

**FOOD DERIVED FROM INSECT-PROTECTED
AND
GLUFOSINATE-AMMONIUM TOLERANT
CORN LINE 1507**

A Safety Assessment

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SUMMARY

Food derived from insect-protected and herbicide-tolerant corn line 1507 has been assessed for safety as a food. This line has been developed primarily for agricultural purposes to provide growers with a variety of corn that is both resistant to attack from major Lepidopteran insect pests, including the European corn borer, and tolerant to glufosinate-ammonium herbicide. The evaluation criteria included characterisation of the transferred genes, analysis of changes at the DNA, protein and whole food levels, stability of the introduced genes, evaluation of intended and unintended changes and assessment of the potential allergenicity or toxicity of any newly expressed proteins.

History of use

Corn (*Zea mays* L.) has undergone substantial genetic breeding by conventional methods over many centuries of cultivation and has been safely consumed as food and feed for thousands of years. Products derived from corn include highly processed corn grain fractions such as flour, high fructose corn syrup, corn oil, breakfast cereals and other products.

The two introduced genes are bacterial in origin. One is derived from *Bacillus thuringiensis* which has an established history of safe use as a biopesticide on agricultural crops, including in the organic farming industry. The second gene is also derived from a common soil bacterium, *Streptomyces viridochromogenes*, which has no known pathogenicity.

Nature of the genetic modification

The two genes introduced into corn line 1507 are *cry1F* (insect-protection) and *pat* (herbicide tolerance). The *cry1F* gene is a synthetic version of a gene from *B. thuringiensis* var. *aizawai*, and encodes a truncated version of an insecticidal protein, Cry1F. This protein specifically targets the larval stage of insect pests of major economic importance in corn.

The *pat* gene is derived from *S. viridochromogenes* and encodes the enzyme phosphinothricin acetyltransferase (PAT), which inactivates the herbicide phosphinothricin and its synthetic form glufosinate-ammonium. The action of the herbicide normally results in plant death due to interference with the plant mechanisms for detoxifying ammonia. However, in corn line 1507, the presence of the PAT enzyme specifically inactivates the herbicide allowing the plants to function normally when sprayed. The herbicide tolerance trait was used also as a selectable marker to facilitate selection of plants with both introduced genes.

Line 1507 contains one complete copy of the transformation cassette incorporating the two linked genes, *cry1F* and *pat*. Expression of the introduced genes is through constitutive promoters, one derived from plants and the other from the cauliflower mosaic virus. Because a purified segment of DNA was used in the transformation, only the genes of interest were transferred. No antibiotic resistance marker genes were transferred to the plants. Molecular and genetic analyses of corn line 1507 indicate that the transferred genes are stably integrated into the plant genome.

Characterisation of novel protein

Expression of the two new genes results in very low levels of the proteins Cry1F and PAT in various tissues of the plant. The mean levels of Cry1F in the edible grain were approximately 100 pg/ug total protein and were similar to plants cultivated across geographical regions.

Expression of the PAT protein in the transformed line was found only at measurable levels in leaf tissue and was below the limit of detection in grain samples, irrespective of the field

locations where the corn was grown and tested. Human exposure to the two introduced proteins through the diet is therefore expected to be at very low levels.

The potential toxicity and allergenicity of the two novel proteins, Cry1F and PAT, were addressed in the assessment. Both introduced proteins were examined for their potential to be toxic to humans, including in acute animal toxicity tests. For Cry1F, no adverse effects were observed in mice at doses up to 576 mg/kg body weight. In a similar study using PAT, no adverse effects were observed in mice at doses up to 5000 mg/kg body weight. In addition, there is no amino acid sequence similarity between the two novel proteins and known toxins recorded in large public domain sequence databases.

The potential allergenicity of the novel proteins was investigated by evaluating whether either of the proteins exhibited any of the physical or biochemical characteristics of known allergens. Neither protein exhibited any significant amino acid sequence similarity with known allergens and both proteins are rapidly digested in simulated mammalian digestive systems. The weight of evidence therefore indicates that neither the Cry1F nor the PAT protein is toxic to humans and neither protein has properties in common with known food allergens.

Comparative analyses

Compositional analyses were completed to establish the nutritional adequacy of food from corn line 1507 compared to that of the conventional counterpart. The results of the compositional analyses on herbicide treated corn plants grown at multiple locations demonstrate that the levels of the important constituents in corn grain (protein, total fat, carbohydrate, ash, fibre, fatty acids, amino acids, minerals and moisture) were similar in corn line 1507 and the non-transformed control corns. In addition, there were no observed differences in results from the analyses of four vitamins (Vitamins B1, B2, E and folate) measured in the transformed and non-transformed corns.

The levels of naturally occurring toxins and anti-nutrients were also assessed. Corn contains no naturally occurring toxins but does contain a number of secondary plant metabolites and trypsin inhibitor as well as a known anti-nutrient. Grain from corn line 1507 and control corn was analysed for five secondary metabolites: inositol, raffinose, p-coumaric acid, furfural, ferulic acid and phytic acid, as well as for trypsin inhibitor activity. The levels of these compounds were either well below the limit of detection or, where detectable, were very similar in both the modified corn line 1507 and the non-transformed controls.

Nutritional impact

Grain from corn line 1507 was shown to be nutritionally equivalent to the non-transformed counterpart in the ability to support typical growth and well-being in rapidly developing broiler chickens, an animal species that is acutely sensitive to nutritional factors in the early stages of growth.

Conclusion

No potential public health and safety concerns have been identified in the assessment of insect-protected, glufosinate ammonium-tolerant corn line 1507. On the basis of all the available data, food derived from corn line 1507 is equivalent to food derived from other commercially available corn in terms of its safety and nutritional adequacy.

FOOD DERIVED FROM INSECT-PROTECTED, GLUFOSINATE-AMMONIUM TOLERANT CORN LINE 1507

A SAFETY ASSESSMENT

BACKGROUND

A safety assessment has been conducted on food derived from corn that has been genetically modified to be protected against insect attack and tolerant to the herbicide glufosinate ammonium. The modified corn is referred to as insect-protected, glufosinate-ammonium tolerant corn line 1507.

The new genetic traits in the corn resulted from the introduction of two new genes encoding the bacterial proteins Cry 1F, conferring resistance to certain insect pests, and phosphinothricin acetyltransferase (PAT), an enzyme conferring tolerance to the synthetic herbicide, glufosinate-ammonium.

Bacillus thuringiensis, a common soil bacterium, produces a number of Cry proteins, known also as Bt proteins, with very selective insecticidal activity. One of the family of Cry proteins, known as Cry1F, has been shown in field research to be effective in controlling certain lepidopteran insect larvae such as those from the European Corn Borer (*Ostrinia nubilalis*), Southwestern corn borer (*Diatraea grandiosella*), black cutworm (*Agrotis ipsilon*) and armyworms (*Spodoptera* sp.). These insects are common pests of corn in the United States where it is intended for this variety to be grown commercially. The Cry1F protein is encoded by the *cry1F* gene derived from *Bacillus thuringiensis* subsp. *aizawai*. The presence of this genetic modification also results in a reduction in moulds and associated mycotoxins in the corn, in addition to the significant control of insect pests.

The PAT enzyme metabolises the herbicide glufosinate-ammonium (or L-phosphinothricin) into an inactive form (OECD, 1999). The enzyme is encoded by the *pat* gene which is derived from *Streptomyces viridochromogenes*, a common soil bacterium.

Corn is used predominantly as an ingredient in the manufacture of breakfast cereals, baking products, extruded confectionery and corn chips. Maize starch is used extensively by the food industry for the manufacture of many processed foods including dessert mixes and canned foods.

Despite the diverse uses of corn products in many foods, corn is a relatively minor crop in both Australia and New Zealand, with a declining area planted over the last decade. When required, products such as high-fructose corn syrup and maize starch are imported from major corn growing regions in the Northern Hemisphere, to meet manufacturing demand.

HISTORY OF USE

Host organism

Maize (*Zea mays* L.), also known as corn, together with rice and wheat, is one of the most important cereal crops in the world with total production of over 590 million tonnes in 2000 (FAOSTAT Database 2001). Almost half of the annual production is grown in the United States.

The majority of grain and forage derived from maize is used as animal feed, however corn also has a long history of safe use as food for human consumption. Corn grain is also

processed into industrial products such as ethyl alcohol (by fermentation), and highly refined starch (by wet-milling) to produce starch and sweetener products. In addition to milling, the maize germ can be processed to obtain corn oil and numerous other more minor products (White and Pollak 1995).

Corn plants usually reproduce sexually by wind-pollination. This provides for natural out-crossing between plants, but it also presents an opportunity for plant breeders to produce hybrid seed by controlling the pollination process. Open pollination of hybrids in the field leads to the production of grain with properties derived from different lines and, if planted, would produce lower yields (Canadian Food Inspection Agency 1994). Instead, by controlling the cross-pollination of inbred lines from chosen genetic pools (using conventional techniques), the combining of desired genetic traits into a controlled hybrid line results in improved agronomic performance and increased yields. This inbred-hybrid concept and resulting yield response is the basis of the modern seed industry in several food commodities including corn.

The commercial production of corn has seen many improvements, particularly since the 1920's when corn varieties were developed by conventional breeding between progeny of two inbred lines to give hybrid varieties that were known to be superior to open-pollinated varieties in terms of their agronomic characteristics. In present agricultural systems, hybrid corn varieties are used in most developed countries for consistency of performance and production. In the case of corn line 1507 hybrids, the presence of the insect-protected and herbicide-tolerance traits will provide producers with additional improvements to the available genetic stock.

Donor organisms

Bacillus thuringiensis

The source of the *cryIF* gene is the common bacterium *Bacillus thuringiensis* subsp. *aizawai*. *B. thuringiensis* are a diverse group of Gram-positive, spore-forming bacteria that were first isolated in 1901, and have proven to be a rich source of insecticidal proteins. Intensive research has identified a growing family of Bt proteins with different insecticidal specificities, including to coleopteran, dipteran and lepidopteran insect orders. While some discoveries are recent, the characterisation of individual Bt proteins and description of their insect specificity and mode of action is well described in the published literature.

The Bt organism has been used safely in spray form as a crop protective agent for at least 40 years (Schnepf *et al.* 1998; U.S. EPA 1996) as a useful alternative or supplement to synthetic chemical pesticide application in commercial agriculture, particularly in the organic farming industry, and in forest management. Several varieties of *B. thuringiensis* have been used as microbial insecticides since 1938 (Merritt 1998). The subspecies *aizawai* is commercially used to control wax moth larvae and various caterpillars, especially the diamondback moth caterpillar (Cornell University 1996).

Streptomyces viridochromogenes

The *pat* gene is derived from the common soil bacterium *Streptomyces viridochromogenes*. The bacterium produces the tripeptide L-phosphinothricyl-L-alanyl-alanine (L-PPT), which was developed as a non-selective herbicide by Hoechst Ag. Over the past decade, the *pat* gene has been introduced into several other genetically engineered food crops to confer tolerance to PPT and the synthetic form glufosinate-ammonium. There have been no adverse effects on human health associated with its use in crops such as canola and several other corn varieties (OECD, 1999 & 2002).

Cauliflower mosaic virus

The 35S promoter and transcription termination sequences used in the genetic construct are derived from the commonly occurring cauliflower mosaic virus (CaMV), a DNA plant virus with a host range restricted primarily to cruciferous plants (ICTV Database 1998) that are common in the food supply. The DNA sequences originating from this virus have no pathological characteristics, other than in association with their target plant species (USDA 1995).

Agrobacterium tumefaciens

The species *Agrobacterium tumefaciens* is a Gram-negative, non-spore forming, rod-shaped bacterium commonly found in the soil. It is closely related to other soil bacteria involved in nitrogen-fixation by certain plants.

Agrobacterium naturally contains a plasmid (the *Ti* plasmid) with the ability to enter plant cells and insert a portion of its genome into plant chromosomes. Normally therefore, *Agrobacterium* is a plant pathogen causing root deformation mainly with sugar beets, pome fruit and viniculture crops. However, adaptation of this natural process has now resulted in the ability to transform a broad range of plant species without causing adverse effects in the host plant.

DESCRIPTION OF THE GENETIC MODIFICATION

Methods used in the genetic modification

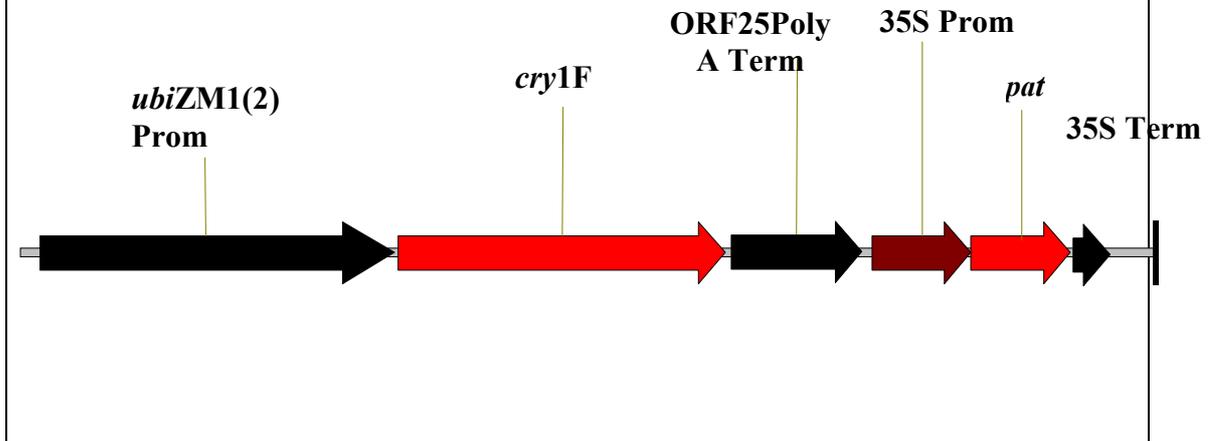
Corn line 1507 was generated by transformation of embryogenic Hi-II corn (*Zea mays*) cells, using a particle acceleration method. A purified linear DNA segment containing the *cryIF* and *pat* coding sequences, together with essential regulatory elements, was used in the transformation process. The DNA segment of 6235 bp was derived from plasmid PHP8999, and contained only the genes of interest. No additional plasmid DNA was used in the transformation event.

Following transformation, the plant embryos were transferred to cultivation medium containing the herbicide glufosinate-ammonium as the selection agent, allowing growth of cells expressing the PAT protein. As expected, the majority of explants were eliminated on this selective medium. Those that survived and produced healthy, glufosinate-ammonium tolerant callus tissue were subsequently regenerated into plants in the greenhouse. Following further testing and selection using European corn borer insects, corn line 1507 was eventually developed, based on the phenotypic characteristics, herbicide tolerance and resistance to lepidopteran insect pests.

Function and regulation of the novel genes

The purified linear segment PH18999A, used in the transformation, is illustrated in Figure 1. The 6235 bp DNA segment comprised two adjacent gene cassettes for expression of the two novel proteins, Cry1F and PAT. The *cryIF* gene is under the regulation of the ubiquitin promoter (ubiZM1(2)) from corn, and a 3' regulatory element derived from *Agrobacterium tumefaciens* (ORF25PolyA). The *pat* gene is regulated by the 35S promoter and the 35S transcription terminator, both from the Cauliflower Mosaic Virus (CaMV). The inserted DNA does not contain an antibiotic resistance gene or bacterial origin of replication sequences.

Figure 1. Arrangement of genetic elements comprising linear transformation segment



Gene cassettes

The DNA components present in the expression cassettes are described in Table 1. Each expression cassette consists of the genes of interest, flanked by regulatory elements derived from either plant or bacterial sources. The regulatory elements are described in the published literature, and their function in plants has been demonstrated (refer to Table 1). The gene sequences for *cry1F* and *pat* have been modified *in vitro* to optimise the production of the corresponding protein in plants (see below for further details on the expression of the genes).

Table 1: Genetic elements in insert PH18999A used to transform corn line 1507

Genetic element	Source	Size (bp)	Function
<i>ubiZM1(2)</i>	Corn (<i>Zea mays</i>)	1986	The ubiquitin promoter (plus 5' untranslated region) from corn (Christensen <i>et al.</i> , 1992) to enable protein expression in plants.
<i>cryIF</i>	<i>Bacillus thuringiensis</i> subsp. <i>aizawai</i>	1818	A truncated version of the coding region of the <i>cryIF</i> gene isolated from <i>B. thuringiensis</i> , in which codon usage has been optimised for expression in plants.
ORF25PolyA	<i>Agrobacterium tumefaciens</i>	714	DNA sequence corresponding to the transcription termination region from the pTi15955 of <i>Agrobacterium tumefaciens</i> (Fraley <i>et al.</i> , 1983).
CaMV 35S promoter	Cauliflower Mosaic Virus (CaMV)	554	Promoter from a common plant virus, directing constitutive protein expression in the plant (Odell <i>et al.</i> 1985)
<i>pat</i>	<i>Streptomyces viridochromogenes</i>	552	The synthetic glufosinate-ammonium tolerance gene, optimised for expression in plants, based on the wildtype phosphinothricin acetyltransferase gene sequence from <i>S. viridochromogenes</i> (Wohlleben <i>et al.</i> , 1988; Eckes <i>et al.</i> 1989; OECD 1999).
CaMV 35S transcription termination element	Cauliflower Mosaic Virus (CaMV)	204	A 3' untranslated region derived from the plant virus, terminating transcription and directing polyadenylation.

cryIF gene

The bacterial *cryIF* gene sequence has been shown to provide high levels of protection against certain insect pests when it is expressed in plants. The gene encodes one of the family of Bt insecticidal proteins, CryIF, that specifically inhibits European and south-western corn borer insects, black cutworm and armyworms.

Higher levels of field resistance in transgenic plants have been previously reported when the coding sequence of the introduced gene is modified to optimise plant codon usage. As naturally occurring Bt genes tend to be A:T rich, while plant genes have higher G:C content, the introduced *cryIF* gene in corn line 1507 has been re-synthesised in the laboratory prior to transformation to optimise expression levels in the plant.

The corresponding amino acid sequence of the Cry1F protein is unchanged by the modified DNA sequence, except for one change at the carboxy terminus of the protein. A leucine residue occurs in place of a phenylalanine residue at position 604 of the 605 amino acids of the plant expressed protein. This single amino acid change results from an intended nucleotide change required to facilitate processing steps in the laboratory. The leucine substitution represents a conservative change in terms of the naturally occurring amino acid at the corresponding position in other Bt proteins.

Under the regulation of the constitutive Ubi-1 promoter element from corn, expression of the *cry1F* gene would be expected in all parts of the plant, conferring insect protection at the whole plant level.

pat gene

Tolerance to the herbicide phosphinothricin (glufosinate-ammonium) has been introduced to a variety of plant species using molecular techniques to insert a copy of the *pat* gene which enables the plant to produce the PAT enzyme. Expression of PAT within the plant cell inactivates L-PPT thereby conferring tolerance to the herbicide (OECD 1999). The use of the *pat* gene in genetically modified corn and canola lines has undergone previous assessment by FSANZ.

As with the insect-tolerance gene, the codon usage pattern of the native *Streptomyces* gene has been modified in the laboratory prior to introduction into the plant. The amino acid sequence of the resulting PAT protein however is not changed (Eckes *et al.* 1989). In corn line 1507, the *pat* gene is under the regulation of the constitutive 35S promoter from CaMV and therefore the new protein is expected to be expressed in all parts of the plant, including the grain.

Characterisation of the genes in the plant

Genomic plant DNA from corn line 1507 was analysed using the standard methodology of Southern hybridisation blots and direct DNA sequencing to examine the presence of the insert, determine copy number, and provide information about the integrity of the inserted sequences. Northern hybridisation blots were also used to determine whether inserted sequences are functional.

Study evaluated:

Glatt, C.M. (2000) Genetic characterisation of maize event 1507: Southern blot analysis. DuPont de Nemours Company, Newark, Delaware, USA

Multiple Southern hybridisation blots were used to determine the nature and number of *cry1F* and *pat* gene insertions present in corn transformation event 1507.

The test material was root tissue taken from plants of two different generations, designated as T1S1 generation and BC4 generation, during the breeding of corn line 1507. The T1S1 generation seed consisted of the original transformed Hi-II corn line crossed to an elite inbred line to give an F1 hybrid, then self-crossed to give T1S1 seed. The BC4 generation seed consisted of the fourth backcross generation of the original transformed Hi-II line. Plants of both generations were grown in the glasshouse and root samples (four replicates) obtained for genomic DNA extraction and analysis.

Genomic DNA samples prepared from the non-transformed control Hi-II corn and line 1507 were used in the experiments, together with the plasmid DNA (PHP8999) from which the segment used in the transformation was derived. Reporter probes were generated by the use of

specific primers to amplify five defined regions of plasmid PHP8999. The probes used to detect various regions of the transformation cassette were *ubiquitin*, *cry1F*, *CaMV 35S* and *pat*. A probe for the neomycin phosphotransferase (*nptII*) marker gene, which was present in the plasmid but not in the segment used to transform the corn, was also included in the analysis for comparison.

The results from these experiments indicate that one full-length copy of the transformation cassette is present in corn line 1507. The data also indicate that a partial-length insert is present at the same site of insertion (in both generations) and that this additional segment corresponds to a part of the *cry1F* and *pat* coding sequences. The results also confirm that, as expected, corn line 1507 does not contain the antibiotic resistance marker gene from plasmid PHP8999.

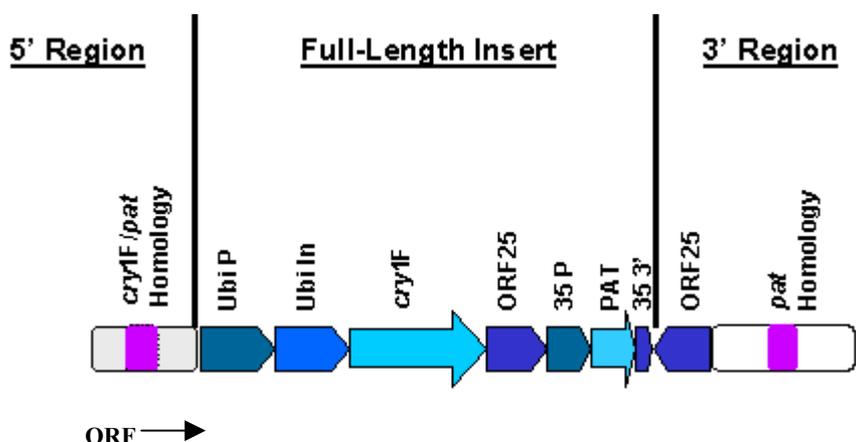
Verification of the nucleotide sequence

Nucleotide sequencing of the newly inserted segment and surrounding genomic regions was completed to confirm the characterisation of corn line 1507. The inserted DNA was amplified using polymerase chain reaction (PCR) methodology. Genomic DNA was extracted from two to three individual plants of corn line 1507 and the unmodified parental corn line (Hi II). PCR products unique to the transformed line were isolated by gel electrophoresis and sequenced directly, or sub-cloned into plasmid vectors and sequenced.

Analysis of the sequence data has identified that the transformation resulted in the insertion of one full-length copy of the gene cassette, together with partial fragments of the cassette at both the 5' and 3' ends, in the region adjacent to the site of insertion. A partial segment (335 bp) of the *cry1F* coding sequence was detected at the 5' end of the insert. In addition, a small fragment of the *pat* gene (comprising only the 5' portion of the gene) is also present at both the 5' (at least 19 bp) and 3' (188 bp) ends of the inserted DNA. Immediately adjacent to the 3' end of the full-length cassette, the nucleotide sequence corresponds to the majority (550 bp of the total 714 bp) of the ORF25 transcription termination element in the reverse orientation.

The sequence data enabled the construction of a complete map of the insert including the identification of restriction enzyme sites that give rise to DNA fragments that were correspondingly detected in the Southern Hybridisation analyses. The simplified map of the inserted DNA in corn line 1507 is depicted in Figure 2.

Figure 2. Diagram of inserted genes in corn line 1507



The nucleotide sequence and Southern blot data demonstrate that corn line 1507 contains one full-length copy of PHI8999A, one partial copy of *cry1F* in the 5' region, and two partial copies of *pat*; one in the 5' region and one in the 3' region relative to the insertion event.

Analysis of border regions

Overall, approximately 10,000 bp of DNA, covering the entire insert and extending approximately 2,500 nucleotides into plant genomic DNA at the 5' end and almost 2,000 nucleotides into plant genomic DNA at the 3' end of the insert, was sequenced. Homology searches were carried out to assist with identification of the border regions. In addition to homology searching using the BLAST program, the 5' and 3' border sequence was analysed for potential open reading frames (ORFs).

PCR analysis was used to compare the sequence in the 5' border region of corn line 1507 to the equivalent region in the unmodified parental corn line (Hi-II) used in the transformation. The sequence data revealed two ORFs in this region that are present in both the unmodified Hi-II line and corn line 1507, demonstrating that they are not novel to the transformed line. A third ORF (see Figure 2), spanning a total of 681 bp from the 5' *cry1F* fragment to the start of the *ubiZM1(2)* promoter, is unique to corn line 1507 and is characterised by the presence of short fragments of the transformation cassette, including the very small fragment of the *pat* coding sequence, interspersed with corn genomic sequence.

There is no evidence to indicate that the third ORF, 5' to the full length insert, could give rise to a protein product. The sequence is without many of the critical gene expression elements known to be associated with expression of stable proteins. Analysis of upstream sequences failed to detect any consensus promoter elements, and the G/C content of the ORF is low (46%) compared with the average for corn genes (56%). This latter property is known to adversely affect protein expression relative to native maize coding sequences. Northern blot analysis confirmed the prediction that there is no corresponding protein expression in the plant (see below).

As an added measure of assessment, the putative amino acid sequence arising from this ORF was analysed for homology with known allergenic proteins. No significant homology was found based on the criteria for a minimal domain size of identity across 8 contiguous amino acids.

Homology searching was conducted also on the 3' border sequence, again using the GenBank public databases and the BLAST program. These analyses confirmed the presence of 550 bp of the ORF25 termination element in the reverse orientation to the inserted DNA cassette, 520 bp of corn genomic sequences followed by a 188 bp fragment of the *pat* gene, then further corn DNA sequences. There are no significant ORFs (longer than 300 bp) occurring in the 3' border region in corn line 1507.

Northern blot analyses

In order to determine whether there is any expression resulting from the presence of partial *cry1F* and *pat* gene sequences, or the inverted ORF25 termination fragment, RNA was analysed by Northern blot for the presence of corresponding transcripts. Total RNA from leaf tissue of corn line 1507 (4 plants) and non-GM control corn (5 plants) was extracted for use in the experiments.

Based on the information gained from the nucleotide sequencing results, multiple genetic probes were prepared. No hybridising bands other than those corresponding to the expected full-length transcripts were observed with any of the probes. The northern blot results are therefore consistent with the conclusion that the partial copies of *cry1F* or *pat* occurring in the flanking regions to the full-length insert are not expressed as unique RNA transcripts in corn line 1507.

In addition, separate Northern blots were carried out to determine whether the potential ORF occurring in the 5' border region is producing a unique RNA transcript indicative of a level of expression in the plant. Sequence homology determined that this 681 bp ORF comprises 121 bp of the partial *cry1F* gene, 320 bp of a partial maize chloroplast *rpoC2* gene, and the adjoining sequence up to and including the first 72 bp of the *ubiZM1* promoter in the full-length insert.

Total RNA from leaf tissue of corn line 1507 (9 plants) and non-GM corn (5 plants) was prepared in the same manner as for the previous Northern blot experiments. In this case, the probes corresponded to the 320 bp fragment of the maize chloroplast *rpoC2* gene, and a positive control probe. No hybridisation signal was visible in either corn line 1507 or the non-GM control with the *rpoC2* probe, but a strong signal was detected in all samples using the control probe. These results demonstrate that this potential ORF, novel to corn line 1507, is not producing a detectible RNA transcript and is therefore not expressed in the plant.

Stability of the genetic changes

Study evaluated:

Song, P., Ernest, A.D., and Collins, A. (2002). Polymerase Chain Reaction (PCR) Analysis of *B.t.* Cry1F Maize Line 1507 and Its Hybrids. Laboratory Study GH-C 5371.

The presence of the transferred genes in corn line 1507 was investigated over multiple generations to ascertain genetic stability. The results from several rounds of backcrossing and self-crossing demonstrate that the *cry1F* and *pat* genes are stable in this line over at least six generations.

Observations of the phenotype indicated that the transgenes are inherited as dominant genes according to Mendelian segregation patterns. This method of analysis involved spraying each generation with glufosinate-ammonium to score and eliminate null segregants (those plants not containing a copy of the transgene). Further segregation data were obtained from plants derived from the F1 generation on the basis of herbicide tolerance, and later also challenged

with neonate European corn borers. All of the plants determined to be tolerant to glufosinate-ammonium were also found to be resistant to European corn borer infestation.

Using PCR methodology, the presence of the same *cry1F* and *pat* gene sequences in corn line 1507 was confirmed in two independent hybrids derived from the T1S1 generation, confirming the stability of the introduced genes in the corn genome. Southern blot experiments, where similar hybridisation results were obtained for both the T1S1 and BC4F1 generations, also support the conclusion that the genetic modification is stable over multiple generations.

Antibiotic resistance genes

Antibiotic resistance genes can be present in some transgenic plants as a result of their use as marker genes in the laboratory or in the field. It is generally accepted that there are no safety concerns with regard to the presence in the food of the antibiotic resistance gene DNA *per se* (WHO 1993). There have been concerns expressed however that there could be horizontal gene transfer of antibiotic resistance genes from ingested food to microorganisms present in the human digestive tract and that this could compromise the therapeutic use of some antibiotics.

Transformation of corn line 1507 was achieved using a specific segment of plasmid DNA corresponding to the genes of interest in conjunction with essential controlling elements. As transformed plant cells were selected using the introduced herbicide tolerance trait, no antibiotic resistance marker genes were necessary for this genetic modification. The molecular analyses have confirmed that no antibiotic resistance genes were transferred to corn line 1507.

Breeding pedigree

Details were provided on the selective breeding program undertaken with the transformed line to demonstrate the production of a variety of elite corn lines with various commercial applications.

The *cry1F* and *pat* genes were transformed into the original parental line known as Hi-II, which was subsequently known as maize line 1507. The genetic makeup of this transformed line was 100% Hi-II. Maize line 1507 was crossed to an elite inbred line, so the resulting progeny contained 50% Hi-II germplasm and 50% elite inbred germplasm. Based on Mendelian genetics, only 50% of the progeny would contain the *cry1F/pat* genes (positive plants) and 50% of the progeny would not contain the new genes (null segregants).

The positive plants, with 50% Hi-II germplasm and 50% elite inbred germplasm, are then crossed again (or backcrossed) to the elite inbred. The resulting progeny contain 25% Hi-II germplasm and 75% elite germplasm. This process is repeated until the elite germplasm is very close to 100% and the *cry1F* and *pat* genes are also present.

High yielding hybrid corn seed sold to farmers is produced by crossing two distinct inbred corn lines. Each inbred corn line has a different genetic background that allows the hybrid seed to be optimised for a specific geographical region where corn is grown. Seed companies may sell over 100 different hybrid seed products requiring the development of hundreds of inbred corn lines. A new gene, such as *cry1F* in corn line 1507, is introduced into the many different inbred lines through conventional backcrossing.

Summary and conclusions from molecular characterisation

Corn line 1507 was produced using particle acceleration to insert a linear DNA segment of approximately 6.2 kb, comprising two bacterial genes and their defined controlling elements necessary for expression in plants. The encoded genes are *cry1F* (conferring insect protection) and *pat* (conferring tolerance to glufosinate-ammonium). Glufosinate-ammonium tolerance was used as a selectable marker for the transformation event and there was no transfer of any antibiotic resistance marker genes.

The insertion event was characterised using a range of molecular techniques including Southern and Northern hybridisation blots and DNA sequencing. The results of these analyses indicate that one complete, functional copy of the transformation cassette is present in corn line 1507. Using these tools, it was shown also that certain DNA rearrangements were present at both ends of the full-length insert, comprised of generally small fragments of both the *cry1F* and *pat* genes and the ORF25 transcription termination element. All of the studies on the 5' and 3' border regions indicate that the additional gene fragments do not result in detectable RNA expression products in the plant. Detailed studies, specifically targeting the putative open reading frame in the 5' border region, also failed to detect any RNA production.

Genetic rearrangements are known to occur with high frequency at the site of insertion of novel DNA during plant transformation, particularly using particle acceleration techniques. In this case, nucleotide sequencing was used to fully characterise the border region between corn genomic DNA and inserted DNA, providing a comprehensive picture of the molecular features that define corn line 1507. The conclusion from these analyses is that only the full-length *cry1F* and *pat* genes are expressed in the transformed plants.

Detailed phenotypic and molecular analyses demonstrate that the two new genes are physically stable and are inherited from one generation to the next according to predicted Mendelian patterns of inheritance.

CHARACTERISATION OF NOVEL PROTEIN

Biochemical function and phenotypic effects

Studies evaluated:

Evans, S.L. Equivalency of Microbial and Maize Expressed Cry1F Protein; Characterisation of Test Substances for Biochemical and Toxicological Studies. Project ID MYCO98-001. Completed October, 1998.

Schafer, B.W. and Schwedler, D.A. Characterisation of the Recombinant Cry1F protein derived from *Pseudomonas fluorescens* and Transgenic Maize. Laboratory study GH-C 5294. Completed September, 2001.

Gao, Y. Equivalency between transgenic corn-produced and microbially-derived Cry1F protein. Dow AgroSciences, 2002.

Corn line 1507 contains two new bacterial proteins, Cry1F and PAT. Several techniques have been used to study the sites and level of expression of these two proteins in the modified plants.

Field studies representative of the conditions and growth stages corresponding to commercial corn production were undertaken during the 1998-1999 growing season in Chile, and during the 1999 growing season in France, Italy and the USA. The expression levels of Cry1F and PAT proteins in leaf, pollen, silk, stalk, whole plant, grain and senescent whole plant tissues

from corn line 1507 and a non-GM control with comparable background genetics were measured using ELISA (Enzyme Linked Immunosorbent Assay), specifically developed for each protein. Western blot analysis was used to further characterise the specificity of the newly expressed proteins.

Cry1F

B. thuringiensis (Bt) occurs naturally in the soil and on plants including trees, vegetable crops and cotton. From intensive study of Bt species, four major classes of insecticidal protein genes (*cry1*, *cry2*, *cry3* and *cry4*) have been identified that are useful for the control of pest species among certain of the insect orders. This includes proteins that encode lepidoptera-specific (Cry1), lepidoptera- and diptera-specific (Cry2), coleoptera-specific (Cry3) and diptera-specific (Cry4) proteins respectively (Chambers *et al.*, 1991). Cry1F was isolated from *B. thuringiensis* subsp. *aizawai* and is distinctly different in protein sequence and insecticidal specificity from the other Cry1 proteins (Chambers *et al.*, 1991).

The mode of action of the insecticidal crystal proteins is to cause the death of susceptible insect larvae through the combined effects of tissue damage and a lack of feeding. Upon ingestion, the protein is solubilized and, in some cases, proteolytically processed by insect gut proteases to yield an active truncated toxin moiety. The activated protein toxin interacts specifically with gut receptors and leads to disruption of the osmotic balance of the cells in the insect midgut, ultimately leading to the death of the larvae.

In its natural form, Cry 1F is produced as a large protoxin of 1174 amino acids. Following solubilisation and proteolytic processing in the gut of susceptible insect larvae, the active toxin moiety corresponds to approximately 600 amino acids at the N-terminal end of the full-length protein. Although precise cleavage has not been shown, the activated toxin is estimated to correspond to amino acids 28-612, based on laboratory data and computer simulations. Therefore, to confer insect resistance, a truncated form of the *cry1F* gene, encoding only the active toxin moiety, was inserted into the corn plants.

The complete amino acid sequence of both the full-length Cry 1F protoxin from *B. thuringiensis*, and the truncated version used in corn line 1507 has been provided. The truncated Cry1F protein is identical to amino acids 1-605 of the N-terminal domain of the native Cry1F protoxin, with the exception of a single amino acid substitution, leucine in place of phenylalanine, at position 604 (F₆₀₄L).

The amino acid change was made to facilitate production in the laboratory of large quantities of a microbially-produced Cry1F/Cry1A(b) chimeric protein that was used as a source of the Cry1F moiety required for toxicology studies. The chimeric protein is a fusion of the gene sequence coding for the Cry1A(b) C-terminal domain with the gene sequence coding for the Cry1F core toxin. The decision to use F₆₀₄L substitution was based on the occurrence of leucine in the homologous position of other Cry1 proteins, and is therefore a conservative substitution.

The Cry1F/Cry1A(b) chimeric protein produced in *Pseudomonas fluorescens* strain MR872 enables high levels of expression of soluble protein. The MR872 fusion protein is subsequently enzymatically cleaved *in vitro* with trypsin to obtain the Cry1F core protein. The amino acid sequence of the microbially-derived Cry1F protein (MR872) used in the toxicology studies was also submitted for comparison with the native and plant produced versions. Protein sequencing data showed that the N-terminal amino acid in both the plant derived Cry1F and the trypsin-processed microbial Cry1F corresponds to residue 28. On the basis of further experimental evidence, the biochemical characteristics and biological activity of the trypsin-released Cry1F core protein are equivalent to the corn-expressed core Cry1F protein.

Although the DNA sequence of the *cry1F* transgene was altered from the native gene sequence to enable higher levels of expression of the core Cry1F protein in plants, there is no change in amino acid sequence compared to the corresponding wildtype protein sequence, except for the specific single amino acid change outlined above.

PAT

L-PPT, the active ingredient in glufosinate-ammonium herbicide, binds to, and inactivates, the enzyme glutamine synthetase in plants preventing the detoxification of excess ammonia which ultimately results in plant death.

The activity of the PAT protein has been described in detail (OECD, 1999). The PAT enzyme is specific in catalysing the conversion of L-PPT to an inactive form, N-acetyl-L-PPT, which does not bind to the enzyme glutamine synthetase. The expression of PAT in corn line 1507 therefore results in the conversion of herbicide to the inactive form, allowing the detoxification of ammonia to continue in the plant in the presence of the herbicide. Plants expressing the PAT enzyme are therefore tolerant to the herbicide, enabling treatment of surrounding weeds without harm to the crop.

The *pat* gene from *S. viridochromogenes* encodes a polypeptide of 183 amino acids, and the mature PAT protein is known to be a homodimer of approximately 43 kDa in the native form (Wehrmann *et al.*, 1996).

The *pat* gene has been resynthesised in the laboratory with a codon usage optimised for expression in plants. The synthetic *pat* gene encodes the same amino acid sequence as the native gene, and when expressed in plants, confers tolerance to glufosinate-ammonium (Eckes *et al.*, 1989). Effective expression has been reported in numerous plant species including *N. tabacum*, *L. esculentum*, *M. sativa* as well as important food crops such as *B. napus* (canola) and *Z. mays* (corn).

Characterisation of the novel proteins expressed in corn line 1507

Studies evaluated:

Alarcon, C and Marshall, L., 2000. Characterisation of proteins as expressed in Bt Cry1F maize tissues. Performing Laboratory: Pioneer Hi-Bred International Inc., Johnston, Iowa. Study Number PH199-023

Gao, Y. and Collins, R.A., 2002. Gel-Electrophoresis, Western Blot, and ELISA of Truncated Cry1F Delta-endotoxin Following Heat Treatment. Dow AgroSciences Regulatory Laboratories, Indianapolis, USA. Study Number GH-C 5366.

Western blot techniques were used to examine biochemical properties including molecular weight and immunoreactivity of the CRY1F and PAT proteins expressed *in planta* comparatively against the respective microbially-derived protein produced in the laboratory. For this purpose, polyclonal antibodies that recognise multiple antigenic epitopes were used. Protein was extracted from a range of samples including leaf, pollen, grain and whole plant tissues from field grown corn line 1507 plants and a non-transformed control grown in Chile during the 1998/99 growing season.

Cry1F

The results of the analyses of Cry1F protein expression in plant tissues demonstrated that under denaturing conditions the Cry1F protein was detected as two bands with almost identical mobility (a doublet) of approximately 65 to 68 kDa in leaf, pollen, grain and whole plant tissue. No other bands indicative of a partial Cry1F protein or a fusion protein of greater

molecular size were observed. Due to the presence of the known enzyme cleavage sites near the amino-terminus of the protein, the doublet is expected to have resulted from limited N-terminal processing by a plant protease with trypsin-like specificity.

PAT

In its native form, the PAT protein is known to be a homodimer of approximately 43 kDa, comprised of two identical components of approximately 22-23 kDa (Wehrmann *et al.*, 1996, OECD, 1999). The immunoreactivity of the PAT protein extracted from leaf, grain, pollen and whole plant tissues derived from corn line 1507 was compared on a Western blot (under denaturing conditions) with that of a microbially-expressed PAT protein produced in the laboratory.

Plant-expressed PAT, of equivalent electrophoretic mobility to the microbially-produced protein, was detected as a band of approximately 22 kDa only in leaf tissue from corn line 1507. There was no detectable PAT protein present in pollen, whole plant, or grain from the transformed line. These results are consistent with the levels and relative distribution of PAT protein detected by ELISA (see below). No other bands indicative of a partial PAT protein or a larger fusion protein with distinctive electrophoretic mobility were observed in these tissues from 1507 corn plants.

As expected, the Western blots did not detect immunoreactive bands corresponding to either of the novel proteins in the untransformed control corn tissue, using polyclonal antibodies.

Protein expression analyses

The introduced genes are each under the regulation of a constitutive promoter. However, at tissue level, the expression of either novel protein can vary and may be below the limit of detection. Three separate studies were undertaken to directly measure the levels of both novel proteins in a range of plant tissues derived from corn line 1507 and a non-GM control, when grown in different geographical locations representative of major commercial corn production regions.

Study evaluated:

Stauffer, C. and Rivas, J., 1999. Quantitative ELISA Analysis of Cry1F and PAT Expression Levels in and Compositional Analysis of Maize Inbred and Hybrid Lines 1362 and 1507. Performing Laboratory: Pioneer Hi-Bred International Inc., Johnston, Iowa. Study Number 98-09-RA-NGLP-012.

The test system for this study consisted of four field sites located in the major corn growing regions of Chile, considered to be environmentally similar to corn growing regions in the United States where corn line 1507 would be a suitable agricultural product. At each site, multiple (usually 20) leaf, pollen, silk, stalk and grain samples were taken from five discrete plants. Whole plant and senescent whole plant samples consisted of three plants pooled together. CRY1F and PAT protein levels were measured in each of the samples using specific ELISAs developed for each protein.

Test seed from corn line 1507 (inbred and hybrid lines) were used in the planting of the field sites. The non-GM control seed was derived from Hybrid A_M and Inbred A_M that were representative of the transformed line in terms of their genetic background. Agricultural practices for growing the test and control plants were typical for producing corn in the regions chosen for this study. Chemical and fertilizer applications were appropriate for each location, and all test lines were sprayed with glufosinate-ammonium (Liberty®) using a hand spray at approximately the V5-V6 stage of development. The concentration of the active ingredient was 150 g/L, which is approximately four times the recommended label rate.

Total soluble protein in the corn tissue preparations was measured, using bovine serum albumin (BSA) as the protein standard. Reference standards were prepared in the laboratory from microbially-expressed Cry1F (truncated toxin) and PAT proteins. The Cry1F protein was purified from *Pseudomonas fluorescens* (strain MR872) that contained a gene encoding the truncated Cry1F toxin. Characterisation of the standard was accomplished by electrophoretic mobility and amino acid analysis. The PAT protein was purified from recombinant *E. coli* (strain BL21) containing the *pat* gene. Characterisation of the PAT reference standard was accomplished by electrophoretic mobility (silver stain), sequencing and amino acid analysis. Both assay systems used polyclonal rabbit antibodies specific to the respective test protein.

The results of the ELISAs show that Cry1F was expressed in the transformed line at detectable levels in all collected tissues. The highest levels of expression of Cry1F were measured in the stalks (approximately 600 pg/μg total protein) while the lowest levels were detected in the corn silks (approximately 54 pg/μg total protein). The edible grain contained approximately 100 pg/μg total protein.

In contrast, expression of the PAT protein in the transformed line was found in only one of the leaf samples (21.4 pg/μg total protein); expression in all other plant tissues was below the limit of detection (<20 pg/μg total protein). As expected, expression of the Cry1F and PAT proteins was not detected in the non-transformed control plants.

Study evaluated:

Stauffer, C., 2000. Quantitative ELISA Analysis of poCry1F and PAT Protein Expression Levels, Composition and Efficacy of Hybrid Lines 1360 and 1507 – EU Field Sites. Performing Laboratory: Pioneer Hi-Bred International Inc., Johnston, Iowa. Study Number PH199-005.

A second study was conducted using samples collected from three locations in France and three locations in Italy, all in major corn growing regions of the European Union. At each location in Italy, the trial consisted of transformed corn plants sprayed with glufosinate-ammonium, corn line 1507 unsprayed, and a non-transformed control hybrid with a genetic background representative of the transformed line. At the locations in France only, the trial involved corn line 1507, not sprayed with the herbicide, and the appropriate non-transformed control hybrid.

Tissue samples were collected from both the transformed and non-transformed lines as follows: leaf at V9 stage, whole plant at V9 stage, pollen, silk, stalk, whole plant at R1 stage, whole plant at R4 stage, grain and senescent whole plant. In addition, whole plant forage (R4 stage) and grain were collected from the glufosinate-ammonium sprayed plots. Grain was collected when plants were physiologically mature, corresponding to the time of typical commercial grain harvest. All tissue samples were analysed for Cry1F and PAT protein levels using the specific ELISA techniques. As before, each protein standard was characterised by electrophoretic mobility, sequencing and amino acid analysis.

The field studies show that expression of the Cry1F protein in transformed corn line 1507 occurs at measurable levels in all plant material sampled and tested. As found in the Chilean study, the stalks registered the highest expression level. However, in the European study, the lowest levels of Cry1F protein were measured in the grain (approximately 90 pg/μg total extracted protein). Overall, the pattern of expression in various plant tissues and at various stages of plant development in the studies conducted in Europe were similar to the results from the Chile studies. There was no significant difference in the expression level of Cry1F measured in the grain from sprayed (90.3 pg/μg) compared to unsprayed (96.4 pg/μg) plants.

The expression levels of the PAT protein in corn line 1507 were also similar in both studies; the amounts of PAT present were below the limit of detection of the assay (20 pg/μg total extracted protein) for all tissues and whole plant samples except for the leaf samples which again contained low levels (approximately 40 pg/μg total extractable protein). As expected, expression of the Cry1F and PAT proteins was not detected in any samples from the non-transformed control plants.

Study evaluated:

Zeph, L., 2001. Quantitative ELISA Analysis of poCry1F and PAT Protein Expression Levels in Hybrid and Inbred Lines of TC1507 and an Inbred Line of TC1360. Performing Laboratory: Pioneer Hi-Bred International Inc., Johnston, Iowa. Study Number PH 199-001.

A third study was conducted on samples obtained from four locations in the Midwestern corn growing regions of the United States. As before, plant tissues including leaf, pollen, silk, stalk and grain were collected from both corn line 1507 and a non-transformed control at each location. In addition, whole plant samples (entire plant except roots) were harvested approximately four weeks after pollination. Senescent whole plant samples, including ears, were harvested when the plant tissue had turned brown and dried.

The transgenic hybrid and inbred lines (test lines) used in this study were obtained from seed produced during the process of backcrossing the original transformant with elite inbred lines. The non-transgenic hybrid and inbred lines (control lines) were similar to the test lines in terms of background genetics but did not express Cry1F or PAT. As the population of plants in the test plots was segregating for the *pat* and *cry1F* genes, it was necessary to identify positive plants expressing both novel proteins. The test lines were therefore treated with glufosinate-ammonium herbicide by leaf painting plants at approximately the V4 to V5 stage of development. Since the two inserted genes are linked, plants exhibiting tolerance to glufosinate-ammonium herbicide are considered to also express the Cry1F protein.

In both the hybrid and inbred transgenic lines, expression of the Cry1F protein was found at measurable levels in all test tissues sampled. The results of this study are similar to those found in the other studies where it was observed that levels of Cry1F protein expression were highest in the stalks and lowest in either leaf, pollen or grain tissues. Although in this study the Cry1F protein expression was lowest in silk tissue, the edible grain contained only slightly higher levels than the silks, with 116 pg/μg total extractable protein (TEP) and 231 pg/μg TEP for the hybrid and inbred plants respectively. In addition, the pattern of expression of the Cry1F protein throughout the plant in both hybrid and inbred lines was identical.

Expression of the PAT protein was only found in leaf tissue samples (hybrid test lines) at detectable levels up to approximately 54 pg/μg TEP. These levels are nevertheless sufficient to confer tolerance to glufosinate-ammonium herbicide at the level of the whole plant. In all other tissues, the levels of PAT protein were below the limit of detection (20 pg/μg TEP). These results are comparable to those obtained in the other studies. As expected, expression of the Cry1F and PAT proteins was not detected in any samples from the control plants.

Potential toxicity of novel proteins

CryIF

Studies evaluated:

Evans, S., 1998. Equivalency of Microbial and Maize Expressed Cry1F Protein; Characterisation of Test Substances for Biochemical and Toxicological Studies. Mycogen Corporation Study ID MYC098-001.

Kuhn, J.O. 1998. Cry1F *Bacillus thuringiensis* subsp. *aizawai* Delta-endotoxin – Acute Oral Toxicity Study in Mice, conducted by STILLMEADOW, Inc. 12852 Park One Drive, Sugar Land, Texas 77478. Laboratory Study Number 4281-98.

Brooks, B.S. 2000. PAT Microbial Protein (FL): Acute Oral Toxicity Study in CD-1 Mice, conducted by Toxicology and Environmental Research and Consulting, The Dow Chemical Company, Michigan, USA. Laboratory Study ID 991249.

To obtain quantities of the Cry1F protein sufficient for biochemical and toxicological tests, the protein was produced microbially in the laboratory from large-scale cultures of *Pseudomonas fluorescens* strain MR872.

A range of analyses was subsequently undertaken to establish that the microbially-produced protein is equivalent to the protein produced by the transformed corn plants. The data collected demonstrate that the plant-derived protein exhibits characteristics expected of the *cryIF* gene product with respect to molecular weight, immunoreactivity, apparent lack of post-translational modification, N-terminal amino acid sequence and bioactivity on susceptible insect larvae.

Acute oral toxicity

An acute oral toxicity study using laboratory mice was conducted to examine the potential toxicity of a single dose of the core Cry1F protein. The test substance, Cry1F delta endotoxin from *B. thuringiensis* subsp. *aizawai*, was administered by gavage in a 2% aqueous solution of carboxymethyl cellulose to each of 10 albino mice, 5 males and 5 females. The dose of test protein was equivalent to 576 mg/kg body weight. An individual dose was calculated for each animal based on its fasted body weight and administered in a total volume of 33.7 ml/kg, given as two half doses approximately one hour apart. Observations for mortality and clinical or behavioural signs of toxicity were carried out at least three times on the day of dosing (Day 0) and twice daily thereafter for 14 days. Individual body weights were recorded just prior to dosing and on days 7 and 14. At study termination, the animals were killed and examined for gross necropsy and any abnormalities recorded.

No animal deaths occurred during the course of the study and, with the exception of one female, all animals recorded normal relative weight gains at the mid-point and end of the study. There were no adverse clinical or behavioural signs of pathology and gross necropsy conducted at termination of the study revealed no observable abnormalities. The study conclusion was that the oral LD₅₀, as indicated by the data, was determined to be greater than 576 mg/kg in male and female mice.

Comparison of amino acid sequence with other proteins

The comparison of amino acid sequence of an introduced protein with that of known protein toxins is another means of evaluating the potential toxicity of a novel protein. A protein identified as having significant sequence similarity to a known toxin can then be further assessed using traditional toxicological approaches. Therefore, computer alignment analyses

of the amino acid sequence of the truncated Cry1F protein (present in corn line 1507) against database entries with over 900,000 protein sequences were performed.

As expected, the results of the amino acid comparison showed that Cry1F has significant sequence similarity with other Bt insecticidal proteins such as Cry 1A(b), a protein already approved for use in transgenic corn. In general, the family of Bt proteins is considered to lack mammalian toxicity based on studies that examine environmental and occupational exposures, and a history of safe use. Three other proteins from various sources were identified as having some limited degree of similarity with Cry1F, however none of these are known protein toxins.

PAT

Studies evaluated:

Stauffer, C. and Rivas, J., 1999. Quantitative ELISA Analysis of Cry1F and PAT Expression Levels in and Compositional Analysis of Maize Inbred and Hybrid Lines 1362 and 1507. Performing Laboratory: Pioneer Hi-Bred International Inc., Johnston, Iowa. Study Number 98-09-RA-NGLP-012.

Brooks, B.S. 2000. PAT Microbial Protein (FL): Acute Oral Toxicity Study in CD-1 Mice, conducted by Toxicology and Environmental Research and Consulting, The Dow Chemical Company, Michigan, USA. Laboratory Study ID 991249.

Acute oral toxicity

An acute oral toxicity study in mice was conducted using microbially-derived PAT protein (84% purity) prepared as a reference standard also for use in other analyses. The test substance was administered by gavage tube to each of 10 CD-1 mice (5 males and 5 females) at a dosage equivalent to 5000 mg PAT protein /kg body weight, suspended in aqueous methylcellulose. Due to the calculated delivery volume exceeding 2 ml/100g body weight, the required dose was delivered in two halves approximately one hour apart. During the course of the study, detailed clinical observations and individual animal weights were recorded.

All of the mice survived for the two-week study period. There were no clinical observations associated with toxicity, and all animals, with the exception of one female, gained normal body weight. At the conclusion of the study, there were no gross pathologic lesions found in any of the test animals. It was concluded that the acute oral LD₅₀ of microbially-derived PAT protein in mice is greater than 5000 mg/kg.

The results and conclusions from this study are consistent with those obtained from other toxicology studies using the PAT protein. FSANZ has previously assessed scientific data in relation to the potential for toxicity of PAT in other genetically modified food commodities such as corn¹ and canola. The conclusion from these previous assessments is that the safety of the PAT protein has been well established. The synthetic *pat* gene used in corn line 1507 is identical to the one used in the other commodities, providing additional supporting evidence for the lack of toxicity of the encoded PAT protein.

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A372: Food derived from glufosinate-ammonium tolerant and fertility controlled canola

A375: Food derived from glufosinate-ammonium tolerant corn line T-25

A380: Food derived from insect-protected and glufosinate-ammonium tolerant DBT418 corn

Potential allergenicity of novel proteins

The potential allergenicity of novel proteins is evaluated using an integrated, step-wise, case-by-case approach relying on various criteria used in combination, since no single criterion is sufficiently predictive of either allergenicity or non-allergenicity. The assessment focuses on the source of the novel protein, any significant amino acid similarity between the novel protein and that of known allergens, and the structural properties of the novel protein, including susceptibility to degradation in simulated digestion models. Applying such criteria systematically, provides reasonable evidence about the potential of the newly introduced proteins to act as an allergen. The two new proteins in corn line 1507 were evaluated according to these criteria.

Source of novel protein

The Cry1F insecticidal protein is encoded by the *cry1F* gene, derived from the soil bacterium *B. thuringiensis* subsp. *aizawai*. Microbial formulations of Bt are perhaps the most well-known and widely used biopesticides and have a long history of safe use.

The subspecies *aizawai* is commercially used to control wax moth larvae and various caterpillars, especially the diamondback moth caterpillar (Cornell University, 1996).

The PAT enzyme introduced into corn line 1507 is naturally present in the soil bacterium *S. viridochromogenes*, a species not considered as pathogens of plants, humans or other animals (OECD, 1999). As described earlier in 4.3.2, PAT is present as a novel protein in several approved GM commodities (canola, corn) and is not associated with allergenicity.

Sequence comparison to known allergens

Study evaluated:

Meyer, T. 1999. Comparison of Amino Acid Sequence Similarity of Cry1F and PAT Proteins to Known Allergen Proteins. Pioneer Hi-Bred International Inc. Johnston, Iowa. Study Number PH199-013.

A comparison of the amino acid sequence of the introduced proteins to that of known allergens can provide information on the extent to which an introduced protein is structurally similar to a known allergen. This is based on the identification of contiguous identical sequence matches that may be immunologically significant. This information may therefore suggest whether the introduced protein has allergenic potential.

A database was compiled using the Wisconsin Genetics Computer Group (GCG) sequence analysis program to search standard DNA and protein sequence databases. The results of the sequence alignment demonstrate that neither the Cry1F nor PAT proteins share significant amino acid sequence homology with known allergen proteins. This result was expected for the PAT protein, as it has been the subject of previous safety assessments.

Digestibility of Cry1F and PAT proteins

Typically, most food allergens tend to be stable to the peptic and acidic conditions of the digestive system if they are to reach and pass through the intestinal mucosa to elicit an allergic response (Kimber *et al.*, 1999; Metcalfe *et al.*, 1996). Various physicochemical properties of the Cry 1F and PAT proteins were investigated, including the susceptibility of these proteins to proteolytic degradation in conditions that mimic digestion.

Cry1F

The *in vitro* digestibility of bacterially derived Cry1F protein was determined in laboratory experiments where the protein was exposed to simulated mammalian gastric fluid either with varying amounts of pepsin for a set incubation period, or a set amount of pepsin incubated in a time course experiment ranging from 0 to 60 minutes. The samples were analysed by SDS-PAGE and visualised by Coomassie blue staining.

The results of the experiments show that Cry1F was completely digested to amino acids and small peptides within 5 minutes at molar ratios approximating 1:100 (Cry1F:pepsin). In separate experiments, the same molar ratio of 1:100 effected nearly complete proteolysis of the Cry 1F within one minute. In the mammalian gut, further digestion occurs in the duodenum where pancreatic enzymes (lipase, amylase and the serine protease, trypsin) continue the degradative process. The results of the simulated upper gastrointestinal digestion with pepsin therefore indicate that Cry1F is readily degraded in environments simulating mammalian digestion.

PAT

A separate study was conducted to evaluate the stability of the PAT protein in a simulated gastric system containing 0.3% (weight /volume) pepsin, over various time intervals ranging from 5 seconds to 10 minutes. Following electrophoresis of reaction mixtures, all protein and digested fragments were visualised by Coomassie blue staining.

The gels show that PAT degrades to below detectable levels within 5 seconds of exposure to simulated gastric fluid (SGF), thus demonstrating very low stability in a normal mammalian digestive environment.

Glycosylation

As protein allergens are often glycosylated, studies were carried out to determine whether Cry1F is a glycoprotein. The potential glycosylation of Cry1F protein *in planta* and from a microbial source was examined by a sensitive immuno-blot technique used for glycoprotein detection. The studies provided no evidence for post-translational modification involving carbohydrates of the Cry1F in corn-derived extracts.

Heat stability

The stability of the Cry1F protein to heat was tested under bioassay conditions. Aqueous formulations of the microbially-produced truncated Cry1F protein were incubated at various temperatures (60° C, 75° C and 90° C) for 30 minutes. The positive control formulation was treated at 4°C for the same period. The formulations were then applied to the surface of artificial insect diet in bioassay trays. Each well on the tray was infested with a neonate larva of tobacco budworm (*Heliothis virescens*). Insect mortality and weight were measured after 6 days exposure to the treated diet.

There was 96% growth inhibition of the larvae feeding on the diet coated with Cry1F protein pre-treated at the control temperature (4°C). Using protein that had undergone pre-treatment at temperatures of 75°C and 90°C, the percentage mortality was reduced to zero, with 8% and 3% larval growth inhibition respectively. At the intermediate temperature of 60°C, the protein gave rise to 25% mortality of insect larvae with 93% growth inhibition.

These results demonstrate that Cry1F is labile to temperatures above 60°C, and that the resultant denaturation of the protein leads to a concomitant reduction in insecticidal activity.

Summary and conclusions

Two bacterial proteins are expressed in corn line 1507 – Cry1F and PAT. Analyses of the modified line indicate that both proteins are present at low levels in some plant tissues and occur below the limit of detection in other parts of the plant. Using antibodies specific for each protein, Western blot data show the presence of immunoreactive bands corresponding to the proteins of the expected size and mobility in the transformed corn when compared to laboratory-purified protein standards.

The levels of the novel proteins in plant tissues from corn line 1507 grown at multiple sites in locations in Chile, Europe and the United States were measured using protein-specific ELISA techniques. PAT protein was detected at very low levels only in leaf tissue.

In all other plant tissues tested, including the edible grain, the amount of PAT protein was below the limit of detection of the assay system. The mean levels of Cry1F in the edible grain were uniformly low across the three geographical areas, measuring 90, 100 and 116 pg/ μ g total protein. When tested, there was no significant difference in the expression level of Cry1F measured in grain from either herbicide-sprayed (90.3 pg/ μ g total protein) or non-sprayed plants (96.4 pg/ μ g total protein).

A range of biochemical and bioinformatic studies were conducted to determine whether Cry1F and PAT exhibit the potential to be either toxic or allergenic when present in foods. Both of these proteins are derived from bacterial sources that are not known to be either toxic or allergenic. The Bt organism has been used safely as a naturally occurring biopesticide over a long period, and the PAT protein has been present in a range of agricultural crops produced over the last decade with no apparent adverse effects. The protein expression studies indicate that dietary exposure to either of the introduced proteins would be very low. Neither protein exhibits significant amino acid sequence similarity with known toxins or allergens. Both proteins are readily broken down in conditions that mimic human digestion. Furthermore, no adverse effects were observed when the proteins were administered by gavage to mice at doses greatly exceeding the likely human level of exposure through consumption of corn products.

The biochemical and physicochemical investigations on the novel proteins therefore strongly support the conclusion that Cry1F and PAT are unlikely to be either toxic or allergenic to humans.

COMPARATIVE ANALYSES

Studies evaluated:

Stauffer, C. and Rivas, J., 1999. Quantitative ELISA Analysis of Cry1F and PAT Expression Levels in and Compositional Analysis of Maize Inbred and Hybrid Lines 1362 and 1507. Pioneer Hi-Bred International Inc., Johnston, Iowa. Study Number 98-09-RA-NGLP-012.

Stauffer, C. and Zeph, L., 2000. Compositional Analysis of Maize MPS Hybrid Line 1507. Pioneer Hi-Bred International Inc, Johnston, Iowa and Woodson-Tenet Laboratories Inc, Delaware, Iowa. Study Number 98-09-RA-NGLP-012.

Stauffer, C. 2000. Quantitative ELISA Analysis of poCry1F and PAT Protein Expression Levels, Composition and Efficacy of Hybrid Lines 1360 and 1507 – EU Field Sites. Pioneer Hi-Bred International Inc, Johnston, Iowa. Study Number PH199-005.

The key constituents in corn have been evaluated in order to compare corresponding data from corn line 1507 expressing Cry1F and PAT proteins, the non-transformed counterpart and

published literature values obtained for conventional varieties of corn. This evaluation includes a study of the major constituents that are characteristic of whole corn grain, taking account of the natural variation in composition that is known to occur due to genetic variability and geographical or environmental factors. As a reference tool, the OECD has produced a consensus document on compositional considerations for new varieties of maize, which looks at key food (and animal feed) nutrients, anti-nutrients and secondary plant metabolites (OECD, 2002).

Nutrient analyses

Two major studies were conducted at different geographical areas to determine the compositional profile of key corn tissues collected from corn line 1507 and appropriate non-transformed control lines grown under field conditions.

The initial study was conducted at four trial sites located in the major corn growing regions of Chile. Plant tissue samples were collected from a hybrid line derived from 1507, an inbred line derived from 1507 and control lines designated as Hybrid A_M and Inbred A_M. The test lines were segregating for the two transgenes, *cry1F* and *pat*, and were sprayed with glufosinate-ammonium herbicide at approximately the V5-V6 stage of development. Plants that were damaged by the herbicide were considered to lack both transgenes and were removed from the plots. The control lines are not genetically modified and have background genetics representative of the test lines.

At physiological maturity, whole plant forage, consisting of the pooled material from three self-pollinated whole plants, and grain samples from the hybrid test and control lines were harvested for compositional analyses. The nutrient analyses included fat, protein and fibre content and moisture and ash analyses. In addition, grain was measured for fatty acid and amino acid profile, mineral content (calcium, phosphorus, copper, iron, magnesium, manganese, potassium and zinc), vitamin content (vitamins B1, B2, E and folic acid), tocopherols and the anti-nutrient substances phytic acid and trypsin inhibitor. The analyses were conducted at Woodson-Tenent Laboratories according to the methods of the Association of Official Analytical Chemists (AOAC).

The results obtained for the proximate analysis of the grain samples are presented in Table 2. Levels of these components in corn line 1507 and its non-GM counterpart were comparable, and also within previously reported ranges for corn grain. A small difference in the percentage of fat between the GM line and its comparator was statistically significant ($p < 0.05$) but values were within the literature reported range for that variable.

Table 2: The means and p-values (across all sites) for the proximate analysis of grain from corn line 1507 and a control corn hybrid from samples collected in the 1998/1999 field trials in Chile.

Variable (% dry weight)	Corn Line 1507 (mean)	Control Line (mean)	P-value	Literature range*
Fat	3.83	3.94	0.046	3.1-5.7
Protein	11.20	11.32	0.611	6.0-12
ADF	3.55	3.68	0.250	3.0-4.3
NDF	10.47	10.08	0.315	8.3-11.9
Ash	1.51	1.50	0.335	1.1-3.9
Carbohydrates**	83.45	83.23	0.352	63.3-89.7

* Watson, 1982 and 1987.

** Carbohydrates calculated as the % dry weight less % protein, fat and ash.

The results for the proximate analyses on the forage samples collected during the same trial also show no statistically significant differences between corn line 1507 and the non-GM control samples (data not presented).

Amino acid analysis

The levels of eighteen amino acids in the grain from corn line 1507 and the non-GM control were compared, and the results are presented in Table 3 below. There were two values, cysteine and methionine, where the difference between the two lines was found to be statistically significant, but the magnitude of the difference was small and not considered to be biologically significant as the values fall within the ranges previously reported in the literature.

Table 3: Amino acid composition of corn grain. Values are means expressed as a percentage on a dry weight basis.

Amino acid	Corn Line 1507 (mean)	Non-GM control (mean)	p-value	Literature range a b
Glycine	0.39	0.40	0.150	0.26-0.47 0.24-0.41
Threonine	0.40	0.41	0.302	0.29-0.39 0.21-0.37
Valine	0.51	0.52	0.902	0.21-0.52 0.25-0.67
Isoleucine	0.40	0.40	0.952	0.26-0.40 0.19-0.39
Leucine	1.42	1.43	0.880	0.78-1.52 0.43-1.35
Phenylalanine	0.56	0.57	0.479	0.29-0.57 0.04-0.54
Histidine	0.29	0.30	0.822	0.20-0.28 0.21-0.32
Lysine	0.32	0.32	0.522	0.20-0.38 0.19-0.36
Arginine	0.44	0.45	0.672	0.29-0.59 0.28-0.55
Cysteine	0.21	0.23	<0.0001	0.12-0.16 0.13-0.27
Methionine	0.19	0.20	0.020	0.10-0.21 0.12-0.26
Tryptophan	0.08	0.08	0.065	0.05-0.12 0.05-0.10
Serine	0.54	0.55	0.390	0.42-0.55 0.25-0.46
Alanine	0.84	0.85	0.727	0.64-0.99 0.37-0.81
Glutamic Acid	2.14	2.18	0.472	1.24-1.96 0.89-2.02
Proline	1.01	1.03	0.679	0.66-1.03 0.43-1.01
Aspartic Acid	0.77	0.81	0.102	0.58-0.72 0.37-0.80
Tyrosine	0.20	0.20	0.954	0.29-0.47 0.17-0.31

a: Watson, 1982

b: Data from analyses of 22 commercial Pioneer® Hybrids

Fatty Acid Analysis

Corn oil is an excellent source of polyunsaturated fatty acids, with a high level of the essential fatty acid linoleic acid (18:2). In addition, it has naturally low levels of the saturated fatty acids, palmitic acid (16:0, 11%) and stearic acid (18:0, 2%). It is known also that corn oil from cooler regions has a higher proportion of unsaturated fatty acids than corn oil from warmer areas, which appears to be an adaptation to climatic conditions. However, genotype has a greater influence on fatty acid composition than any environmental factor. The biochemical variability for fatty acid composition among corn genotypes is known to cover a broad range.

Analyses of five major fatty acids in corn grain were conducted: palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3). There was a small statistically significant difference between corn line 1507 and the non-GM control in all of these measurements except for levels of palmitic acid that were similar in both lines. However, the values for these fatty acids in corn line 1507 and the control were within the literature reported range for maize.

Mineral analysis

The levels of nine minerals (calcium, phosphorus, copper, iron, magnesium, manganese, potassium, sodium and zinc) were analysed in grain from corn line 1507 and the non-GM control line. The levels of sodium however, were below the limit of quantitation in all samples. For the remaining eight mineral components, no significant differences between the transformed and non-transformed lines were detected. There was also no difference between either of the lines and the range of values found in the literature (from Watson, 1982).

In addition to a published range, data were compiled on calcium levels in 22 current commercially grown hybrids (Pioneer®). The modifications made by the testing laboratory (Woodson-Tenent) to the method for determining calcium levels typically results in lower amounts of calcium than those reported in the literature. It was therefore considered more accurate to compare the tested lines to the compiled data from the Pioneer hybrids which were analysed by the same method, rather than to previously published data produced using a different method of analysis.

Vitamin analyses

Grain from corn line 1507 and the control corn was analysed for content of four vitamins. Vitamin B1, B2, total tocopherols (Vitamin E) and folic acid levels in both transformed and non-transformed lines were determined for comparison to the published literature range. The results show small statistically significant variations between the transformed and non-transformed corn in terms of the total tocopherols (slightly higher in corn line 1507) and in vitamin B1 levels (slightly lower in corn line 1507). However, the values obtained for all vitamin analyses were within the respective range previously reported in the literature (Watson 1982 and 1987). It should be noted that there is no typical range available for folic acid in grain, although an average value of 0.3 ppm is reported (Watson, 1987). Levels of folic acid in corn line 1507 and the control corn were not significantly different.

European field trial

Another study was conducted at locations in France and Italy. Corn line 1507 and a non-GM control corn with genetics representative of the test line, were grown also in field sites in locations in France and Italy. At each of the sites in Italy, plots comprising transformed plants sprayed with glufosinate-ammonium herbicide, unsprayed transformed plants, and unsprayed

control plants were cultivated. Glufosinate-ammonium herbicide was not applied to any of the plants at the trial sites in France. Grain and whole plant tissue samples were collected for measurement of various compositional and nutritional parameters.

As in the Chilean field trials, the nutrient analyses included moisture, fat, protein, ash, fibre and carbohydrates for whole plant samples. Grain samples were analysed for moisture, crude fat, crude protein, ash, fibre, and carbohydrate content, as well as fatty acid and amino acid composition, levels of minerals, certain vitamins, and the anti-nutrient compounds phytic acid and trypsin inhibitor. In addition, tocopherol levels in the grain were measured.

The forage samples were dried to between 7% and 14% moisture before processing. The grain was dried to between 9% and 12% moisture before shelling. Laboratories at Woodson-Tenent determined the exact moisture content for each sample so that the results could be reported on a dry weight basis.

The results of the proximate analyses on grain are presented in Table 4 below. The values are estimated means (across all sites) and carbohydrate levels are arithmetically calculated using measured percentages of protein, fat and ash.

Table 4: Proximate analysis of grain across all sites.

Variable (% dry weight)	Corn line 1507 (unsprayed) (mean ± SE)	Corn line 1507 (sprayed) (mean ± SE)	Non-GM control (mean ± SE)	Literature Range*
Fat	4.21 ± 0.12	4.41 ± 0.14	4.41 ± 0.12	3.1 – 5.7
Protein	11.73 ± 0.24	12.04 ± 0.28	10.98 ± 0.24	6.0 – 12.0
ADF	2.37 ± 0.17	2.52 ± 0.18	2.29 ± 0.17	3.0 – 4.3
NDF	10.16 ± 0.30	10.54 ± 0.35	10.13 ± 0.30	8.3 – 11.9
Carbohydrates	82.46 ± 0.57	81.97 ± 0.25	83.00 ± 0.28	63.3 – 89.7
Ash	1.60 ± 0.04	1.67 ± 0.05	1.56 ± 0.04	1.1 – 3.9

* Watson, 1982 or Watson, 1987.

The results show that grain from transformed corn line 1507, either sprayed or unsprayed, has similar levels of the proximate components to the non-transformed control corn line. Furthermore, with the exception of ADF (Acid Detergent Fibre), the measurements were all within the respective literature range for that variable. The levels of ADF in both the control and transformed line were both slightly lower than the published range indicating a small genotypic variation that is not noteworthy.

The proximate analyses on the forage samples in the European study also show no statistically significant differences between corn line 1507 and the non-GM control samples (data not presented). Furthermore, there was close correlation in the proximate results obtained from both the Northern and Southern hemisphere studies for both the grain and the forage.

Mineral analysis

As was observed in the field trials in Chile, the levels of eight minerals (calcium, phosphorus, copper, iron, magnesium, manganese, potassium and zinc) present in the grain from corn line 1507, either from sprayed or unsprayed plants, and the non-GM control, reveal no differences of any biological significance between the comparators. Furthermore, all of the values were within the reported literature range, noting that the accepted range for calcium levels was derived from data obtained from an analysis of 22 commercial hybrid corn lines (not

genetically modified). Sodium levels were again below the limit of quantitation for all the lines, including the non-GM control.

Amino acid analysis

The results of the amino acid analysis of the grain from the transformed and non-transformed lines grown in the European field trials are presented in Table 5.

There are small statistically significant ($p < 0.05$) differences between corn line 1507 and its comparator in the amino acid levels indicated in Table 5 (bold print). In general, individual amino acid levels in corn line 1507 are marginally elevated when compared to those of the control line, although the measurements show no difference between the sprayed and unsprayed GM plants. In addition, the levels of threonine and glutamic acid in 1507 corn, unsprayed and sprayed, were marginally higher than the respective published literature range.

In general, the data indicate that protein levels in corn line 1507 are marginally higher than in the non-GM control corn, although the difference is small and not statistically significant. This difference is reflected in slightly elevated measurements of several of the amino acids in the transformed line that are considered typical for the genetic background of this particular corn.

Fatty acid analysis

To allow a comparison between studies, the same five major fatty acids in corn grain were analysed in the European study: palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3). There was no statistically significant difference between the transformed line (sprayed or unsprayed) and the non-transformed counterpart in the observed levels of these five fatty acids. All samples tested were also within the previously established literature range for the respective fatty acid component.

Vitamin analysis

As in the Chilean study, grain from corn line 1507 and the control was analysed for its content of four vitamins: thiamine hydrochloride (B1), riboflavin (B2), folic acid and total tocopherols. The levels of these vitamins in the GM line were not different from the control, whether the plants were sprayed or unsprayed.

Table 5: Amino acid composition of corn grain. The values are estimated mean values across all sites \pm standard errors. Data presented as percentage of dry weight.

Amino acid	Corn Line 1507 (unsprayed)	Corn Line 1507 (sprayed)	Non-GM Control	Literature range a b
Glycine	0.41 \pm 0.0090	0.42 \pm 0.0102	0.38 \pm 0.0090	0.26-0.47 0.24-0.41
Threonine	0.41 \pm 0.0080	0.41 \pm 0.0094	0.37 \pm 0.0080	0.29-0.39 0.21-0.37
Valine	0.51 \pm 0.0106	0.52 \pm 0.0125	0.47 \pm 0.0106	0.21-0.52 0.25-0.67
Isoleucine	0.41 \pm 0.0098	0.41 \pm 0.0116	0.36 \pm 0.0098	0.26-0.40 0.19-0.39
Leucine	1.38 \pm 0.03	1.41 \pm 0.04	1.23 \pm 0.04	0.78-1.52 0.43-1.35
Phenylalanine	0.55 \pm 0.018	0.56 \pm 0.014	0.49 \pm 0.012	0.29-0.57 0.04-0.54
Histidine	0.31 \pm 0.0065	0.32 \pm 0.0076	0.29 \pm 0.0065	0.20-0.28 0.21-0.32
Lysine	0.32 \pm 0.008	0.33 \pm 0.009	0.31 \pm 0.008	0.20-0.38 0.19-0.36
Arginine	0.47 \pm 0.012	0.48 \pm 0.014	0.44 \pm 0.012	0.29-0.59 0.28-0.55
Cysteine	0.22 \pm 0.004	0.23 \pm 0.005	0.22 \pm 0.004	0.12-0.16 0.13-0.27
Methionine	0.20 \pm 0.0035	0.21 \pm 0.0041	0.20 \pm 0.0035	0.10-0.21 0.12-0.26
Tryptophan	0.10 \pm 0.0035	0.10 \pm 0.0037	0.09 \pm 0.0035	0.05-0.12 0.05-0.10
Serine	0.55 \pm 0.012	0.56 \pm 0.014	0.50 \pm 0.012	0.42-0.55 0.25-0.46
Alanine	0.83 \pm 0.018	0.85 \pm 0.022	0.74 \pm 0.018	0.64-0.99 0.37-0.81
Glutamic Acid	2.12 \pm 0.050	2.18 \pm 0.060	1.90 \pm 0.050	1.24-1.96 0.89-2.02
Proline	1.00 \pm 0.0212	1.04 \pm 0.0258	0.92 \pm 0.0217	0.66-1.03 0.43-1.01
Aspartic Acid	0.79 \pm 0.0157	0.81 \pm 0.0186	0.71 \pm 0.0157	0.58-0.72 0.37-0.80
Tyrosine	0.21 \pm 0.0048	0.21 \pm 0.0057	0.19 \pm 0.0048	0.29-0.47 0.17-0.31

a Watson, 1982.

b Data from analysis of 22 Pioneer® brand hybrids.

Key toxicants

In general, more than 70% of edible corn grain is composed of starch, with smaller amounts of protein, oil and other nutritionally valuable substances. There are no known naturally occurring toxins in corn. While mycotoxins can be detected in corn, these are metabolites produced by fungal contamination of corn kernels as a result of production and storage under adverse conditions. They are not a natural component of sound corn.

Key anti-nutrients and secondary metabolites

Corn contains insignificant levels of anti-nutrient compounds. The levels of trypsin inhibitor in particular are known to be very low (Del Valle *et al.*, 1983; Watson, 1987). Lectins, carbohydrate-binding proteins with haemagglutination activity, have been found at low levels in the endosperm and germ. Phytic acid is also present in low amounts in corn, and levels in maize grain vary from 0.45 to 1.0% of dry matter (Watson, 1987).

Grain from corn line 1507 and the control corn, collected from sites in both field trials, was analysed for five secondary metabolites. The secondary metabolites measured were inositol, raffinose, p-coumaric acid, furfural and ferulic acid. The trypsin inhibitor activity of the transformed and non-transformed corn grain was also compared using an enzyme activity assay (limit of detection was 2000 TIU/g dry weight of sample). In addition, data on the levels of phytic acid were provided. As the sites in Italy were sprayed with glufosinate-ammonium, the data available are for both sprayed and unsprayed plants. A literature range was available only for raffinose and phytic acid, as other compounds occur at such low levels that they are not generally measured.

The levels of trypsin inhibitor and furfural were below the limit of quantitation. The levels of the other metabolites and phytic acid in corn line 1507 are similar to the levels found in the untransformed control grain. There were no observed differences between the sprayed and unsprayed plants and all values were within the literature reported range where that was available.

Summary and conclusions from compositional analyses

In general, examination of data from the comparative analyses of both the Chilean and European studies, generated over a period of two years, reveal no compositional differences of biological significance in the grain from the transformed line and the non-transformed control. Several observed differences in some nutrient components such as amino acids are not indicative of an overall pattern of change arising from the genetic modification. Whereas measurements are similar across sites for the transformed line, the compositional analyses for the control line are marginally lower overall in the European study. The data are explained by the known natural variation in composition that arises due to a broad range of factors that influence plant growth and biochemistry. In addition, the spraying of corn line 1507 with glufosinate-ammonium herbicide does not have a measurable effect on the composition of the grain.

NUTRITIONAL IMPACT

Animal feeding studies

In assessing the safety of a genetically modified food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and well-being. In most cases, this can be achieved through an understanding of the genetic modification and its consequences together with an extensive compositional analysis of the food. Where, on the basis of available data, there is still concern or doubt in this regard, carefully designed feeding studies in animals may provide further reassurance that the food is nutritionally adequate.

Such studies may be considered necessary where the compositional analysis indicates significant differences in a number of important components or nutrients, or where there is concern that the bioavailability of key nutrients may be compromised by the nature of the genetic changes to the food.

In this case, the nutritional performance of corn line 1507 was investigated with a feeding study using chickens. Rapidly growing broiler chickens are sensitive to changes in nutrient quality in diets, and therefore serve as a useful model species to evaluate the wholesomeness of protein/amino acid sources.

Feeding study in Broiler Chickens

Study evaluated:

Zeph, L., 2000. Nutritional Equivalency of B.t. Cry1F Maize – Poultry Feeding Study. Performing Laboratories PARC Institute Inc., 30 North Harrison Street, Easton, MD 21601. PARC Trial Number 98-PIO-25a-BB, Study Number PHI99-010.

This study compares the performance and processing parameters of rapidly growing broiler chickens (*Gallus domesticus*) raised on a diet containing either corn line 1507, a non-transformed control corn line (Hybrid 7250) or four commercially available reference corn lines, over approximately 42 days. The non-transformed control hybrid line has a genetic background representative of the test line, but is not genetically modified and does not express either the Cry1F or PAT proteins.

A total number of 245 healthy male chickens (Cobb x Cobb) were randomly assigned to the various treatment groups. All diets were formulated to meet nutritional recommendations, and consisted of a commercial corn/soy ration. From days 0-20, chickens were fed a starter diet containing 54.21% w/w corn while from days 21-42, chickens were fed a grower/finisher diet containing 57.03% w/w corn. These rations applied to all treatments. Both the solid diets and water were provided to the chickens *ad libitum* and no medication was administered during the course of the study.

To ensure the integrity of the grain used in the study, sub-samples of the test corns (hybrid corn 1507 and the control) were analysed for expression of Cry1F using a specific ELISA. This assay system confirmed the presence of the novel protein in grain from corn line 1507 and its absence in the control grain. In addition, samples of whole corn grain, starter and grower diets for each treatment were analysed for nutrient composition (proximates, amino acids, calcium and phosphorous). The results demonstrate a close similarity in composition of all diets and dietary components in the study.

During the course of the study, the birds were examined daily for general health, and any abnormal observations were recorded. Individual body weight was recorded on days 0 and 42, and body weight gain over the period (trial days 0 to 42) was calculated from these data. Standard statistical methods were applied in the calculation for feed conversion over the starter and grower/finisher periods. The results are presented in Table 6 below.

Table 6: Summary of performance measures; values are means for all animals per treatment.

○ Parameter	Diet Treatment					
	Ref #1	Ref #2	Ref #3	Ref #4	Control hybrid 7250	Hybrid corn line 1507
Mortality %	5.71	5.71	2.86	5.71	2.86	5.71
Body weight (kg) day 0	0.044	0.043	0.043	0.043	0.044	0.043
Body weight (kg) day 42	1.730	1.739	1.738	1.728	1.739	1.757
Daily gain (g per bird per day)	0.040	0.040	0.040	0.040	0.040	0.041
Feed Conversion (body wt. corrected)	1.797	1.806	1.808	1.804	1.802	1.775

The results of the broiler feeding study show there were no differences in parameters tested between birds fed a diet containing corn line 1507 and four non-transformed reference lines or a control line. These results support the results of the compositional analyses and indicate that corn line 1507 is equivalent to non-transformed corn in the ability to provide adequate nutrition to rapidly growing broiler chickens.

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