2336, and that, for each event, the *cp4 epsps* gene cassette was incorporated at a different locus. The notifiers also used Southern blot analysis to conclude that the integrity of the *cp4 epsps* gene cassette was maintained and that alfalfa events J101 and J163 do not contain any detectable backbone sequence from the PV-MSHT4 vector.

2.1 The notifiers assessed the genetic stability of the insertions in J101, in J163, and in a population resulting from the conventional breeding of J101 and J163 (dihomogenic

population). The notifiers explained that, for the single event populations, both chi square analysis of phenotype segregation and Southern blot analysis of genotype segregation indicate a stable, one-locus Mendelian inheritance pattern and that, for the dihomogenic populations, chi square analysis of the Syn1 generation indicates normal Mendelian inheritance for the two independent loci.

The notifiers stated that alfalfa is an outcrossing autotetraploid plant with eight sets of chromosomes and that varieties of alfalfa are composed of a heterogenous group of individuals. As a consequence of the genetics associated with conventional breeding of the J101 and J163 alfalfa lines, the *cp4 epsps* gene copy number is variable with advancing generations, ranging from zero to eight, for populations carrying both events.

4.0 Introduced Protein

4.1 Identity and Function

The expressed gene product in events J101 and J163 is a protein, CP4 EPSPS, derived from *Agrobacterium* sp. strain CP4. It is a single polypeptide 455 amino acids long and structurally and functionally similar to native plant EPSPS enzymes. Naturally occurring plant EPSPS, which catalyzes an essential step in aromatic amine synthesis essential to plant viability, is inhibited by the herbicide glyphosate. Compared to alfalfa EPSPS, the CP4 EPSPS protein has a much lower affinity for glyphosate, the active component of Roundup herbicide. Consequently, plant cells producing CP4 EPSPS are tolerant to glyphosate treatment.

The notifiers purified the CP4 EPSPS protein from the forage tissue of alfalfa plants containing either event J101 or event J163 and characterized the introduced protein using N-terminal sequence analysis, matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot analysis, and glycosylation analysis.

4.2 Expression Level

The notifiers estimated the expression level of the CP4 EPSPS protein in J101 and in J163 by enzyme-linked immunosorbent assay (ELISA). They determined that the mean levels of CP4 EPSPS protein in forage from alfalfa plants containing event J101 and plants containing event J163, collected across two seasons and from six field sites, were 257 and 270 g/g of tissue fresh weight (tfw), respectively.

5.0 Safety Assessment of the Introduced Protein

The notifiers presented data and information to support the safety of the CP4 EPSPS protein for human and animal consumption. They provided the following arguments in support of CP4 EPSPS protein safety:

Agrobacterium species are not known for human or animal pathogenicity; *Agrobacterium* sp. *cp4*

epsps has a history of use as a genetic donor in numerous glyphosate-tolerant crops, which humans and animals have consumed since 1996.

The CP4 EPSPS protein is functionally equivalent to native plant EPSPS protein except for its affinity for glyphosate.

Based on an amino acid homology search of several toxin databases, the CP4 EPSPS protein does not have biologically relevant structural similarities to protein toxins known to cause adverse health effects in humans or animals.

CP4 EPSPS protein is present at low levels: it represents only a relatively small portion of the total protein (0.49 percent in J101 and 0.52 percent in J163) in alfalfa. Fresh alfalfa forage contains 5.2 percent total protein levels and dried alfalfa contains approximately 20 percent protein by weight.

No treatment-related adverse effects were observed in an acute toxicity test in which mice were gavaged with doses of up to 572 milligrams of CP4 EPSPS1 per kilogram of body weight. According to the notifiers, this represents a safety factor for cows of approximately 26 and 27, for J101 and for J163 respectively, given a 630 kg cow consuming 13 kg of alfalfa dry matter per day.

The notifiers also assessed the allergenic potential for CP4 EPSPS protein produced from events J101 and J163. They concluded that the CP4 EPSPS protein from glyphosate-tolerant alfalfa lines J101 and J163 does not pose a significant allergenic risk based on 1) the absence of known reports of allergies to *Agrobacterium* species, which is the source of the *cp4 epsps* coding sequence transferred to the recipient plant lines, 2) the absence of immunologically relevant sequences, as determined by comparison of the amino acid sequence of the CP4 EPSPS protein to sequences in several allergen databases, and 3) the susceptibility of CP4 EPSPS protein to rapid proteolytic digestion in simulated gastric fluid.

6.0 Compositional Assessment

The notifier states that alfalfa has a long history as a feed source for many animal species, but its greatest use is for dairy and beef cattle. Greater than 95 percent of alfalfa is used as animal feed on farms and is consumed as pasture, greenchop, silage, or dried forage. A small amount of alfalfa is sold and fed as dehydrated pellets. Human food uses of alfalfa are minor and are consumed as compressed leaf material for dietary supplements and herbal teas or as fresh sprouts.

6.1 Justification of Comparable Lines

In their submission, the notifiers stated that, because alfalfa is an outcrossing autotetraploid, seed generated through self-pollination of a single genotype of alfalfa is not of adequate vigor for use in safety assessment studies. Consequently, they used a control population of null segregants (segregants lacking both the glyphosate-tolerant phenotype and the *cp4 epsps* genotype) from the transformation protocol. The notifiers described how these "near isogenic" null segregants were

developed in parallel with J101 and J163 and, in turn, were used to develop control alfalfa populations for use in compositional equivalence evaluations. Statistically significant differences between the transgenic and control alfalfa populations were declared at the 5 percent level of significance (i.e., where the p-value is less than 0.05).

6.2 Compositional Analysis

The notifiers compared the composition of simplex (single copy, single event) glyphosate-tolerant alfalfa lines J101 and J163 to the null segregant control line, using second cutting forage samples grown at five replicated field sites across the alfalfa-producing regions of the United States and harvested at the early to late bloom stage. A randomized complete block design with four blocks at each location was used. Twelve commercially available conventional alfalfa varieties, used to establish commercial compositional ranges, were grown at these sites, with four varieties grown at each of the five field sites. Forage samples were analyzed for 35 nutritional components including proximates (protein, fat, ash and moisture), acid detergent fiber (ADF), neutral detergent fiber (NDF), amino acids, various minerals and carbohydrates (by calculation).

The notifiers developed a tolerance interval using twelve different varieties of commercially available, conventional alfalfa to establish comparable ranges for compositional constituents. Using these ranges, the notifiers calculated the tolerance interval to contain, with 95 percent confidence, 99 percent of the values contained in the population of commercial alfalfa varieties. The notifiers used this 99 percent tolerance interval to determine if the test range was within the variance of a population of reference alfalfa varieties.

The notifiers stated that statistically significant differences were observed for the level of some analytes in comparison to the control population. For line J101, the mean level of cystine was greater than, and the mean levels of glutamic acid and tyrosine were less than, the null segregant controls. For line J163, the mean levels of cystine, acid detergent fiber (ADF), and neutral detergent fiber (NDF) were greater than, and the mean levels of histidine, lysine, and tyrosine were less than, the null segregant controls. However, the notifiers further noted that while the analyte levels were different, these levels were still within Monsanto's and Forage Genetics' commercial compositional ranges and their tolerance interval derived from data on conventional species grown at the same sites, and comparable to published literature values. The notifiers stated that the mean levels of iron and ash for the control and all varieties were unusually high but that these high mean levels could be attributed to high levels measured at one site. Consequently, repeat measurements were made for iron and ash the following year, resulting in levels similar to other sites and values found in the literature. The notifiers also found several differences in the level of nutrients within the locations, but except for the nutrients already cited, these were not consistent across locations. The notifiers therefore, concluded that these differences are unlikely to be biologically meaningful.

6.3 Levels of Naturally Occurring Toxicants and Anti-nutrients

The notifiers cited lignin, an insoluble amorphous macromolecule, as a key anti-nutritional factor in alfalfa where high lignin concentrations decrease alfalfa digestibility. The notifiers measured lignin levels in the forage of plants containing either event J101 or J163 as well as null segregant and commercial varieties. From these measurements, they concluded that the levels of lignin in J101-containing alfalfa were not statistically different from the levels of lignin in the control alfalfa population. The notifiers observed a statistically significant increase in the levels of lignin in J163-containing alfalfa when compared to the levels in control alfalfa. However, the notifiers noted that these levels were within their tolerance interval, established using data derived from conventional species and similar to published literature values. In summary, the notifiers concluded that the lignin levels in glyphosate-tolerant alfalfa are comparable to lignin levels in conventional alfalfa.

The notifiers reviewed the literature and concluded that coumestrol is the most estrogenically active phytoestrogen in alfalfa. The notifiers conducted field trials to measure coumestrol at four locations using a randomized complete block design with four replicates at each location. The combined statistical analysis of all the data showed no statistically significant differences. There were some location differences, but these were not consistent across all locations. The notifiers concluded that coumestrol levels in alfalfa lines J101 and J163 are equivalent to those in the null segregant controls and conventional alfalfa forage.

Digestive bloat constitutes a high health risk to cattle grazing on lush alfalfa pastures or fed fresh greenchop alfalfa. Saponins and soluble forage proteins are suggested as contributing agents to frothy bloat (ruminal tympany) in ruminants. The notifier concluded that a search of the literature did not reveal evidence showing that these compounds are the causative agents for bloat. Further, there are no reliable methods or databases on the level of saponins and soluble forage proteins in alfalfa.

In conclusion of their assessment, the notifiers state that alfalfa event J101 and event J163, and the feeds and foods derived from them, are as safe and nutritious as current commercial varieties of alfalfa and the comparable feeds and foods derived from them.

7.0 Conclusions

Monsanto and Forage Genetics have concluded that their glyphosate-tolerant alfalfa event J101 and event J163, and the feeds and foods derived from them, are not materially different in safety, composition, or any other relevant parameter from alfalfa now grown, marketed, and consumed. At this time, based on Monsanto's and Forage Genetics' description of its data and information, the Agency considers this consultation on alfalfa event J101 and event J163 to be complete.

C. Hendrickson W. D. Price

---

[1] The CP4 EPSPS protein used in this study was produced in *E. coli*. Monsanto and Forage Genetics note that the CP4 EPSPS protein produced in *E. coli* is biologically, chemically, and functionally equivalent to the CP4 EPSPS protein produced in plants.

FDA/Center for Food Safety & Applied Nutrition
Hypertext updated by jmf/rxm February 2, 2005