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EXECUTIVE SUMMARY
to
Application to Food Standards Australia New Zealand for the inclusion of Lepidopteran-protected maize MON 89034 in Standard 1.5.2 - Food Derived from Gene Technology

Submitted by:

Monsanto Australia Limited
Level 12 / 600 St Kilda Road,
Melbourne Victoria 3004

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EXECUTIVE SUMMARY

Maize (*Zea mays* L.) is susceptible to feeding damage from insect pests resulting in significant economic losses. In 1997, Monsanto commercialised the first-generation YieldGard® Corn Borer (hereafter referred to as MON 810), which produces a Cry1Ab protein that provides effective protection against damage caused by lepidopteran insect pests, especially the European corn borer (ECB, *Ostrinia nubilalis*) and the corn earworm (CEW, *Helicoverpa zea*). The benefits of MON 810 and other Bt maize products have included more effective control of lepidopteran larval pests and lower levels of harmful mycotoxins in Bt maize, which has resulted in improved food and feed safety of maize by reducing insect damage and subsequent fungal infections that produce mycotoxins. This reduction has been consistently demonstrated in countries around the world where corn borers are the predominant insect pests (Clements et al., 2003; Dowd, 2000 and 2001; Hammond et al., 2002 and 2004; de la Campa et al., 2005; Bakan et al., 2002; Magg et al., 2002; Munkvold et al., 1999; Munkvold, 2003; Papst et al., 2005; Pietri and Piva, 2000; Wu, 2006). Furthermore, the use of MON 810 and other Bt maize products has reduced the use of chemical insecticides (Carpenter et al., 2004). Within ten years of the first Bt maize product introduction, the safe and effective use of Bt maize has been adopted globally on over 17 million hectares (James, 2005) to control several primary insect pests of maize in 12 countries.

Recently, Monsanto Company has developed MON 89034 as a second generation product to provide enhanced benefits for the control of lepidopteran pests of maize. MON 89034 will effectively address a maize grower's need to control a wider spectrum of lepidopteran pests, strengthen the insect resistance management, further reduce the potential for mycotoxins in grain, enable more efficient plant breeding of this multi-genic trait into superior hybrids compared to MON 810, and potentially reduce the refuge acreage required for resistance management purposes.

By producing effective levels of two insecticidal proteins, Cry1A.105 and Cry2Ab2, MON 89034 increases the durability of the product against the primary lepidopteran pests of maize. In addition, the individual proteins extend the spectrum of control against lepidopteran insects commonly present in maize fields. Specifically, the Cry1A.105 protein provides increased activity against fall armyworm (FAW, *Spodoptera sp.*) and black cutworm (BCW, *Agrotis ipsilon*) compared to Cry1Ab. The Cry2Ab2 protein provides improved control over Cry1Ab products from damage caused by CEW.

The wider spectrum of activity in MON 89034 will also potentially contribute to the further reduction of mycotoxins in grain that result from fungal invasion after insect feeding damage.

Taken together, adoption of MON 89034 is likely to enhance the economic benefits to farmers and improve the quality of grain and the safety of derived food and feed products. In addition, MON 89034 was developed to allow the efficient introgression of two insect protection traits into improved maize germplasm, which will reduce the time

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and costs for new improved variety introductions into the marketplace. MON 89034 was developed using a single transformation vector containing both the *cry1A.105* and *cry2Ab2* genes. This approach, known as vector stacking, increases the efficiency of breeding multiple traits into new maize hybrids, thereby providing growers an earlier access to improved germplasm containing these traits rather than through conventional inbred stacking.

MON 89034 was produced by *Agrobacterium*-mediated transformation of maize with the PV-ZMIR245 vector, which is a binary vector containing two separate transfer DNA's (2T-DNA). The first T-DNA, designated as T-DNA I, contains the *cry1A.105* and the *cry2Ab2* expression cassettes. The second T-DNA, designated as T-DNA II, contains the *nptII* (neomycin phosphotransferase II) expression cassette. During transformation, both T-DNAs were inserted into the genome. The *nptII* selectable marker gene was used for the selection of transformed cells in the presence of neomycin. A significant proportion of the cells selected for resistance to neomycin due to the presence of T-DNA II will also contain T-DNA I. Once the transgenic cells were identified, the selectable marker gene was no longer needed. Traditional breeding was used to produce plants that only contained the *cry1A.105* and *cry2Ab2* expression cassettes (T-DNA I) and did not contain the *nptII* expression cassette (T-DNA II), thereby, producing marker-free maize MON 89034.

Molecular characterisation of MON 89034 by Southern blot analyses was conducted to determine: (1) the number of inserts and copies in the genome; (2) intactness of the genetic elements within the insert; (3) absence of the T-DNA II encoding the selectable marker; (4) absence of backbone sequences; and (5) stability of the inserted DNA across multiple generations. Results demonstrated that the DNA inserted into the maize genome is present at a single locus and contains one functional copy of the *cry1A.105* and the *cry2Ab2* expression cassettes. All genetic elements were shown to be present in the inserted DNA as expected. However, the *e35S* promoter, which regulates expression of the *cry1A.105* gene, was modified and the Right Border sequence present in PV-ZMIR245 was replaced by a Left Border sequence in MON 89034. There were no other elements, either full length or partial, present other than those associated with the intended insert, and no backbone plasmid DNA or *nptII* sequences were detected. PCR and DNA sequence analyses provided the complete DNA sequence of the insert and confirmed the organisation of the elements within the insert. The stability of the integrated DNA and absence of the T-DNA II and backbone sequences in multiple generations of MON 89034 was also confirmed. The heritability of the *cry1A.105* and *cry2Ab2* genes was confirmed by segregation analysis of several generations of MON 89034. These results are consistent with the conclusion of a single active site of insertion that segregates according to the Mendelian laws of genetics.

Detailed biochemical characterisation of the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034 confirmed their identity and equivalence to the corresponding *E. coli*-produced proteins. The characterisation was based on (1) the source organism from which the two Cry proteins are derived; (2) identity and function; and (3) physicochemical and functional equivalence to the *E. coli*-produced protein standards.

Cry1A.105 is a chimeric protein comprised of domains I and II from Cry1Ab and Cry1Ac, domain III from Cry1F (Bt subsp. *aizawai*), and the C-terminal portion from Cry1Ac (Bt subsp. *kurstaki*). Domains I and II of Cry1A.105 are 100% identical in amino acid sequence to domains I and II of both Cry1Ab and Cry1Ac, domain II is 99% identical to domain III of Cry1F, and the C-terminal portion is 100% identical to the C-terminal portion of Cry1Ac. The overall amino acid sequence identity to the Cry1Ab, Cry1Ac, and Cry1F proteins is 90.0%, 93.6%, and 76.7%, respectively. The Cry1A.105 protein produced in MON 89034 is structurally and functionally similar to Cry1A proteins produced in a number of biotechnology-derived crops (e.g., YieldGard Corn Borer corn, Bollgard® cotton and Bollgard II® cotton) that have a demonstrated history of safe use.

The Cry2Ab2 protein produced in MON 89034 is derived from the Bt subspecies *kurstaki* and its amino acid sequence differs from that of the wild-type protein by a single amino acid. The Cry2Ab2 protein has 88% amino acid sequence identity to the Cry2Aa protein which is present in commercial microbial pest control products such as Dipel® and Crymax®. The Cry2Ab2 proteins produced in MON 89034 and Bollgard II cotton have an identical amino acid sequence.

The characterisation of the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034 confirmed that these proteins are equivalent to the respective *E. coli*-produced protein standards used in safety studies. Since the *in planta* expression of the Cry1A.105 and Cry2Ab2 proteins is low, it was necessary to produce these proteins in the high-expressing recombinant host organism, *E. coli*, so that they could be used in safety studies. The *E. coli*-produced proteins were engineered to have the identical amino acid sequences as their counterparts expressed in MON 89034. The MON 89034- and *E. coli*-produced proteins were then evaluated to ensure that they were physicochemically and functionally equivalent based on the following analytical tests: (a) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to estimate approximate molecular weight; (b) western blot analysis to demonstrate identity and immunoreactivity; (c) N-terminal sequence analysis or western blot analysis to examine the intactness of the N-terminus; (d) matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to establish protein identity by peptide mapping; (d) glycosylation analysis to determine the presence or absence of covalently-linked carbohydrates; and, (e) insect bioassay to assess functional equivalence. These analyses provided a detailed characterisation of the Cry1A.105 and Cry2Ab2 proteins isolated from MON 89034 and confirmed their equivalence to the *E. coli*-produced Cry1A.105 and Cry2Ab2 proteins.

The assessment of potential allergenicity and toxicity showed there was a reasonable certainty of no harm to mammals from exposure to the Cry1A.105 and Cry2Ab2 proteins. These assessments were based on: (a) an evaluation of potential allergenicity based on the source of the protein, structural similarities to known allergens, *in vitro* digestibility in simulated digestive fluids; and, (b) an evaluation of potential toxicity based on history

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of use, similarity to known toxins or biologically active proteins, and evaluation of acute toxicity to mammals.

As mentioned previously, *Bacillus thuringiensis*, the donor organism for these two Cry proteins, has been used commercially for over four decades to produce microbial pesticides, and there are no confirmed cases of allergic reactions to Cry proteins. Results of extensive bioinformatics assessments using FASTA sequence alignment and eight-amino acid sliding window searches showed that the Cry1A.105 and Cry2Ab2 proteins do not share any amino acid sequence similarities with known allergens, gliadins, glutenins, or protein toxins that have adverse effects to mammals. Assessment of the in vitro digestibility in simulated gastric fluid (SGF) showed that the Cry1A.105 and Cry2Ab2 proteins are rapidly digested, with greater than 95% to 99% of the proteins, respectively, being digested in less than 30 seconds. Proteins that are rapidly digestible have a lower risk of causing allergic reactions or resulting in toxicity when consumed. Mice acute oral toxicity studies demonstrate that the Cry1A.105 and Cry2Ab2 proteins are not acutely toxic and do not cause any adverse effects even at maximum attainable dose levels of 2072 and 2198 mg/kg body weight for the Cry1A.105 and Cry2Ab2 proteins, respectively. The independent safety assessment for each of the Cry proteins in mice was considered appropriate and adequate based on the extensive history of safe use of mixtures of Cry proteins present in Bt microbial pesticides.

Tissues of MON 89034 were collected from field trials conducted at five sites in the U.S. during 2005. Tissues from the different growth stages of the maize plant were collected throughout the growing season and analyzed by enzyme-linked immunosorbent assay (ELISA). The mean Cry1A.105 levels across sites were 520 µg/g dwt in young leaf, 42 µg/g dwt in forage, and 5.9 µg/g dwt in grain. The mean Cry2Ab2 levels across sites were in 180 µg/g dwt in young leaf, 38 µg/g dwt in forage, and 1.3 µg/g dwt in grain. In general, the levels of the two Cry proteins declined over the growing season.

Compositional assessment of the grain and forage from MON 89034 demonstrated that it is nutritionally and biologically equivalent to its conventional counterpart, LH198 x LH172. Compositional data on key nutrients, anti-nutrients and other components were collected for the forage and grain from MON 89034 and conventional control maize, grown at five field sites in the U.S. during 2004. Five conventional, commercial maize reference hybrids were also grown at each site, for a total of 15 references. Composition data from the references was used to establish a range of variability described by a 99% tolerance interval for each component analyzed. Statistical comparisons of 61 components from MON 89034 and the control were conducted for the combination of all five sites (i.e., the combined-site) and for each individual site. The overall data set was examined for evidence of biologically relevant changes. Evaluation of the data, including the results of statistical analysis, leads to the conclusion that MON 89034 is compositionally and nutritionally equivalent to conventional maize.

No statistical differences were observed in 58 of 61 combined-site site comparisons made between MON 89034 and the conventional control. The three differences observed were generally small (3.4 – 19.2%), considering the range of natural variability, and the mean levels and ranges of MON 89034 were well within the 99% tolerance intervals for

commercial maize. For the individual site analyses, there were no statistical differences that were consistently observed across all sites. Furthermore, the means and ranges of all components from MON 89034 showing a statistical difference were within the 99% tolerance intervals of conventional maize and/or within the International Life Sciences Institute Crop Composition Database.

In conclusion, the data and information presented in this summary demonstrate that the foods derived from MON 89034 are as safe and nutritious as the comparable foods derived from conventional maize.

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