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CENTER FOR FOOD SAFETY AND APPLIED NUTRITION

CFSAN/Office of Food Additive Safety
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Biotechnology Consultation Note to the File BNF No. 000079

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Subject: Roundup Ready® Creeping Bentgrass Event ASR368

Keywords: creeping bentgrass, *Agrostis stolonifera* L., glyphosate (N-phosphonomethyl-glycine), glyphosate-tolerant, herbicide tolerant, CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS), *Agrobacterium* sp. CP4 strain, Roundup Ready(R)

1. Background

In a submission dated September 13, 2002 and a supplement dated August 18, 2003, Monsanto Company (Monsanto) and The Scotts Company (Scotts) provided summary data and information supporting their safety assessment of glyphosate-tolerant creeping bentgrass (*Agrostis stolonifera* L.) transformation event ASR368. Monsanto has successfully completed prior consultations with the agency on other glyphosate-tolerant plants containing the same introduced gene, including soybean (BNF 000001), canola (BNF 000020, BNF 000077), cotton (BNF 000026), corn (BNF 000035, BNF 000051, BNF 000071), and sugar beet (BNF 000056).

2. Intended Technical Effect and Feed Use

The intended effect of this genetic modification of *A. stolonifera* is to confer tolerance to the herbicide glyphosate. Monsanto and Scotts achieved this by transformation of the parent bentgrass line B99061R with a 5-enolpyruvylshikimate-3-phosphate synthase (*epsps*) gene from *Agrobacterium* sp. CP4 strain. The EPSPS protein encoded by this gene is resistant to glyphosate inhibition.

The place of origin for creeping bentgrass (*A. stolonifera*) is either Eurasia or North America, and wild varieties can be found on both continents. Seeding varieties have been bred for use on golf course putting greens since the middle of the 20th century, and this application remains the primary use of the species. *A. stolonifera* is not generally used for commercial or residential ground cover due to the intensive input required to maintain its aesthetic character. There is

minor use of creeping bentgrass straw consisting mainly of stems remaining after seed harvest as a low quality animal feed. *A. stolonifera* is also known to grow in natural pastures, particularly in the northwestern United States.

3. Method of Development

3.1 Parental Line

Event ASR368 was generated in *A. stolonifera* L. line B99061R. According to the developers, B99061R was selected because it responds well to tissue culture and biolistic transformation.

3.2 Genetic Modifications

The plasmid vector PV-ASGT08 was used to generate event ASR368. The vector backbone contains the pBR322 replication origin, which allows the replication of the vector in the intermediate host E. coli. The vector backbone also contains the neomycin phosphotransferase II (*nptII*) gene. The DNA segment of the PV-ASGT08 vector intended for transformation, designated PV-ASGT08L, contains two *cp4 epsps* gene cassettes. The synthetic *cp4 epsps* coding sequence in both cassettes is derived from *Agrobacterium* sp. strain CP4 and begins with an appended chloroplast transit peptide coding sequence (ctp2) derived from the *Arabidopsis thaliana epsps* gene. The CTP2 transit peptide directs the CP4 EPSPS protein expressed in event ASR368 to chloroplasts. The *ctp2 cp4 epsps* coding sequence in each cassette is followed by the 3' nontranslated region of the nopaline synthase gene from *Agrobacterium tumefaciens* that provides the transcription termination signal. The upstream regulatory sequence of the first cassette consists of an enhanced 35S promoter from the cauliflower mosaic virus (CaMV) and the intron of the hsp70 gene (heat shock protein) from *Zea mays*. The upstream sequence of the second cassette consists of the 5' region of the rice (*Oryzae sativa*) actin1 gene which contains the promoter, transcription start site and first intron. Two distinct promoters were used in order to drive expression of the gene product in both vegetative and reproductive tissues.

The PV-ASGT08 vector was propagated in *E. col* and digested with the restriction enzyme HindIII to separate the backbone from the DNA segment PV-ASGT08L. The PV-ASGT08L segment was subsequently purified, precipitated onto gold particles, and introduced into the recipient variety B99061R by particle acceleration. The transformed tissue was isolated by selection with glyphosate, plants were regenerated from tolerant callus tissue, and ASR368 was isolated from among these plants.

3.3 Insert Characterization and Stability

The developers characterized event ASR368 using Southern blot analysis, polymerase chain reaction (PCR) and DNA sequencing. According to the developers, the results of this characterization support the conclusion that a single copy of PV-ASGT08L was incorporated into the nuclear genome of *A. stolonifera* line B99061R at a single locus. The developers assessed the stability of the inserted DNA in three generations using segregation studies and Southern blot analysis and concluded that the insert was stable.

4. Introduced Protein

4.1 Identity and Function

The introduced substance produced by event ASR368 is a synthetic protein derived from *Agrobacterium* sp. strain CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS). EPSPS is naturally present in plant chloroplasts and catalyzes a critical step in the synthesis of aromatic amino acids. The plant variants of EPSPS bind the herbicide glyphosate which blocks their catalytic activity. CP4 EPSPS has a much lower affinity for glyphosate and is therefore resistant to glyphosate-mediated inhibition. A transit peptide (CTP2) derived from the *A. thaliana* EPSPS protein has been added in order to direct CP4 EPSPS to the chloroplast, which is the normal site of plant EPSPS expression. The transit peptide is cleaved from the mature protein once it reaches the chloroplast.

4.2 Expression Level

The developers tested event ASR368 for expression of CP4 EPSPS using Western blots and enzyme-linked immunosorbent assay (ELISA). The developers determined that the mean expression level of the CP4 EPSPS protein in plants collected from 4 field sites was 68.6 (+/- 17.3) micrograms/gram fresh weight.

5. Safety Assessment of the Introduced Protein

The developer presented studies and data to support the safety of the CP4 EPSPS protein for animal consumption. Similar studies and data had previously been presented by Monsanto to support the safety of this protein for consumption by humans and animals. Safety data include a history of safe consumption, acute toxicity studies, comparative database searches, and simulated digestion studies.

Monsanto conducted studies to determine whether the CP4 EPSPS protein is a potential toxin. Monsanto reports that the CP4 EPSPS protein is readily digested in simulated gastric fluid and simulated intestinal fluid. The company also reports that no treatment-related adverse effects were observed in acute toxicity tests in which mice were gavaged with doses of up to 572 milligrams of CP4 EPSPS per kilogram of body weight.

The developers searched protein databases for sequences homologous to CP4 EPSPS and concluded that the CP4 EPSPS protein is not homologous to known protein toxins. They note that EPSPS proteins with significant homology to the ASR368 CP4 EPSPS protein are found in crop plants as well as fungal and bacterial food sources. They also note that the donor organism (*Agrobacterium* sp. strain CP4) is not known for human or animal pathogenicity. Finally, the developers report that a CP4 EPSPS protein more than 99% similar to that from ASR368 is present in other glyphosate-tolerant food crops that were the subject of prior consultations with FDA. These crops were planted on 44.8 million acres worldwide in 2001.

The developers state that because of the studies and data discussed above and because this CP4 EPSPS protein has a history of safe human and animal consumption, ASR368 creeping bentgrass is considered as safe for use as animal feed as conventional creeping bentgrass straw (forage).

6. Feed Assessment

6.1 Justification of Comparable Feed and Historic Uses

The developers selected the parental creeping bentgrass (*A. stolonifera* L.) line B99061R as the primary comparator. Seven other commercially available creeping bentgrass varieties were used for further comparison with the transformed line. Creeping bentgrass is present in European and North American pastures and meadows as a volunteer colonizer, but is rarely sown for forage. Its primary use for over a hundred years has been as a playing surface on golf courses. The straw produced during seed harvest is sometimes used as a low quality maintenance feed for ruminant animals.

6.2 Nutrient Analysis

The developers compared the composition of the bioengineered ASR368 line to the parental control line B99061R using forage samples from four replicated sites collected during the 2000-2001 field season. Three other control commercial varieties were concurrently grown and sampled at each field site. Samples were also taken from four additional commercial varieties grown at a separate nonreplicated site. The developers analyzed key components of creeping bentgrass including proximates (protein, fat, ash, moisture, and carbohydrates), acid detergent fiber (ADF), neutral detergent fiber (NDF), crude fiber, minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc). Results were reported as means for the bioengineered line and B99061R control, and as ranges for the seven commercial reference varieties.

Monsanto conducted a combined statistical analysis of the analytical results obtained for samples from all of field trials and found no statistically significant differences ($p < 0.05$) in the levels of all measured components between the bioengineered ASR368 line and the control B99061R line. Monsanto also conducted a statistical analysis on the data from each site and found statistically significantly higher levels of moisture and phosphorus at the Illinois site, and statistically significantly lower levels of NDF at the Illinois and Oregon sites in the ASR368 line relative to the B99061 control line. However, these differences were not consistent over all the locations, did not affect the combined statistical analysis, and were within the 99% tolerance interval generated by Monsanto for the seven commercial varieties. In addition, all values were within the range Monsanto determined for the commercial varieties.

6.3 Levels of Naturally Occurring Toxicants and Antinutrients

The developers discussed the possibility of exposure to toxic or deleterious substances naturally found in <>*A. stolonifera*. The developers report that no such substances were discovered in a search of the literature.

7. Conclusions

The developers have concluded that forage from the <>*A. stolonifera* line containing event ASR368 is not materially different in composition, safety, or any other relevant parameter from conventional creeping bentgrass other than its tolerance to glyphosate. At this time, based on the data and information provided by Monsanto and Scotts, the agency considers the developers' consultation on Roundup Ready(R) creeping bentgrass event ASR368 to be complete.

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