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NOTIFICATION TO MARKET PRODUCTS CONTAINING GENETICALLY MODIFIED ORGANISMS IN ACCORDANCE WITH DIRECTIVE 2001/18/EC

1507 MAIZE

Submitted by:

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SECTION 2

INFORMATION REQUIRED IN NOTIFICATIONS CONCERNING RELEASE OF GENETICALLY MODIFIED HIGHER PLANTS (ANNEX IIIB, DIRECTIVE 2001/18/EC)

A. GENERAL INFORMATION

1. Name and address of the notifier

(a) Name of the notifier:

This is a joint notification submitted by:

Pioneer Hi-Bred International, Inc. as represented by Pioneer Overseas Corporation.

Mycogen Seeds, c/o Dow AgroSciences LLC.

(b) Address of notifiers:

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(c) The notifiers are:

Domestic manufacturer

Pioneer Hi-Bred and Mycogen Seeds are developers of the technology and producers of 1507 maize seed (inbreds and hybrids).

Importer

Yes, as importer of seed.

(d) In case of import:

(i) Name of manufacturers

Pioneer Hi-Bred International, Inc.

Mycogen Seeds, c/o DowAgroSciences LLC.

(ii) Addresses of manufacturers

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Des Moines, IA 50309
U.S.A.

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2. Name, qualifications and experience of the responsible scientist(s)

This is a joint notification submitted by Pioneer Hi-Bred, as represented by Pioneer Overseas Corporation, and Mycogen Seeds, c/o Dow AgroSciences LLC. Pioneer Hi-Bred is the primary contact for this submission and therefore all correspondence should be sent to the responsible scientist at Pioneer Overseas Corporation:

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3. Title of the project

Notification to market products containing genetically modified 1507 maize for all uses including cultivation in the EU.

(a) Member State of notification:

Spain.

(b) Notification number:

C/ES/01/01

(c) Name of the product (commercial and other names):

The product described in this notification is *B.t.* Cry1F maize line 1507, referred to as 1507 maize. It consists of maize product consisting of or derived from seed of 1507 maize genetically modified to express CRY1F protein, conferring resistance to certain lepidopteran insect pests, and PAT protein, conferring tolerance to glufosinate-ammonium herbicide. The maize product also consists of progeny derived from conventional breeding between 1507 maize with any traditionally bred maize. Commercial names will be assigned to 1507 maize seed at the time of market introduction.

In accordance with the OECD guidance for the designation of a unique identifier for transgenic plants (ENV/JM/MONO(2002)7), the unique identification code assigned to 1507 maize is DAS-Ø15Ø7-1.

(d) Date of acknowledgment of notification:

11 July 2001

(e) Scope of notification:

This notification is for consent to market genetically modified 1507 maize products in accordance with Part C of Directive 2001/18/EC. The scope of this notification is for all uses of 1507 maize including cultivation of 1507 maize seed (inbreds and hybrids) in the EU. The proposed uses of grain and other products of 1507 maize, arising from imports and cultivation, will be the same as for any other maize. The use of 1507 maize for human food is considered in a separate application submitted in accordance with Regulation (EC) No. 258/97.

The period for the first consent is requested for a maximum period and should end ten years after inclusion date of the first variety of 1507 maize on an official

national catalogue of plant varieties in accordance with Council Directives 70/457/EEC and 70/458/EEC.

B. INFORMATION RELATING TO THE RECIPIENT OR PARENTAL PLANTS

1. Complete name

(a) Family name:	Gramineae
(b) Genus:	*Zea*
(c) Species:	*Z. mays* L.
(d) Subspecies:	None
(e) Cultivar/breeding line:	Line Hi-II
(f) Common name:	Maize; corn

2. (a) Information concerning reproduction :

(i) Mode(s) of reproduction

Maize (*Zea mays* L.) is the only species usually included in the genus Zea, of the family Gramineae. It is a highly domesticated agricultural crop with well-characterised phenotypic and genetic traits. It reproduces sexually by wind-pollination and being a monoecious species has separate male staminate (tassels) and female pistillate (silk) flowers. This gives natural outcrossing between maize plants but it also enables the control of pollination in the production of hybrid seed. Typical of wind-pollinated plants, a large amount of redundant maize pollen is produced for each successful fertilisation of an ovule on the ear. Wind movements across the maize field cause pollen from the tassel to fall on the silks of the same or adjoining plants. Measuring about 0.1 mm in diameter, maize pollen is the largest of any pollen normally disseminated by wind from a comparably low level of elevation.

(ii) Specific factors affecting reproduction

As a wind-pollinated, monoecious species, reproduction is by self pollination and fertilisation and, cross-pollination and fertilisation, with frequencies of each normally determined by proximity and other physical influences on pollen dispersal.

Reproductive factors such as tasselling (pollen production), silking, and pollination are the most critical stages of maize development.

Repeated cycles of self-pollination leads to homogeneity of the genetic characteristics within a single maize plant (inbred). Controlled cross-pollination of inbred lines from chosen genetic pools combines desired genetic traits resulting in a hybrid with improved agronomic performance and yield increase. This inbred-hybrid concept and improved yield response is the basis of the modern maize seed industry.

Maize varieties planted by EU farmers are almost entirely hybrids. Open pollination of hybrids in the field leads to the production of grain with properties from different lines and, if planted, would produce lower yields than those obtained with hybrids (Canadian Food Inspection Agency, 1994).

(iii) Generation time

Maize is an annual crop with a cultural cycle ranging from as short as 10 weeks to as long as 48 weeks covering the period of seedling emergence to maturity (Shaw, 1988). This variance in maturity allows maize to be grown over a range of climatic conditions.

(b) Sexual compatibility with other cultivated or wild plant species, including the distribution in Europe of the compatible species

There are no other cultivated or wild plant species sexually compatible with maize in the EU. Maize plants will intra-pollinate and transfer genetic material between maize except for certain popcorn varieties. The extent of pollination between maize will depend upon prevailing wind patterns, humidity and temperature.

It is generally considered that teosinte (*Zea mays* ssp. *mexicana*) is an ancestor of maize, although opinions vary as to whether maize is a domesticated version of teosinte. However, teosinte is an ancient wild grass found in Mexico and Guatemala and it is not present in the EU.

3. Survivability

(a) Ability to form structures for survival or dormancy

Maize is a non-dormant annual crop and seeds are the only survival structures. Natural regeneration of maize from vegetative tissue is not known to occur.

(b) Specific factors affecting survivability

Survival of maize seed is dependant upon temperature, moisture of seed, genotype, husk protection and stage of development. Maize seed can only survive under favourable climatic conditions. Freezing temperatures have an adverse effect on germination of maize seed and it has been identified as a major risk in limiting production of maize seed (Shaw, 1988). The generative phase of maize is supported by short day conditions. Further, maize is a C_4 plant and therefore its vegetative growth is sensitive to low temperatures. Chlorosis will occur at temperatures below 15°C. Moreover, the minimum temperature for germination of 8 to 10°C will restrict its survival and reproduction capabilities mainly to the Southern European geographical zones. Temperatures above 45°C have also been reported as damaging to the viability of maize seed (Craig, 1977).

4. Dissemination:

(a) Ways and extent of dissemination

Maize dissemination occurs via kernel (seed/grain) and pollen. Maize has been domesticated for thousands of years and as a result maize dispersal of individual kernels does not occur naturally.

Pollen shedding from the tassels takes place over a period of 10 to 15 days. Pollen grains are round, heavy and with a high water content, characteristics that limit their dispersal and attachment to plant surfaces, such as leaves. Generally, viability of shed pollen is 10 to 30 minutes, although it can remain viable for longer time under favourable conditions (Canadian Food Inspection Agency, 1994). However, dispersal of maize pollen tends to be limited as it is influenced by the large size and rapid settling rate of the pollen. Deposition of maize pollen has been found to rapidly decline from 2.3×10^7 grains m^{-2} at a 1 m offset from the field edge to 7.1×10^3 grains m^{-2} at 60 m: this represents a decline in pollen concentrations of over four orders magnitude extending from radial distances of 1 m to 60 m from the field edge (Raynor *et al.*, 1972).

(b) Specific factors affecting dissemination

Mechanical harvesting and transport are ways of disseminating grain and insect or wind damage may cause mature ears to fall to the ground and avoid harvest. Regardless of these routes of dissemination, maize cannot survive without human assistance in non-agricultural habitats in the EU. Because of its highly domesticated nature, maize seed requires the semi-uniform soil conditions resulting from cultivation in order to germinate and establish in agricultural habitats (Canadian Food Inspection Agency, 1994).

5. Geographical distribution of the plant

Maize is grown over a wide range of climatic conditions because of its many divergent types. However, survival and reproduction in maize is limited by cool conditions (Shaw, 1988). Practically no maize can be grown where the mean mid-summer temperature is <19°C or where average night temperature falls below ~13°C. The majority of maize is produced between latitudes 30 and 55 degrees, with relative little grown at latitudes higher than 47 degrees anywhere in the world. The greatest maize production occurs where the warmest month isotherms range between 21 and 27°C and the freeze-free season lasts 120 to 180 days. Summer rainfall of 15 cm is the lower limit for maize production without irrigation. There is no upper limit of rainfall for growing maize, although excess rainfall will decrease yields. Maize has been cultivated in Europe starting in Spain since the 16th century.

6. **In the case of plant species not normally grown in the Member State(s), description of the natural habitat of the plant, including information on natural predators, parasites, competitors and symbionts**

Maize is normally grown in the EU and its natural habitat consists of the relatively well characterized agricultural environment. Maize is known to interact with other organisms in the environment including insects, birds, and mammals. It is susceptible to a range of fungal diseases and insect pests, as well as competition from surrounding weeds.

7. **Other potential interactions, relevant to the GMO, of the plant with organisms in the ecosystem where it is usually grown, or elsewhere, including information on toxic effects on humans, animals and other organisms**

Maize is extensively cultivated in the EU and has a history of safe use. Maize grain and forage, or derived products of maize, are not considered to have toxic effects on humans, animals and other organisms (Del Valle, 1983).

C. INFORMATION RELATING TO THE GENETIC MODIFICATION

1. Description of the methods used for the genetic modification

The particle acceleration method (high-velocity microprojectiles) (Klein *et al.*, 1987) was used to introduce a purified linear DNA fragment (PHI8999A, 6235 bp; Figure 1) containing the *cry*1F and *pat* coding sequences and the necessary regulatory components into maize cells resulting in maize event 1507. The plant regenerated from these maize cells expressed the CRY1F protein and the PAT protein and is referred to as *B.t.* Cry1F maize line 1507, or simply as 1507 maize. Details of the transformation are described below.

Immature embryos isolated from the Hi-II maize ears harvested soon after pollination were cultured on callus initiation medium for several days. On the day of transformation, microscopic tungsten particles were coated with the purified PHI8999A insert (Figure 1; Table 1), containing the *cry*1F and *pat* genes, and they were accelerated into the cultured embryos, where the insert DNA was incorporated into the maize cell genome. In particular, the *nptII* gene was not part of the 6235 bp linear DNA fragment (PHI8999A) that was purified and used in the transformation. The *nptII* gene is therefore not present in 1507 maize, as confirmed by the molecular analyses carried out with Southern blots (see Point **D.2.a.**).

After transformation, the embryos were transferred to callus initiation medium containing the herbicide glufosinate-ammonium as the selection agent for the expression of PAT protein. Individual embryos were kept physically separate during culture, and the majority of explants died on the selective medium.

Those embryos that survived and produced healthy, glufosinate-ammonium tolerant callus tissue were assigned unique identification codes representing putative events, and were continually transferred to fresh selection medium. Maize plants were regenerated from tissue derived from each unique event and transferred to the greenhouse. Leaf samples were taken for molecular analysis to verify the presence of the inserted genes by PCR and to confirm expression of the CRY1F protein by ELISA. Plants were then subjected to a whole plant bioassay using European corn borer insects. Positive plants were crossed with inbred lines to obtain seed from the initial transformed plants. A number of lines were evaluated in the field. Maize line 1507 (event 1507) was selected for its agronomic characteristics and excellent resistance to European corn borer and other lepidopteran insect pests.

Other GM maize lines were also obtained in the same manner, such as *B.t.* Cry1F maize line 1360, or simply 1360 maize. The 1360 maize is of particular relevance to this notification as it was selected as an appropriate source of maize expressed CRY1F protein (*i.e.*, representative of similar events, such as 1507 maize) for comparison with microbially-derived CRY1F protein produced in *Pseudomonas fluorescens* to demonstrate the equivalence between maize expressed and microbially-derived CRY1F proteins (Evans, 1998; Annex 25). The intention was to be able to produce sufficient quantity of CRY1F protein from a recombinant microbial source (*i.e.*, microbially-derived CRY1F protein)

for a broad range of studies. This was necessary because it is not possible to derive high amounts of adequate CRY1F protein from any maize line transformed with insert PHI8999A, such as 1507 or 1360 maize.

2. Nature and source of the vector used

No vector was used for the transformation of 1507 maize. As mentioned above (Point **C.1.**), a linear DNA fragment containing the *cry*1F and *pat* coding sequences together with the necessary regulatory components only (Figure 1; Table 1) was used for transformation by particle acceleration. No additional DNA sequences were used for introducing the insert.

The insert was obtained from plasmid PHP8999 (Figure 2) following digestion of the plasmid DNA with the restriction enzyme *Pme*I. As a result, two linear fragments of DNA were obtained: a 6235 bp fragment, *i.e.* the intended insert containing the *cry*1F and *pat* genes; and a 3269 bp fragment not used in transformation. A detailed description of the organization, size and function of the genetic material present in the 3269 bp fragment is provided in Table 2. The 6235 bp (PHI8999A) fragment was subsequently purified by agarose gel electrophoresis and used in the transformation of 1507 maize.

3. Size, source (name) of donor organism(s) and intended function of each constituent fragment of the region intended for insertion

The insert PHI8999A consisted of a linear DNA fragment of 6235 bp containing the synthetic version of plant optimized and truncated *cry*1F gene from *Bacillus thuringiensis* sbsp. *aizawai* with transcription directed by the ubiquitin promoter *ubi*ZM1(2) from *Zea mays* and with a termination sequence derived from ORF25PolyA from *Agrobacterium tumefaciens* extrachromosomal plasmid pTi15995. The insert also contains the synthetic version of plant optimized phosphinothricin acetyltransferase gene sequence, *pat*, from *Streptomyces viridochromogenes* with transcription directed by CaMV 35S promoter and CaMV 35S terminator, from cauliflower mosaic virus.

A restriction map of insert PHI8999A is shown on Figure 1, and a complete description of the size, position, source of donor organism and intended function of the DNA sequences contained in the insert, together with appropriate references, is presented in Table 1.

The size of truncated *cry*1F gene in the intended insert is 1818 bp and has been optimized for plant codon usage. It codes for amino acids 1-605 of the CRY1F protein from *Bacillus thuringiensis* sbsp. *aizawai* and includes the active core of the native CRY1F protein (Chambers, *et al.*, 1991; Figure 3). A change in the coding sequence was made to introduce a *Xho*I restriction site at the 3' end of the truncated *cry*1F gene. This change was designed so that it resulted in a single and conservative amino acid substitution in maize expressed CRY1F protein, leucine at position 604 instead of phenylalanine ($F_{604}L$ conservative substitution). The choice to use the $F_{604}L$ substitution was also based on the occurrence of leucine in the homologous position of other CRY1 proteins (e.g. CRY1AC) from *Bacillus thuringiensis* sp.

Expression of truncated *cry*1F is regulated by the 1986 bp long *ubi*ZM1(2) promoter from *Zea mays* (Figure 4). This promoter is identical to the Ubi-1 promoter for maize ubiquitin gene, originally described by Christensen *et al.* (1992). Termination of transcription of truncated *cry*1F is controlled by the 714 bp long terminator for the mannopine synthase or *mas1* gene from *Agrobacterium tumefaciens* extrachromosomal plasmid pTi15995. The *mas1* gene was initially identified as ORF25 by Barker *et al.* (1983), and hence the terminator sequence used in the modification of 1507 maize was named ORF25PolyA. It comprises base pairs 21728 to 22441, both inclusive, of the Ti plasmid sequence (Barker *et al.*, 1983), and its sequence is given in Figure 5.

The sequence of the *pat* gene in insert PHI8999A is 552 bp long and has been optimized for expression in plants. It is based on the native *pat* gene from *Streptomyces viridochromogenes* (OECD, 1999; Annex 6), and it codes for phosphinothricin acetyltransferase protein, PAT, which provides tolerance to glufosinate-ammonium herbicide. *Streptomyces viridochromogenes* is a common soil bacterium that produces the tripeptide L-phosphinothricyl-L-alanyl-alanine (L-PPT), which was developed as a non-selective herbicide by Hoechst Ag. (Sykes, 1998).

The CaMV 35S promoter and terminator sequences from cauliflower mosaic virus regulate expression of *pat* and consist of 554 and 204 bp, respectively, as described in Table 1. Cauliflower mosaic virus is a DNA caulimovirus with a host range restricted primarily to cruciferous plants (ICTV Database, 1998). It has a double stranded DNA genome within which two distinct promoters, producing 19S and 35S transcripts, have been identified (Kay *et al.*, 1987). The 35S promoter and its variants with enhanced transcriptional activity are constitutively active in several plant species, including maize.

Open reading frame analysis

Theoretically, mRNA can contain more than one start codon for protein translation. These are commonly referred to as open reading frames (ORFs). In the maize genome, smaller ORFs in the alternate frames of a known gene sequence are common. Typically ORFs that could potentially code for proteins of less than 150 amino acids are widespread in the maize genome. However, it is less common to find ORFs in maize that could potentially result in expression of proteins of greater than 200 amino acid residues. Therefore, in searching for potential ORFs in the *cry*1F and *pat* coding sequences introduced into 1507 maize, no significant concern would be associated with ORFs of less than 100 amino acids (300 bp) since these are commonly found in maize.

An analysis of the PHP8999 plasmid, which contains the PHI8999A sequence used in transformation of 1507 maize, for the presence of potential ORFs, both sense and anti-sense, identified no ORFs in the *cry*1F and *pat* coding sequences that could encode proteins of greater than 100 amino acids. A potential ORF of 630 bp spanning part of the ORF25PolyA terminator and the CaMV 35S promoter sequences was identified (Figure 6), however, there are no regulatory sequences next to this potential ORF, named ORF4, that could direct its

expression in 1507 maize. In particular, the sequences directly upstream of this potential ORF4 have not been identified as promoter-like sequences that might drive transcription and translation of the potential ORF4.

Based on the fact that potential ORFs are common throughout the maize genome together with the absence of regulatory sequences in the proximity of the potential ORF4 of 630 bp spanning part of the ORF25PolyA terminator and the 35S promoter in 1507 maize, we can conclude that there is no significant concern associated with the presence of this new potential ORF4 in 1507 maize.

D. INFORMATION RELATING TO THE GENETICALLY MODIFIED PLANT

1. Description of the trait(s) and characteristics which have been introduced or modified

The 1507 maize has been genetically modified (GM) to express the proteins CRY1F and phosphinothricin-N-acetyltransferase (PAT). Expression of the CRY1F protein confers season-long resistance against certain lepidopteran pests, such as the European corn borer (*Ostrinia nubilalis*) and *Sesamia* spp. It is expressed constitutively and when cultivated, it provides control against insect pest damage. Expression of the PAT protein confers tolerance to application of glufosinate-ammonium herbicide. It is expressed constitutively serving as a selectable marker and when cultivated, the maize plant will tolerate field application rates of 1600 g a.i./ha of glufosinate-ammonium without showing any phytotoxicity symptoms. Tolerance to the herbicide provides for an alternative in weed management.

CRY1F protein for insect pest resistance:

The biological activity of CRY1F protein has been tested on a range of lepidopteran insects, including certain pests that feed on maize plants. The tests were conducted by exposing the insects to artificial dietary substrates that had been treated with aqueous formulations of microbially-derived CRY1F protein. Results from these laboratory tests show that the European corn borer (*Ostrinia nubilalis*), fall armyworm (*Spodoptera frugiperda*), corn earworm (*Helicoverpa zea*) and black cutworm (*Agrotis ipsilon*) are susceptible to CRY1F protein (Table 3) (Evans, 1998; Annex 25). In a separate study, the lesser cornstalk borer (*Elasmopalpus lignosellus*), southwestern corn borer (*Diatraea grandiosella*) and sugarcane borer (*Diatraea saccharalis*) were also found to be susceptible to microbially-derived CRY1F protein, whereas there was no activity against western corn borer (*Diabrotica virgifera virgifera*), corn leafhopper (*Dalbulus maidis*) and corn leaf aphid (*Rhopalosiphum maidis*) (Table 4) (Herman and Korjagin, 1999). In addition, laboratory tests have been carried out to determine the survival and development of Mediterranean corn borer larvae fed on 1507 maize leaf disks and on the whole plant (Castañera, 2001) (Annex 16). The results obtained in these laboratory tests show that 1507 maize plants expressing CRY1F protein are highly effective against the Mediterranean corn borer (*S. nonagrioides*), with both types of feeding assays (leaf discs and whole plant) yielding similar results.

Furthermore, the efficacy of 1507 maize hybrids against a range of insect pests has been determined and compared with conventional maize controls under field conditions and the results were found to be consistent with laboratory tests (Table 5).

A separate field study was carried out in 2000 in France to evaluate 1507 maize and non-GM control after application of a synthetic insecticide (Karate Xpress, active ingredient lambda-cyhalothrin) commonly used to control European corn borer infestation (Vernier, *et al.*, 2001b; Annex 34). The 1507 maize showed

significant efficacy to control both European corn borer and pink stalk borer (*Sesamia nonagrioides*) and it provided better protection from both pests than traditional insecticide applications (Table 6).

Additional field trials were carried out at two separate locations in 2002 in Spain, Calatorao and Montañana, in the region of Aragón for the purpose of assessing the agronomic behaviour and efficacy of the 1507 maize plants under different conditions of infestation from the target insect pests ECB and/or *Sesamia* spp. The trials involved two replicates from each of the following ten entries: 1507 maize hybrids from four different genetic backgrounds together with their respective non-GM control maize hybrids with comparable genetic background, and two commercially available non-GM maize hybrids as additional controls. Neither the 1507 maize plants nor the non-GM control maize were sprayed with chemical insecticides. Insect tunnelling lengths (cm) were measured in the stalks of samples of 10 plants per plot and the mean values were calculated for the two replicates planted at each of the two locations.

A summary of the results obtained for the 1507 maize and appropriate non-GM control maize with comparable genetics has been presented in Table 23. The results confirmed that the two locations suffered significantly different insect pest pressure in terms of presence of insect pest species causing and the resulting damage. The overall insect pest pressure was much stronger at Calatorao than at the Montañana location. *Sesamia* spp. was the predominant insect pest at Calatorao, with up to 95% of the larvae identified during splitting of the stalks as *Sesamia* spp., and only 5% identified as ECB larvae. However, at Montañana 50% of the larvae were identified as ECB and 50% as *Sesamia* spp. In addition, Figures 31 to 34 provide actual photographs taken during the field trials which show a comparison of insect damage on 1507 maize versus non-GM control of comparable genetic background. These findings have confirmed the efficacy of 1507 maize against populations of ECB and *Sesamia* spp. and the relevance of 1507 maize against such economically important insect pests in the absence of insecticide application.

In summary, the results show that 1507 maize provides much improved control against lepidopteran insects pests, such as European corn borer and pink stalk borer, in comparison to conventional (non-GM) maize under a range of environmental conditions.

PAT protein for glufosinate-ammonium tolerance:

The active ingredient in glufosinate-ammonium herbicide is L-phosphinothricin (L-PPT). L-PPT binds to glutamine synthetase in plants preventing the detoxification of excess ammonia resulting in plant death. The activity of the PAT protein (phosphinothricin-N-acetyltransferase) is specific to catalysing the conversion of L-PPT to N-acetyl-L-PPT. This is an inactive form which does not bind to glutamine synthetase (De Block *et al.* 1987). The expression of PAT protein in 1507 maize allows the detoxification of ammonia to continue thereby conferring tolerance to the herbicide glufosinate-ammonium. Field trials have shown that 1507 maize is tolerant to field application rates of 1600 g a.i./ha of glufosinate-ammonium herbicide without showing any phytotoxicity symptoms.

This rate is equivalent to four times the recommended field application rate of the glufosinate-ammonium herbicide.

The activity of the PAT protein has been described in detail by the OECD (1999): "Consensus document on general information concerning the genes and their enzymes that confer tolerance to phosphinothricin herbicide" (Annex 6).

No other traits have been introduced or modified in 1507 maize as confirmed by the observations of 1507 maize when cultivated in a range of maize growing regions such as USA, Chile, European Union and Central and Eastern Europe (see Points **D.4.** and **D.13.**).

2. Information on the sequences actually inserted/deleted

(a) Size and structure of the insert and methods used for its characterization, including information on any parts of the vector introduced in the GMHP or any carrier or foreign DNA remaining in the GMHP

The size and structure of the insert present in 1507 maize has been characterized by Southern blot and DNA sequence analyses, as described below, and based on a series of detailed studies (Glatt, 2000; see Annex 7; see also Annexes 9, 10, 11 and 12).

The genetic material inserted in 1507 maize can be divided into three separate major sections:

i) The 5' border sequence, comprising the flanking region of maize genomic DNA and other DNA segments (regions 1 to 8; Table 18);

ii) The almost full-length PHI8999A insert, corresponding to sequence of bp 11 to 6196 of PHI8999A insert used in the transformation of 1507 maize (region 9 in Table 18); and,

iii) The 3' border sequence, comprising the flanking region of maize genomic DNA and other DNA segments (regions 10 to 15; Table 18).

In summary, the Southern blot and sequence analyses demonstrate that the genetic material inserted in 1507 maize consists of an almost full-length copy of the linear fragment used in the transformation (*i.e.*, 6186 bp from the 6235 bp of insert PHI8999A, containing the *cry*1F and *pat* genes together with the regulatory sequences necessary for their expression). In addition, the plant insert contains the following non-functional fragments:

- one fragment (335 bp) of the *cry*1F gene (region 4 in Table 18), with no *ubi*ZM1(2) promoter sequence, and one fragment (15 bp) of the *cry*1F gene (region 7c), both located at the 5' end of the almost full-length insert;
- two fragments (201 bp and 138 bp long, respectively) of the *pat* gene (regions 7a and 7b, respectively), without regulatory sequences associated,

located at the 5' border and, one fragment (188 bp) of the *pat* gene, located at the 3' border (region 13);
- one fragment (118 bp) of the polylinker region and *ubi*ZM1(2) promoter sequence located at the 5' border (region 8);
- one fragment (550 bp) of the ORF25PolyA terminator sequence in inverted position located immediately at the 3' end of the almost full-length insert (region 10).

The 1507 maize does not contain the *nptII* gene nor any other detectable fragments from the portion of plasmid PHP8999 that was not intended for transformation of 1507 maize. Maize genomic DNA flanking regions at both the 5' and 3' borders of the 1507 maize insert have been sequenced and characterised in detail. In addition, analysis by PCR amplification has confirmed the presence of both maize genomic flanking regions in non-GM Hi-II maize used in the transformation of 1507 maize.

The base pairs missing from the almost full length insert are the following: base pairs 1-10 at the 5' end of the PHI8999A linear DNA fragment used in the transformation of 1507 maize, and base pairs 6197 to 6235 at the 3' end of the PHI8999A linear DNA fragment. These base pairs have probably been lost during the transformation process of 1507 maize. However, the loss of base pairs from the ends of linear DNA fragments during the transformation of plants is not unusual and can be expected.

As described in detail in **Point C.3** and Table 1, the missing base pairs correspond to the polylinker regions found at both ends of the linear DNA fragment used in the transformation. These polylinker regions have been purposely built in and correspond to sequences of DNA containing appropriate restriction sites in order to allow the cloning of the necessary adjacent genetic elements. Their function is thus limited to the manipulation of the DNA sequence in the laboratory prior to the actual transformation of maize. In particular, the location of the missing base pairs confirms that they are not relevant, and that their absence will not give rise to any adverse effect on the expression or stability of the *cry*1F and *pat* genes introduced in 1507 maize. Furthermore, the expression of the *cry*1F and *pat* genes and the stability of the genetic modification in 1507 maize has been confirmed by the detailed analyses discussed below and in **Points D.3.** and **D.5.**, which include Southern, Western blot and segregation analyses, respectively, and as confirmed by the phenotypic and agronomic characteristics of the 1507 maize plants (**Point D.4.**) and by the results of the Northern and RT-PCR analysis discussed below.

Southern analysis:

Leaf samples were obtained from plants of two different generations during the breeding of 1507 maize: designated as T1S1 generation and BC4 generation. The T1S1 generation seed consisted of the original transformed Hi-II maize line crossed to an elite inbred to give an F1 hybrid, and then selfed to give T1S1 seed. The BC4 generation seed consisted of the fourth backcross generation of the original transformed Hi-II maize line. Plants of both generations were

grown in the glasshouse and leaf samples obtained for genomic DNA extraction and analysis.

Plasmid PHP8999 DNA, genomic DNA from Hi-II maize, and genomic DNA from 1507 maize T1S1 and BC4 generations were digested with the restriction enzymes *Pme*I, *Hin*dIII, *Pst*I, *Bam*HI, *Eco*RI, and *Bam*HI combined with *Eco*RI. Genomic DNA lots within each 1507 maize generation were pooled prior to digestion. Representative aliquots of each digest were separated on five individual agarose gels and transferred to nylon membranes for hybridization with specific DNA probes. A description of the DNA probes used in this study is shown in Table 7 and their location on plasmid PHP8999 is shown on Figure 7.

The expected results for each digestion and hybridization are shown in Table 8, and the observed results for each digestion and hybridization are shown in Table 9. The expected fragment sizes in Table 8 considers that the DNA fragment used to transform 1507 maize (insert PHI8999A) was inserted into the genome as a single intact copy.

As expected, the *Pme*I restriction site is lost during transformation because the specific sequence required for *Pme*I digestion (GTTT/AAAC) is not likely to be present at the point of integration into the maize genome. Therefore, the expected and observed hybridization fragments are larger than 6235 bp (Tables 8 and 9).

Confirmation for presence of *cry*1F gene:

The digestions with *Hin*dIII, *Pst*I, and *Bam*HI were conducted for the purpose of characterizing the *cry*1F gene and its *ubi*ZM1(2) promoter in 1507 maize. The *Hin*dIII restriction enzyme cuts at the 5' end of the *ubi*ZM1(2) promoter and the 3' end of the *cry*1F coding sequence. The purpose of the *Hin*dIII digestion was to determine whether the full-length *cry*1F gene is present with its promoter intact. The *Pst*I digestion was intended to provide further information on whether the *ubi*ZM1(2) promoter was intact as this enzyme cuts essentially at both ends of this promoter. *Bam*HI digestion was intended to provide information on whether the *cry*1F coding sequence was intact as this enzyme cuts at both the 5' and 3' end of this coding sequence.

The expected results for the *cry*1F gene can be summarized as follows. *Hin*dIII digestion was expected to produce a 3890 bp fragment containing the *ubi*ZM1(2) promoter and *cry*1F gene. This expected fragment was observed after hybridization with probes specific for the *ubi*ZM1(2) promoter and *cry*1F (Figures 8 and 9). The *Pst*I digestion supports the conclusion that *ubi*ZM1(2) promoter is intact resulting in the expected 1986 bp fragment when genomic DNA was hybridized with the *ubi* probe (Figure 8). Finally, the expected 1828 bp fragment was present when genomic DNA was digested with *Bam*HI and hybridized with the *cry*1F probe, which indicated an intact *cry*1F coding sequence is present (Figure 9). Table 9 summarizes these results for the *cry*1F gene and its promoter.

The evidence for an additional copy of the *cry*1F sequence is based on results of the *Hin*dIII and *Pst*I digestions followed by hybridization with the *cry*1F and *ubi* probes as shown in Table 9. *Hin*dIII digestion and hybridization with the *cry*1F probe resulted in two bands: one of the expected 3890 bp size and a second, representing an additional copy, that is larger and estimated at ~4000 bp in size (Figure 9). Hybridization of the *Hin*dIII digest with the *ubi* probe resulted in one band of the expected 3890 bp size and failed to reveal the ~4000 bp fragment (Figure 8). This indicates that the promoter region is either absent in this additional copy or it is not intact. A small portion of the *ubi*ZM1(2) promoter cannot be detected by the *ubi* DNA probe used in this study because the *ubi* probe was prepared with a fragment of the *ubi*ZM1(2) promoter extending from 120 bp to 1707 bp (Table 7). Therefore, an approximately 300 bp region of the *ubi*ZM1(2) promoter that is 5' to the *cry*1F gene cannot be detected with this probe. None of the other digestions were designed to provide evidence for the presence or absence of the *ubi*ZM1(2) promoter on the additional *cry*1F gene. Interpretation of hybridization results with the *ubi* probe is made difficult by the fact that the *ubi*ZM1(2) promoter was isolated from maize and therefore is present in the non-GM control maize plants. This results in hybridizing bands that appear in the lanes containing DNA from the non-GM control and 1507 maize (Figure 8 and Table 9). Nevertheless, the results of the *Hin*dIII digestion support the conclusion that the *ubi*ZM1(2) promoter on the additional copy of the *cry*1F coding sequence is either absent or not intact.

Confirmation for presence of *pat* gene:

The digestions with *Eco*RI, *Bam*HI, and the combination *Bam*HI/*Eco*RI, were conducted for the purpose of characterizing the *pat* gene and its CaMV promoter in 1507 maize. The *Eco*RI enzyme cuts at the 5' end of the CaMV 35S promoter and at the 3' end of the CaMV 35S terminator for the *pat* gene and was expected to result in a 1329 bp fragment if an intact copy of the *pat* gene and its CaMV 35S promoter and terminator is present in 1507 maize. The *Bam*HI enzyme cuts at the 5' end of the *pat* gene and within approximately 150 bp of the 3' end of this gene. An additional digestion with the combination *Bam*HI/*Eco*RI was conducted to determine whether a 546 bp fragment corresponding to the CaMV 35S promoter could be detected after hybridization with the CaMV 35S promoter DNA probe.

The results for the *pat* gene can be summarized as follows. The expected 1329 bp *Eco*RI fragment was observed after hybridization with the CaMV 35S and *pat* DNA probes (Figures 10 and 11). The presence of an intact CaMV 35S promoter was confirmed because the expected 546 bp fragment was observed with the combined *Bam*HI/*Eco*RI digestion (Figure 10). The presence of an intact *pat* gene was confirmed because the expected fragments were observed after *Bam*HI digestion followed by hybridization with the *pat* DNA probe (Figure 11). Finally, *Hin*dIII digestion was expected to produce 2170 bp fragment containing the CaMV 35S promoter, *pat* gene, and CaMV 35S terminator if the sequences were present as full-length copies. This expected fragment was observed after hybridization with the CaMV 35S and *pat* DNA probes (Figures 10 and 11). Table 9 summarizes these results for the *pat* gene and its promoter and terminator.

Confirmation for absence of *nptII* gene and sequences of plasmid PHP8999 not intended for transformation:

The *Pme*I DNA fragment used to transform 1507 maize was obtained from plasmid PHP8999. The portion of the plasmid that was used as the insert for transformation did not contain the kanamycin resistance gene, *nptII*. To further verify that 1507 maize does not contain the *nptII* gene, genomic DNA was hybridized with an *nptII* probe (Table 7). The Southern blot is shown in Figure 12. As expected, no bands hybridizing to the *nptII* DNA probe were detected, which confirmed that the *nptII* gene for kanamycin resistance is not present in 1507 maize.

Additional Southern blot analyses of 1507 maize have been carried out to provide further evidence on the absence of all sequences of plasmid PHP8999 that were not intended for transformation of 1507 maize.

Genomic DNA samples of eight 1507 maize plants from two different generations (T1 and BC4, early and late generations, respectively) were digested with *Hin*dIII restriction enzyme and probed with *i)* whole-length plasmid PHP8999 (9054 bp); *ii)* 3.9 kb *Hin*dIII fragment corresponding to part of insert PHI8999A; and, *iii)* 2.2 kb *Hin*dIII fragment corresponding to another part of insert PHI8999A. Figure 13 shows the location of these probes relative to insert PHI8999A. As expected, only bands relating to the *Hin*dIII fragments corresponding to parts of insert PHI8999A have been obtained. Figure 14 shows the results of Southern blot analyses of 1507 maize probed with plasmid PHP8999 (blot *i)* in Figure 14) and with the 3.9 kb and 2.2 kb *Hin*dIII fragments obtained from plasmid PHP8999 (Blots *ii)* and *iii)* in Figure 14, respectively).

Assuming that unintended integration of plasmid PHP8999 had taken place in 1507 maize, a ~3.4 kb band would be expected in the Southern blot probed with the whole length PHP8999 probe (Blot *i)* in Figure 14). However, there is no evidence for the presence of the ~3.4 kb *Hin*dIII fragment corresponding to the part of plasmid PHP8999 that was not intended for transformation of 1507 maize.

No unexpected bands of a smaller size (<3.4 kb) have been observed either. The ~2.2 kb and ~1 kb bands observed in the Southern blots are recognized by the 2.2 kb *Hin*dIII probe corresponding to part of insert PHI8999A (Figure 14, blot *iii)*). The ~3.9 kb band observed corresponds to the 3.9 kb *Hin*dIII fragment expected from the insert PHI8999A. In addition, the largest fragment of ~6.7 kb observed is recognized by the ~3.9 kb probe corresponding to part of insert PHI8999A, and may correspond to the additional *cry*1F copy plus flanking maize genomic sequence.

ORF analysis of the 1507 maize insert sequence:

Analysis of the 1507 maize insert sequence for the presence of potential open reading frames (ORFs) has been carried out. In the maize genome, smaller ORFs in the alternate frames of a known gene sequence are

common. Typically, ORFs that could potentially code for proteins of less than 150 amino acids are common in maize. However, it is less common to find ORFs in maize that could potentially result in expression of proteins of greater than 200 amino acid residues. Therefore, in searching for potential ORFs in the 5' and 3' border sequences and in the rest of the sequence of 1507 maize insert, no significant concern would be associated with ORFs of less than 100 amino acids (300 bp) since these are commonly found in maize.

As shown in Figure 20, three potential ORFs longer than 300 bp (100 amino acids) were identified on the 1507 maize insert 5' border sequence: ORF1 spanning bases 362-691 (330 bp total); ORF2 spanning bases 433-780 (348 bp total); and, ORF3 spanning bases 1896-2648 (753 bp total) extending from near the end of region 4 to region 7b (Figure 20).

A potential open reading frame, ORF4, was identified in the full-length insert of 1507 maize partially spanning the ORF25PolyA terminator and the CaMV35S promoter sequences (Figure 20). This potential ORF of 630 bp (210 amino acid residues) is also present in the PHI8999A sequence used in the transformation of 1507 maize. However, there were no ORFs longer than 300 bp identified in the 3' border sequence of the 1507 maize insert (regions 10 to 15).

Both ORF1 and ORF2 are contained within regions 1 and 2 of the 1507 maize insert which correspond to maize genomic DNA. This has been confirmed by PCR analysis showing that regions 1 and 2 are present in non-GM Hi-II maize used in the transformation of 1507 maize, as discussed in detail below.

PCR analysis:

PCR analysis was used to determine whether the regions with no known homology in the 5' and 3' border sequences of the 1507 maize insert are present in the non-GM Hi-II maize used in the transformation to produce 1507 maize and thus represent the borders with maize genomic DNA. The PCR results obtained confirm the presence of region 1, region 2 and region 15 in non-GM Hi-II maize (Figures 21 and 22). In addition, and as discussed above, ORF1 and ORF2 are contained within regions 1 and 2 of the 1507 maize insert and therefore the PCR results also confirm the presence of ORF1 and ORF2 in non-GM Hi-II maize.

In order to confirm the presence of the regions 1 and 2, with no known homology, in the genomic DNA of 1507 maize and non-GM Hi-II maize, five different PCR analyses were carried out. Three reactions were designed to amplify DNA within regions 1 through 3 (Table 19). The first reaction was entirely within region 1 of the 5' border of 1507 maize insert (Reaction A - 300 bp amplicon), the second reaction spanned region 1 to region 3 (Reaction B - 768 bp amplicon) and the third spanned region 2 to region 3 (Reaction C - 424 bp amplicon). The expected amplicons were present in both 1507 maize and in the non-GM Hi-II maize (Figure 21). Reactions D and E were designed as

specific primer pairs for the 1507 maize insert and should not produce an amplicon in the non-GM Hi-II maize. The results, shown in lanes 12 – 17 of Figure 21, indicate that both reactions D and E are specific for 1507 maize and produce an amplicon of the expected size. The PCR results show that the undescribed sequence (region 1 and region 2), which contains ORF1 and ORF2, is present in the non-GM Hi-II maize. These results also show that regions 2 and 3 are contiguous in the non-GM Hi-II maize. The DNA sequences amplified in reactions A, B, and C are not unique to the 5' border of 1507 maize insert but are also present in the genomic DNA of non-GM Hi-II maize.

A separate PCR analysis was used to determine if the region with no known homology at the end of the 3' border of 1507 maize insert (region 15 in Table 18) is present in non-GM Hi-II maize used for transformation to produce 1507 maize. Successful PCR amplification of region 15 in both 1507 maize and non-GM Hi-II maize revealed that region 15 was present in maize genomic DNA. Five different PCR analyses were carried out on genomic DNA prepared from 1507 maize and non-GM Hi-II maize (Table 20). Three reactions were designed to amplify DNA within region 15 of the 3' border; reaction H - 175 bp amplicon, reaction I – 134 bp amplicon, and reaction J – 107 bp amplicon. The expected amplicons were present in both non-GM Hi-II maize and in 1507 maize as shown in lanes 10 through 20 of Figure 22. Reactions F and G were designed as specific primer pairs for 1507 maize that would span the end of the insert into the 3' border region. In reaction F the forward primer was located in the *pat* gene fragment on the 3' end of the full-length insert (region 13 in Table 18) and the reverse primer was located in the undefined region 15. In Reaction G the forward primer was located in the chloroplast hypothetical protein gene on the 3' end of the full-length insert (region 14 in Table 18) and the reverse primer was located in the undefined region 15. Both Reaction F and Reaction G should produce an amplicon in 1507 maize, but should not produce an amplicon in the non-GM Hi-II maize. The results, shown in lanes 2 - 8 of Figure 22, indicated that both reactions F and G were specific for 1507 maize and produced the expected amplicon. The PCR results show that the undescribed sequence (region 15) on the 3' border of 1507 maize insert is also present in genomic DNA isolated from the non-GM Hi-II maize. The DNA sequences amplified in reactions H, I, and J are not unique to the 3' border of 1507 maize insert but are also present in non-GM Hi-II maize.

As discussed above, the ORF1 and ORF2 sequences lie within regions of maize genomic DNA that show no known homology or high identity (90.5%) to undescribed maize genomic sequence, respectively (see regions 1 and 2 in Table 18). In addition and adjacent to them, a region showing a high degree of identity (86.6 to 89.4%) to retrotransposon LTR sequences has been found (region 3 in Table 18). These transposons and retrotransposon-like sequences are very abundant throughout the maize genome and it has been reported that they comprise half or more of the maize genome (Fedoroff, 2000). This fact on the complexity of the maize genome would make it very difficult to determine by PCR analysis whether the 5' and 3' flanking genomic sequences are in fact continuous in the untransformed maize.

On the other hand and as described in detail in **Point D.4.** below and Figure 18, the originally transformed maize plant undergoes a process of consecutive backcrossing with a selected inbred line in order to ensure that only the intended genetic modification is retained from the original transformed plant and breeded (introduced) into the selected inbred line. During this process of backcrossing natural cross-over recombination may occur in the regions of the insert flanking genomic sequence, which may give rise to re-organization and alterations of those sequences. In any case, whether the 5' and 3' insert flanking genomic sequences are continuous or not in the untransformed maize line Hi-II does not add any scientific evidence about the safety of the genetic modification in 1507 maize. In fact, the genetic modification in 1507 maize can be considered to be safe on the basis of the thorough safety evaluation included in this notification.

Absence of vector backbone sequences in 1507 maize:

Additional Southern blot analysis of 1507 maize has been carried out to provide further evidence on the absence of all vector backbone sequences of plasmid PHP8999 that were not intended for transformation of 1507 maize. Three different DNA probes covering the total backbone sequence as present outside of the *Pme*I fragment of plasmid PHP8999 were used in addition to the full-length *pat* and *cry*1F probes used as positive controls for 1507 maize. The location of the backbone and gene probes relative to plasmid PHP8999 and the size of the probes in base pairs are listed in Table 21 and shown in Figure 23. Backbone Probe 25 is 1245 bp in length and covers the region just outside of the *Pme*I site beyond the CaMV35S terminator to the start of the *nptII* coding sequence from bp 6256 to bp 7500 of plasmid PHP8999. The *kan* probe is 625 bp in length and covers the majority of the *nptII* coding sequence from bp 7494 to bp 8118 of plasmid PHP8999. Backbone Probe 34 is 1476 bp in length and covers the 3' end of the *nptII* coding sequence to just before the *Pme*I site near the ubiquitin promoter from bp 8010 to bp 9485 of plasmid PHP8999.

Southern blots were prepared using genomic DNA isolated from 1507 maize (5 µg) and from non-GM control maize (5 µg) and digested with *Hind*III restriction enzyme. The positive controls included 5 µg of non-GM control maize genomic DNA mixed with 0.5, 1.0, 3.0, and 5.0 approximate gene copy equivalents of plasmid PHP8999 DNA and digested with *Hind* III.

Southern blot analysis results are shown in Figure 24 (*kan* probe), Figure 25 (Backbone Probe 25), Figure 26 (Backbone Probe 34), Figure 27 (full-length *pat* probe) and Figure 28 (*cry*1F probe). The three backbone probes did not detect any hybridizing bands in the two lanes containing 1507 maize genomic DNA (lanes 8 and 9) nor in the control lane (lane 1) but were able to detect the positive control samples down to 0.5 approximate gene copy equivalents.

The probes detected fragments of larger than expected size in lanes containing plasmid PHP8999 DNA at 3.0 and 5.0 approximate gene copy equivalents. The larger bands were due to the presence of partially digested or undigested plasmid DNA that was present as either linear DNA, supercoiled plasmid or plasmid concatamers. Hybridizing bands of larger than expected sizes are commonly seen in high copy number plasmid control lanes. The two positive control

probes, full-length *pat* and *cry*1F, produced the expected hybridizing bands in the two lanes containing 1507 maize genomic DNA (lanes 8 and 9 on Figures 27 and 28).

The Southern blot analysis with the full-length *pat* probe after *Hin*dIII digestion (Figure 27) shows the presence of two bands of approximately 2.1 and 2.3 kb, respectively. The 2.1 kb band corresponds to the *pat* gene in the full-length PHI8999A insert in 1507 maize. The 2.3 kb band corresponds to the fragment of the *pat* gene located at the 3' border of the 1507 maize insert (region 13), as described above. As expected, this 2.3 kb band is not observed with the 3' *pat* probe (Figure 11). This is in agreement with the fact that the fragment of the *pat* gene in region 13 consists of 5' sequence of the *pat* gene that was not part of the 3' *pat* probe (Figure 23).

On the other hand, and as reported above (Figure 9), the Southern blot analysis with the *cry*1F probe after *Hin*dIII digestion (Figure 28) confirms the presence of two bands of approximately 3.9 and 4.1 kb, respectively. The 3.9 kb band corresponds to the *cry*1F gene in the full-length PHI8999A insert in 1507 maize, while the 4.1 kb band corresponds to the fragment of the *cry*1F gene located at the 5' border of the 1507 maize insert (region 4). Digestion with *Pst*I also shows a double band of approximately 0.91 and 0.95 kb and a 7.0 kb band. As explained in **Point D.2.a.**, the double band obtained with *Pst*I (Figure 9 and Tables 8 and 9) corresponds to the *cry*1F gene in the full-length PHI8999A insert in 1507 maize, and the 7.0 kb band corresponds to the additional fragment of the *cry*1F gene located at the 5' border of the 1507 maize insert (region 4).

These additional Southern blot results provide further confirmation that only DNA from within the *Pme*I linear fragment (PHI8999A insert) of plasmid PHP8999 has been incorporated into 1507 maize, and that DNA from other vector backbone sequences not intended for transformation has not been incorporated into 1507 maize.

Northern blot and RT-PCR analyses:

The DNA sequence immediately adjacent and 5' to the start of the almost full-length 1507 maize insert includes the ORF3 sequence. This ORF3 sequence extends from base 1896 to base 2648 of the 5' border sequence, *i.e.* it consists of 753 bp, including the stop codon, codes for a conceptual protein of 250 amino acids (Figure 20) and it spans regions 4 to 7b of the 1507 maize 5' border sequence (Table 18). The DNA sequence homology results from the 5' border region indicate that the ORF3 sequence includes 121 bp of *cry*1F gene, 321 bp of maize chloroplast *rpo*C2 gene, 17 bp of maize chloroplast *trnI* gene, 201 bp of *pat* gene, and 93 bp of *pat* gene complementary sequence.

Northern blot and RT-PCR analyses have been carried out in order to assess transcription from potential open reading frames, ORF3 and ORF4, present in the border and insert sequence of 1507 maize. The original reports have been attached as Annexes 11 and 12, respectively, and a summary of the results obtained is given below.

Northern blot analysis of ORF3 transcription

No hybridization signals corresponding to the ORF3 sequence were observed in mRNA isolated from developing kernels of either 1507 maize or non-GM control maize at the limit of detection of the analysis, which was 0.00125 % of the mRNA fraction. In addition, Northern blot analysis of *cry*1F transcription indicated that only full-length transcript from the *cry*1F gene expression cassette was detected in the mRNA fraction of the developing kernels when hybridized to the *cry*1F probe. The absence of any additional bands containing the *cry*1F sequence provides further confirmation for the absence of transcription of the ORF3 sequence in 1507 maize kernels, as the *cry*1F probe used in the analysis would be expected to detect the 121 bp of *cry*1F sequence contained within the ORF3 sequence. Furthermore, transcription of the *pat* gene in developing kernels was below the limit of detection of the analysis as evidenced by the absence of hybridization signal with the *pat* RNA probe in mRNA samples from 1507 maize kernels. Messenger RNA extraction efficiency, mRNA integrity, and consistency of sample loadings were confirmed by hybridization signals to the maize *dap*A gene probe in 1507 maize and non-GM control mRNA samples.

In summary, the Northern blot analysis indicates that the ORF3 sequence is not present as a unique mRNA transcript in 1507 maize kernels at the limit of detection of the analysis, which was 0.00125 % of the mRNA fraction.

RT-PCR analysis of ORF3 transcription

RT-PCR analysis of ORF3 transcription in 1507 maize kernels has been carried out. Samples of total RNA and mRNA isolated from developing maize kernels (10 days after pollination) were analyzed by RT-PCR for the presence of *cry*1F, *pat*, ORF3 or ORF4 transcripts. The RT-PCR results indicated that PCR product could be detected in 1507 maize total RNA and mRNA samples when primers specific for *cry*1F, *pat*, or ORF4 were used for amplification of the respective target sequences. Primers for *cry*1F and *pat* amplified the expected size PCR products from all 1507 maize RNA samples that underwent successful first-strand cDNA synthesis reactions (Annex 12).

The results obtained from the RT-PCR analysis of ORF3 transcription in total RNA or mRNA isolated from developing kernels of either 1507 maize or non-GM control maize confirmed the absence of ORF3 transcript in 1507 maize kernels, at the estimated limit of detection of the analysis. The limit of detection of the RT-PCR analysis was conservatively estimated to be 20 fg of target RNA per μg of total RNA. Several sets of primers were tested as potential candidates for RT-PCR analysis of the ORF3 sequence in total RNA or mRNA samples from 1507 maize kernels. However, only one set of primers was capable of unambiguously producing a PCR fragment of the expected size, 333 bp, in the 1507 genomic DNA sample used as control, while others amplified non-specific bands even in the non-GM maize control.

On the other hand and as mentioned above, Northern blot analysis on mRNA did not detect the presence of transcript for the *pat* gene in any of the mRNA samples from 1507 maize kernels tested, in contrast to the RT-PCR results that

detected *pat* transcript in all 1507 maize mRNA samples. In addition and as discussed in **Point D.3.** below, PAT protein expression was not detected in 1507 maize kernels from Western blot and ELISA analyses at the relevant limits of detection of these techniques. The combined Northern, RT-PCR, Western, and ELISA results on *pat* gene expression suggest that the *pat* transcript level in developing kernels of 1507 maize is between the relevant limits of detection for the RT-PCR and Northern analyses, showing that at this low level of *pat* transcript no expression of the PAT protein can be detected in 1507 maize kernels.

The genetic elements immediately 5' and 3' to the ORF3 sequence, do not have well defined genetic functions and do not have the characteristics of plant promoter or terminator sequences, respectively (Table 18). This makes it highly unlikely that the ORF3 sequence could be transcribed even at a level lower than that estimated for the limit of detection of the RT-PCR analysis. In conclusion, no significant expression of the ORF3 sequence can be expected to occur in 1507 maize kernels and, as discussed below, even if a negligible amount of ORF3 protein is expressed in 1507 maize kernels there would be no safety concerns as sequence homology searches carried out for the conceptually translated ORF3 sequence, in accordance with the FAO/WHO (2001) recommendations, have confirmed that the ORF3 sequence does not share any immunologically significant amino acid sequence with known allergens and does not encode for any known allergenic, toxic or coeliakie related protein, as detailed on Annexes 13 and 14

Final conclusions on ORF3 transcription

The results from the analysis described in detail above lead to the following conclusions:

- No ORF3 transcript has been detected by Northern or RT-PCR analysis at the limits of detection of these analyses;
- No significant expression of the ORF3 sequence can be expected to occur in 1507 maize kernels; and,
- Even if a negligible amount of ORF3 protein is expressed in 1507 maize kernels, there would be no safety concerns as sequence homology searches carried out for the conceptually translated ORF3 sequence, in accordance with the FAO/WHO (2001) recommendations, have confirmed that the ORF3 sequence does not correspond to any known allergenic, toxic or coeliakie related protein.

Northern blot analysis of ORF4 transcription

Northern blot analysis has been carried out specifically on mRNA samples from developing kernels (10 days after pollination) of 1507 maize in order to evaluate potential transcription of the ORF4 sequence, as described in detail in Annex 11. No hybridization signals corresponding to the ORF4 sequence were observed in mRNA isolated from developing kernels of either 1507 maize or non-GM control maize at the limit of detection of the analysis, which was

estimated to be 0.0005 % of the mRNA fraction. However, Northern blot analysis of *cry*1F transcription indicated that only full-length transcript from the *cry*1F gene expression cassette was detected in the mRNA fraction of the developing kernels when hybridized to the *cry*1F probe. Furthermore, transcription of the *pat* gene in developing kernels was below the limit of detection of this analysis as evidenced by the absence of hybridization signal with the *pat* RNA probe in mRNA samples from 1507 maize kernels. Messenger RNA extraction efficiency, mRNA integrity, and consistency of sample loadings were confirmed by hybridization signals to the maize *dap*A gene probe in 1507 maize and non-GM control mRNA samples.

In summary, the Northern blot analysis indicates that the ORF4 sequence is not expressed as a unique mRNA transcript in 1507 maize kernels at the above mentioned limit of detection of the analysis (0.0005 % of the mRNA fraction).

RT-PCR analysis of ORF4 transcription

RT-PCR analysis of ORF4 transcription in 1507 maize kernels has been carried out and a detailed description has been attached as Annex 12. Samples of total RNA and mRNA isolated from developing maize kernels (10 days after pollination) were analyzed by RT-PCR for the presence of *cry*1F, *pat*, ORF3 or ORF4 transcripts. The RT-PCR results indicated that PCR product could be detected in 1507 maize kernel total RNA and mRNA samples when primers specific for *cry*1F, *pat*, or ORF4 were used for amplification of the respective target sequences. Primers for *cry*1F and *pat* amplified the expected size PCR product from all 1507 maize RNA samples that underwent successful first-strand cDNA synthesis reactions. However, PCR product for ORF4 sequence was generally detected as a weak signal and was not present in all 1507 maize RNA samples. In addition, detection of the PCR product for ORF4 was strongly dependent upon the primer sets used for ORF4 sequence amplification. Taken together, the results obtained from the RT-PCR analysis of ORF4 transcription suggest that any potential transcript containing the ORF4 sequence would be present at or below the limit of detection of the RT-PCR analysis, which was estimated to be in the range of 0.2 fg to 20 fg of target RNA per µg of total RNA depending on the primer set used for PCR (Annex 12).

The genetic elements located immediately 5' to the ORF4 sequence, *i.e.* the *cry*1F gene and the ORF25PolyA terminator, have well defined genetic functions and are not known to function as, nor have the characteristics of plant promoters making it highly unlikely that the ORF4 sequence detected by RT-PCR is a result of independent transcription of ORF4. Furthermore, virtually all plant genes have multiple polyadenylation sites and production of mRNA populations with a variety of 3'-end points in plants, including maize, has been well documented (Rothnie, 1996; Hunt, 1994). The location of the ORF4 sequence in the 1507 maize insert, *i.e.* 3' to the *cry*1F gene, would explain the presence of the ORF4 sequence in read-through transcripts containing the *cry*1F gene and downstream 3' sequence, which would be produced in a relatively very low amount in 1507 maize kernels. Taking into account that transcription termination and polyadenylation are linked and that transcription termination may occur downstream of the polyadenylation signals, production of a very low

amount of read-through mRNA transcripts containing the ORF4 sequence would not be unexpected in 1507 maize kernels, and this could be the most likely source of the ORF4 sequence detected by RT-PCR analysis. Indeed, RT-PCR analysis of 1507 maize kernels detected amplified product of the expected size, starting from the 3' end of the *cry*1F coding sequence and proceeding into the ORF4 sequence, providing strong evidence for the presence of ORF4 sequence on a read-through transcript from the *cry*1F gene (Annex 12).

However, presence of ORF4 sequence on a read-through transcript from the *cry*1F gene would not result in translation of the ORF4 sequence given that plant translational machinery would only translate the first open reading frame on a polycistronic message, *i.e.* the *cry*1F open reading frame in this case (Futterer and Hohn, 1996). Any *cry*1F mRNA transcripts longer than full-length due to read-through transcription would either produce the CRY1F protein of the expected size or a CRY1F protein of larger size, *i.e.* a fusion protein. As discussed in detail in **Point D.3.** below, the CRY1F protein detected in Western blot analysis of 1507 maize plant tissues was present as two bands of nearly identical molecular weight, commonly referred to as a "doublet". The two bands corresponded to approximately 65 and 68 kDa CRY1F proteins and were detected in leaf, pollen, whole plant, and kernel tissue (Alarcon and Marshall, 2000; Annex 8). This doublet results from limited N-terminal processing of maize expressed CRY1F protein by a plant protease with trypsin-like specificity and, the molecular weights of the two bands, 65 and 68 kDa, correspond to the expected sizes of the partially digested and complete CRY1F proteins, which were predicted to be of 65002.03 Da for trypsinolyzed CRY1F protein (residues 28 to 605) and 68204.56 Da for the complete CRY1F protein (residues 1 to 605), respectively. However, no other CRY1F protein bands were observed in 1507 maize and, in particular, bands of larger molecular weight indicative of a fusion protein of greater molecular weight were not observed in 1507 maize tissues.

On the other hand and as mentioned above, Northern blot analysis on mRNA (Annex 11) did not detect the presence of transcript for the *pat* gene in any of the mRNA samples from 1507 maize kernels tested, in contrast to the RT-PCR results that detected *pat* transcript in all 1507 maize mRNA samples. In addition and as discussed in **Point D.3.** below, PAT protein expression was not detected in 1507 maize kernels from Western blot and ELISA analyses at the relevant limits of detection of these techniques. The combined Northern, RT-PCR, Western, and ELISA results on *pat* gene expression suggest that the *pat* transcript level in developing kernels of 1507 maize is between the relevant limits of detection for the RT-PCR and Northern analyses, showing that at this low level of *pat* transcript no expression of the PAT protein can be detected in 1507 maize kernels.

Final conclusions on ORF4 transcription

Based on the detailed findings of RT-PCR results indicating presence of ORF4 sequence in a read-through mRNA transcript of the gene *cry*1F at relatively very low levels in a subset of 1507 maize RNA samples, together with mRNA Northern results indicating absence of detectable ORF4 transcript above the limit

of detection of the Northern (0.0005 % del ARNm), and the fact that plant translational machinery would only translate the first open reading frame on a polycistronic message, we conclude that expression of the ORF4 sequence does not occur to any significant level in 1507 maize kernels. In addition and as discussed above, even if a negligible amount of ORF4 protein is expressed in 1507 maize kernels, there would be no safety concerns as sequence homology searches carried out for the conceptually translated ORF4 sequence, in accordance with the FAO/WHO (2001) recommendations, have confirmed that the ORF4 sequence does not share any immunologically significant amino acid sequence with known allergens and does not encode for any known allergenic, toxic or coeliakie related protein.

Expression of the levels of detection as "number of messenger copies per cell"

The limit of detection percentages to approximate numbers of individual messenger RNA molecules detectable per maize kernel was estimated in order to calculate the level of detection in terms of "number of messenger copies per cell". For this estimation, the efficiency of RNA extraction from the kernels has been valued at 90% of the total RNA. Average extractable total RNA per kernel at this particular growth stage of 10 days after pollination ranges from 48 to 96 µg. Assuming that this upper amount is close to 90% efficiency, the actual amount of total RNA per kernel estimated is ~106 µg.

In order to be able to calculate the level of detection in terms of "number of messenger copies per cell" from the approximate numbers of individual messenger RNA molecules detectable per maize kernel obtained, an estimate of the total number of cells in one developing maize kernel is needed. According to Commuri and Jones (1999) the number of cells in the endosperm of a maize kernel (12 days after pollination) is approximately 300000 (three hundred thousand). Since majority of a maize kernel is made of the endosperm, it is reasonable to use this figure to estimate the level of detection in terms of number of messenger copies per cell.

As described above, the limit of detection of the RT-PCR analysis was determined to be approximately 0.2 fg of target RNA per µg of total RNA or 0.00000002 % of the total RNA. Taking into account that the 630 nt ORF4 synthetic transcript used to calculate the limit of detection has a molecular weight of ~2.08×10^5 Da (630 nucleotides of 330 Da each) we can infer that 0.2 fg of ORF4 transcript would correspond to ~578 RNA molecules or "copies" per µg of total RNA. Using the above assumption of ~106 µg of total RNA per kernel, we can estimate that the limit of detection of the RT-PCR analysis corresponds to 61268 mRNA copies/kernel. In conclusion, based on the number of cells of the endosperm, the limit of detection of the RT-PCR analysis of 1507 maize grain corresponds to an average value of less than one messenger RNA copy per cell. If the number of germ cells was also taken into account in the calculation, the limit of detection of the RT-PCR analysis for the whole kernel would be shown to be even more sensitive than the current estimate.

On the other hand, the limit of detection of the Northern analysis was determined to be between 0.0005 % and 0.00125 % of the mRNA fraction for the ORF4 and

ORF3 transcripts, respectively. As described above, taking into account a 90% efficiency of the RNA extraction from the kernels, the molecular weights of the synthetic transcripts (the 400 nt synthetic ORF3 transcript has a molecular weight of 2.52×10^5 Da) and the fact that mRNA represents ~1% of the total RNA fraction, the limit of detection of the Northern analysis has been estimated to be between ~15 and ~60 million messenger RNA copies per maize kernel for the ORF4 and ORF3 transcripts, respectively. Therefore, the limit of detection of the Northern analysis of 1507 maize grain would correspond, conservatively, to average values of between ~50 and ~200 messenger RNA copies per cell. If the number of germ cells was also taken into account in the calculation, the limit of detection of the Northern analysis for the whole kernel would be shown to be even more sensitive than this estimate.

Overall conclusion:

The results of the molecular characterization described in detailed above supports the conclusion that 1507 maize contains an almost full-length copy of the DNA insert used in the transformation (i.e., 6186 bp from the 6235 bp fragment of insert PHI8999A containing the *cry*1F and *pat* genes) and a limited number of non-functional sequence rearrangements linked to the almost full length insert. The results also support the conclusion that the sequence of plasmid PHP8999 not intended for transformation, and in particular the *nptII* gene, is not present in 1507 maize.

(b) In case of deletion, size and function of the deleted region(s)

Not applicable to 1507 maize.

(c) Copy number of the insert

As described in detail in Point **D.2.a.** above, 1507 maize contains one copy of the full-length insert PHI8999A, and a limited number of non-functional sequence rearrangements linked to the almost full length insert.

(d) Location(s) of the insert in the plant cells and methods for its determination

The insert is integrated into the maize plant genome as confirmed by the molecular characterization of 1507 maize by Southern blot and sequence analyses (described in detail in Point **D.2.a.**) and based on detailed studies (Glatt, 2000; Annex 7; see also Annexes 9, 10, 11 and 12).

3. Information on the expression of the insert

(a) Information on the developmental expression of the insert during the lifecycle of the plant and methods used for its characterization

Expression of CRY1F and PAT proteins in a range of tissues from 1507 maize representing key developmental stages of a typical maize plant (Iowa State University, 1997) was characterized using specific Enzyme Linked Immunosorbent Assay (ELISA) developed for each protein. Samples of leaf

tissue were taken at the V9 or nine leaf stage. Samples of pollen, silk, stalk at approximately the R1 stage (50% pollen shed) of development, and of whole plants at R4 stage (approximately four weeks after pollination) were tested. Mature grain and senescent whole plants were also tested for CRY1F and PAT protein expression. Additional samples of whole plant tissues were taken at selected sites at V9 and R1 stages for CRY1F and PAT expression analyses.

Results from the field trials have been summarized below (Tables 10 and 11) and detailed reports have been attached at Annexes 2 and 4.

Field study in Chile (1998/99):

The study was conducted at four field sites located in the major maize growing regions of Chile. The field sites were near the towns of Buin, Viluco, Graneros and Nancagua. These locations are comparable to regions in North America and Europe where the maize varieties would be suitable as commercial products.

All tissue samples were collected from a single replication of 1507 hybrid and control maize at each site. For the leaf sample, the youngest whorl leaf was collected from five plants when the plants were at approximately the V9 (or nine leaf) stage of development. Pollen, silk and stalk samples were taken from five discrete plants at approximately the R1 stage (50% pollen shed) of development. Grain from five self-pollinated plants was collected after physiological maturity. The whole plant samples (entire plant except roots) consisted of three self-pollinated plants harvested at R4 stage (approximately four weeks after pollination), that were pooled at each site. The senescent whole plant samples, including ears, were harvested when the plant tissue had turned brown and dried. None of the plots were sprayed with glufosinate-ammonium.

Field study in France and Italy (1999):

The study was conducted at three locations in France and three locations in Italy, all situated in the major maize growing regions of the EU. At each location in Italy, the trial consisted of 1507 maize hybrid sprayed with glufosinate-ammonium, 1507 maize hybrid unsprayed (without glufosinate-ammonium), and a non-GM control hybrid with genetics representative of 1507 maize. At each location in France, the trial consisted of 1507 maize hybrid unsprayed (without glufosinate-ammonium), and a non-GM control hybrid with genetics representative of 1507 maize.

All tissue samples were collected from a single replication of 1507 maize and control maize. 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 tissue samples were collected at the different growth stages. Whole plant forage (R4 stage) and grain were collected from the glufosinate-ammonium sprayed plots as well. The samples were evaluated for CRY1F and PAT protein levels using specific ELISA methods developed for each protein.

Results from the field trials in Chile (1998/99) and in France and Italy (1999) confirm expression of CRY1F protein throughout key developmental stages of 1507 maize (Tables 10 and 11).

Expression of PAT protein in 1507 maize does not occur at measurable levels throughout the developmental stages of maize. PAT protein could only be measured at the V9 stage (leaf and whole plant tissues); it was found to be below the limit of detection in R1 or R4 tissues, senescent plant or mature grain.

(b) Parts of the plant where the insert is expressed

As described above, expression of CRY1F and PAT proteins in 1507 maize was analysed in leaf, pollen, silk, stalk, mature grain and whole plant tissues in field trials conducted in Chile in 1998/99 and in France and Italy in 1999.

Field study in Chile (1998/99):

The results demonstrate that expression of CRY1F protein was found at measurable levels in all plant material sampled and tested of 1507 maize (Table 10). Expression of the PAT protein was only found at measurable levels in leaf tissue (V9 stage) samples of 1507 maize. The concentration of PAT in leaves of 1507 maize was from below the limit of detection (LOD) to 40.8 pg/µg total extractable protein. As expected, expression of the CRY1F and PAT proteins was not detected in any samples from the non-GM control plants. The limits of detection were 10 pg/µg total extractable protein for CRY1F, and 20 pg/µg total extractable protein for PAT. A report containing full details of the study is attached at Annex 2.

Field study in France and Italy (1999):

The results demonstrate that expression of CRY1F protein was found at measurable levels in all plant material sampled and tested (Table 11). The levels of expression of CRY1F found in the 1998/99 and 1999 trials were comparable for stalk, whole plant (R4 stage) and grain samples. The apparent differences observed in leaf, pollen and senescent whole plant can be explained by differences in the quality of the samples, which may have caused a decrease in total extractable protein content and thereby a relative increase in CRY1F content. In addition, the differences observed are not consistent with no indication of an obvious trend. For example, leaf expression levels of CRY1F were lowest in samples from Chile, but senescent whole plant samples showed lowest CRY1F levels in samples from the EU.

Expression of the PAT protein was found at measurable levels in the leaf tissue and whole plant (V9 stage) of 1507 maize. The concentration of PAT was from below the LOD to 136.8 pg/µg total extractable protein in leaves of 1507 maize, and from below the LOD to 38.0 pg/µg total extractable protein in whole plant tissue (V9 stage). As expected expression of the CRY1F and PAT proteins was not detected in any samples from the non-GM control plants. The limits of detection were 10 pg/µg total extractable protein for CRY1F, and 20 pg/µg total

extractable protein for PAT. A report containing full details of the field trials and results is attached at Annex 4.

Characteristics of the CRY1F and PAT proteins as expressed in the plant

Western blot analytical techniques were applied to determine if the CRY1F and PAT proteins expressed in 1507 maize plants were of the same molecular weight and immunoreactivity as the microbially-derived proteins (Alarcon and Marshall, 2000; Annex 8). Polyclonal antibodies were used that recognize multiple antigenic epitopes on the protein. Any protein that is smaller (a partial protein) or larger (a fusion protein) in size than expected would be detected as a band of molecular weight that is different from the molecular weight of either the microbially-derived CRY1F or PAT protein.

Leaf, pollen, grain and whole plant tissues from field grown 1507 maize plants were sampled for protein extraction. These tissues were originally obtained during the field studies conducted in the 1998/99 growing season in Chile as described above.

The results of the Western blot analysis of CRY1F protein expression in plant tissues from 1507 maize are shown in Figure 15. The CRY1F protein was detected as two bands of approximately 65 to 68 kDa in leaf, pollen, whole plant, and grain tissue. Expression of CRY1F protein was measurable in all four of these tissues as shown in Table 10. No other bands indicative of a partial CRY1F protein or a fusion protein of greater molecular weight were observed in 1507 maize tissues. In particular, there is no evidence for the presence of a protein derived from the altered open reading frame associated to the additional copy of the *cry*1F gene (See also Point **D.2.a.**, above). No immunoreactive proteins were detected in the negative control tissues, with the exception of a possible weakly reactive band in the negative control for grain tissue. Although this weakly reactive band is not readily visible in the Western blot (Figure 15), it has an apparent molecular weight that is slightly greater than the CRY1F protein, and is probably due to binding of the CRY1F antibody to an epitope present on an endogenous maize protein.

The CRY1F protein detected in Western blot analyses of 1507 maize plant tissues was present as two bands of nearly identical molecular weight, commonly referred to as a "doublet." Protein doublets typically occur during gel electrophoresis if terminal amino acid residues have been removed from the protein as a result of the activity of proteases released during processing of the plant tissue for analysis. In a separate study, N-terminal amino acid sequence analysis of CRY1F protein derived from plant tissue showed that a five amino acid sequence corresponding to the expected N-terminus of proteolytically cleaved CRY1F was obtained (Evans, 1998; Annex 25). The observed sequence was ^{28}STGRL (the superscript denotes the amino acid residue in the protein). This N-terminal sequence would be expected if cleavage of the CRY1F protein occurred during its purification from plant tissue due to the presence of trypsin-like enzyme activity (trypsin cleaves after arginine residues) (See ^{27}R on Figure 16). The N-terminal sequence of the 68 kDa CRY1F protein expressed *in planta* was blocked and therefore could not be sequenced. Hence,

it appears that the doublet results from limited N-terminal processing of maize expressed CRY1F protein by a plant protease with trypsin-like specificity.

Further confirmation of the equivalence between microbially-derived CRY1F expressed in *Pseudomonas fluorescens* and CRY1F protein expressed in 1507 maize has been obtained by Schafer and Schwedler (2001) and Essner (2002) (see Annexes 17 and 18, respectively). The analysis were carried out using SDS-PAGE gel electrophoresis stained with coomassie blue and glycoprotein detection methods, Western blot analysis, and matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The results obtained confirm that microbially-derived CRY1F protein and CRY1F protein expressed in 1507 maize are not glycosylated (Figure 29, Panel C). In addition, molecular weight analysis by MALDI-TOF MS of peptides obtained following denaturation and trypsinolysis of microbially-derived and maize expressed CRY1F protein shows comparable peptide mass fingerprints (Table 28), which strongly substantiates the equivalence between microbially-derived CRY1F protein and CRY1F protein expressed in 1507 maize. The mass spectrometry analysis carried out on CRY1F protein derived from recombinant *Pseudomonas fluorescens* and on CRY1F protein expressed in 1507 maize shows that one mass peak is not present in the case of microbially-derived CRY1F protein but it is present for CRY1F protein expressed in 1507 maize. However, the absence of one or a relatively small number of peptide fragments in one or both of the proteins being compared can be explained by the technical limitations of the technology, and this does not imply that the two proteins are different. Ionization, over digestion, relatively low protein concentration, as discussed below, are some of the factors that could have given rise to the difference observed. The basis for the application of mass spectrometry analyses to the comparison of two proteins is the production and identification of similar patterns of peptide fragments from the test proteins.

The PAT protein is known to be a homodimer of approximately 43 kDa in its native form, and it is comprised of two components of approximately 22 kDa (OECD, 1999; Annex 6). The results of the Western blot analysis of PAT protein expression in plant tissues from 1507 are shown in Figure 17. PAT protein was detected by Western blot analysis in leaf tissue of 1507 maize as a band of approximately 22 kDa under denaturing conditions. No PAT protein was observed in pollen, whole plant, or grain of 1507 maize. These results are consistent with the levels of PAT protein expressed in 1507 maize tissues and the estimated detection limit of the Western blot analysis procedure used in this study. One additional band was observed to react with the PAT antibody in leaf tissue samples, but not in the negative control. This band may represent the ~43 kDa form of the protein that did not denature during gel electrophoresis. All other bands observed in pollen and grain tissue samples had corresponding bands of the same relative mobility in the negative controls; indicating that the polyclonal antibody used to detect the PAT protein also recognized an epitope on a limited number of endogenous maize proteins. The PAT protein standard used in this study also contained bands corresponding to both the 22 kDa PAT protein and the ~43 kDa dimer that did not denature during gel electrophoresis. No other bands indicative of a partial PAT protein or a fusion protein of greater molecular weight were observed in 1507 maize tissues.

4. Information on how the genetically modified plant differs from the recipient plant in mode(s) and/or rate of reproduction, dissemination and survivability

Information concerning reproduction, survivability and dissemination of maize has been provided in Points **B.2.** to **B.4.**, above.

The genetic modification in 1507 maize does not alter maize reproduction, survivability and dissemination characteristics, as described below.

It is important to emphasise that comparisons between non-GM and GM samples are derived from hybrid maize seed with comparable genetic backgrounds (Table 12). Therefore, observed differences are as a result of the genetic modification only, subject to any small differences in the genetic background. In the case of 1507 maize, the 1507 hybrid is of comparable genetic background as the hybrid used for the non-GM control.

The information in Table 12 illustrates the use of non-GM and GM inbreds to derive non-GM and GM maize hybrids with comparable genetic backgrounds. After transformation and subsequent identification and selection of transformation event 1507, a series of GM maize inbreds homozygous for the 1507 modification were produced by sequentially crossing, backcrossing and selfing the progeny carrying the 1507 event with elite non-GM inbreds (Figure 18). Following several rounds of backcrossing, selection and selfing, practically all of the genetic characteristics of the GM inbreds become equally close to those of the elite non-GM inbreds, with the exception of the inserted traits, as intended by the genetic modification.

The 1507 maize has been tested at different locations across key maize growing regions of the USA in 1999, in France, Italy and Bulgaria in 2000, and in Spain in 2002. The agronomic data obtained has been summarized in Tables 13, 14, 24, 25 and 26. The results support the conclusion that there are no unexpected agronomic differences between 1507 maize and non-GM maize, and that 1507 maize has no altered survival, multiplication or dissemination characteristics.

Survival

Cultivated maize has been domesticated to the extent that it can not survive outside managed agricultural environments. In addition, maize does not possess any traits for weediness (Baker, 1974). The genetic modification in 1507 maize results in expression of CRY1F conferring resistance to certain lepidopteran insect pests, such as the European corn borer, fall armyworm and black cutworm, and expression of PAT conferring tolerance to the herbicide glufosinate-ammonium.

However, the survival characteristics of 1507 maize in the environment remain comparable to those of non-GM maize. Resistance against certain lepidopteran insect pests is not sufficient to allow survival of maize outside the agricultural habitat as maize survival in the environment is restricted by a complex

interaction of biotic and abiotic factors (Points **B.2.**, **B.3.** and **B.6.**). In addition, although expression of the PAT protein in 1507 maize confers tolerance to the herbicide glufosinate-ammonium, this is a broad-spectrum herbicide that is not used outside the agricultural habitats. Therefore, tolerance to glufosinate-ammonium should not enhance the potential for survival of 1507 maize in the environment.

Plant vigour and ageing

The field trials carried out since 1998 in different parts of the world have confirmed that 1507 maize shows no unexpected changes of plant vigour or ageing. In fact, visual rating of emergence vigour from spike to one-leaf stage and of vigour at three- to five-leaf stages show that 1507 maize is similar to non-GM maize of comparable genetic backgrounds.

In addition, other agronomic traits indicative of plant vigour and ageing, such as plant height, ear height, stalk lodging, root lodging, top integrity and yield have also been shown to be similar between 1507 maize and non-GM maize with comparable genetic backgrounds.

Pollen production

No changes in pollen production have been observed in field trials of 1507 maize. Also, accumulated maize growing degree days to reach 50% pollen shed are similar between 1507 maize and non-GM maize with comparable genetic backgrounds.

Similarly, accumulated maize growing degree days to reach 50% silking are comparable between 1507 maize and non-GM maize with comparable genetic backgrounds.

Seed production

No unexpected changes in seed production have been observed in field trials of 1507 maize. Grain density, measured as the weight (in kg) of a bushel of grain at 15.5% moisture, of grain from 1507 maize is similar to the grain density from the non-GM maize with comparable genetic backgrounds (Table 13).

Seed viability and germination

No unexpected changes in seed viability or germination have been observed in field trials of 1507 maize. Early stand count establishment, which consists of the average number of plants emerging per plot, is similar between 1507 maize and non-GM maize with comparable genetic backgrounds (Table 13).

Ways of dissemination

The 1507 maize plants do not show different ways of dissemination compared to non-GM maize.

Agronomic characteristics of 1507 maize cultivated in Spain

Additional field trials have been carried out in Spain in 2002. Two sets of field trials representing a total of five different field trials were carried out for agronomic purposes at three separate locations in the region of Aragón.

A first set of trials were carried out at three different locations in order to compare the agronomic characteristics of 1507 maize with those of non-GM maize with a comparable genetic background used as control. The total number of replicates was n = 9. A comparison of germination, accumulated heat units to pollen shed, accumulated heat units to silking, stalk lodging, root lodging, plant height, ear height, final population, stay green, disease incidence, insect damage, and grain moisture has been included in Tables 24 and 25.

In addition, comparative agronomic data was also obtained for four different hybrids of 1507 maize and non-GM control maize. This data was obtained from a second set of field trials at two locations in Spain in 2002. A comparison of yield, moisture at harvest, root lodging, stalk lodging and insect damage has been included in Table 26. The total number of replicates was n = 4.

In summary, a comparison of the agronomic characteristics of 1507 maize and non-GM maize with comparable genetic background cultivated under Spanish conditions further confirms that there are no unexpected adverse differences. In fact and taking into account the significant target pest pressure from ECB and *Sesamia* spp. that occurred in Aragón (Spain) in 2002, the agronomic differences observed in terms of less insect damage, higher yield, higher plant height and ear, and less stalk lodging of 1507 maize plants confirms effective resistance against the attack from target insect pests conferred by the expression of CRY1F protein, as intended by the genetic modification. The slight differences observed in terms of accumulated heat units to pollen shed and silking are due to small differences in the genetic backgrounds of the hybrids.

5. Genetic stability of the insert and phenotypic stability of the GMHP

Data on the Mendelian segregation of inserted genes provides evidence of stable inheritance of the introduced genetic material. The Mendelian segregation of 1507 maize was recorded and analyzed at two stages (Figure 19). The original transformed Hi-II germplasm containing event 1507 (transformant T0) was crossed to an elite inbred to give an F1 hybrid. The F1 hybrid was backcrossed twice to the elite inbred to give BC2F1 seed. Spraying at each generation eliminated plants susceptible to glufosinate-ammonium and resulted in hemizygous seed.

The seed from the BC2F1 generation was planted, and the plants were sprayed with glufosinate-ammonium. The expected segregation ratio was 1:1 (tolerant:susceptible) for glufosinate-ammonium tolerance. This is shown by the BC2F1 data in Table 15.

Later segregation data was obtained from F1 seed as follows: after three backcrosses, 1507 maize seed (BC3F1) was planted and self-pollinated.

Resulting seed (BC3F2) was expected to be 3:1 (tolerant:susceptible) for glufosinate-ammonium tolerance. This seed was planted and sprayed with the herbicide to remove the homozygous susceptible plants. The remaining plants (one part homozygous tolerant and two parts hemizygous tolerant) were crossed to a susceptible inbred to make F1 hybrid seed. This hybrid seed was planted and sprayed with glufosinate-ammonium to check for the expected 2:1 (tolerant:susceptible) ratio. This is shown by the F1 data in Table 15.

After the hybrids were sprayed with glufosinate-ammonium and scored for tolerance, 200 neonate European corn borers were used to infest each F1 plant that survived the glufosinate-ammonium spraying. All of the plants determined to be tolerant to glufosinate-ammonium were also found to be resistant to European corn borer infestation.

In summary, through backcrossing and selfing, as shown in Figure 19, it can be concluded that both the *cry*1F and *pat* genes in 1507 maize are genetically stable for at least six generations.

In addition, these results support the conclusion that 1507 maize is genetically stable and that the *cry*1F and *pat* genes are inherited as Mendelian dominant genes. Results from Southern blot analysis show that the additional copy of the *cry*1F gene was present in the BC4 backcross generation, Point **D.2.a.**, thus supporting the conclusion that it is genetically linked to the insert containing the *cry*1F and *pat* genes.

The Southern analyses summarized in Point **D.2.a.**, confirm that the results of restriction digests were similar for the T1S1 and BC4F1 generations, which provides further confirmation that the inserted DNA has been stably inherited in 1507 maize.

6. Any change to the ability of the GMHP to transfer genetic material to other organisms

Transfer of genetic material from maize plants in Europe is limited to sexually compatible plants of maize grown in agricultural habitats. The genetic modification in 1507 maize does not change the ability of maize to transfer genetic material to other organisms. In particular, there are no sequences in insert PHI8999A that could potentially be involved in transfer of genetic material between maize and other organisms (Table 1).

7. Information on any toxic, allergenic or other harmful effects on human health arising from the genetic modification

Maize is not considered to have harmful effects on human health. Furthermore and as discussed below, the genetic modification in 1507 maize does not introduce any new toxicants or allergenic substances.

As mentioned in Point **C.1.**, in order to have sufficient amounts of purified CRY1F protein for the multiple studies required to assess the safety of maize

expressed CRY1F protein, a form of the protein with equivalent biochemical structure and biological activity to the structure and activity of CRY1F expressed in maize transformed with insert PHI8999A was produced in *Pseudomonas fluorescens* (Evans, 1998; Annex 25).

The amino acid sequence of the microbially-derived CRY1F protein (MR872) used in toxicological studies, is included for comparison in Figure 3. The MR872 designation is given to the chimeric CRY1F/CRY1A(b) protein produced in *Pseudomonas fluorescens*, which consists of the sequence coding for the CRY1A(b) C-terminal domain fused with the sequence coding for the biologically active N-terminal domain of the CRY1F toxin to enable high expression in *Pseudomonas fluorescens*. High expression was possible as the C-terminal domain of the CRY1A(b) protein is involved in facilitating the folding of the active domain of the protein (*i.e.* the N-terminal CRY1F).

The CRY1F/CRY1A(b) protein produced in *Pseudomonas fluorescens* was treated with trypsin to release the active microbially-derived CRY1F protein, which represents amino acids 28 to 612 of the native CRY1F sequence, as confirmed by molecular weight prediction and N-terminal sequencing (Evans, 1998; Annex 25). The positions of putative protease cleavage sites at the start (residues 28 or 31) and at the end (residues 612 or 615) of the active toxin are marked with a ↓ (Figure 3).

The amino acid sequence of the CRY1F protein expressed in 1507 maize is shown in Figure 3. It represents amino acids 1 to 605 of the native CRY1F protein, which is partially cleaved by maize proteases to give a second form of CRY1F representing amino acids 28 to 605, as confirmed by Western blot analyses and N-terminal sequencing of the protein (Point **D.3.**; Evans, 1998; Annex 25). With the exception of a single and conservative amino acid substitution, leucine at position 604 ($F_{604}L$ substitution), it is identical to amino acids 1-605 of the native CRY1F protein which is 1174 amino acids long. This change in the coding sequence was made to introduce a *Xho*I restriction site for fusion of sequences encoding the C-terminal domain of the protein that forms the full length protein. The choice to use the $F_{604}L$ substitution was based on the occurrence of leucine in the homologous position of other CRY1 proteins (e.g. CRY1AC).

The equivalence between microbially-derived CRY1F protein produced by *Pseudomonas fluorescens* and maize expressed CRY1F protein was substantiated with a series of studies comparing their biochemical structure and biological activity, which included N-terminal amino acid sequencing, molecular weight comparison, immunoreactivity, lack of post-translational modification (glycosylation), and comparison of biological activities (Table 16) (Evans, 1998; Annex 25). The maize expressed CRY1F protein used in these studies was purified from an inbred maize plant derived from event 1360. As described in Point **C.1.**, this event was selected as a representative of equivalent maize events produced by the transformation with insert PHI8999A.

Based on these analyses, the microbially-derived CRY1F protein is considered to be equivalent to maize expressed CRY1F, and therefore it could be used as an

appropriate test sample in toxicity studies (Evans, 1998; Annex 25). The results obtained are part of the safety evaluation of the genetic modification in 1507 maize and have been described below.

Toxicity:

The CRY1F protein has specific toxicity against certain lepidopteran insect pests (target organisms). There is no evidence for CRY proteins originating from *Bacillus thuringiensis* to have harmful effects on the health of humans and animals (EPA, 1995a; McClintock *et al.*, 1995; EPA, 1996).

The potential toxicity of the maize expressed CRY1F protein to humans and animals was examined in an acute oral toxicology study where the equivalent microbially-derived CRY1F protein was evaluated for acute toxicity potential in mice (Kuhn, 1998). The highest dose tested was 5050 mg of test material per kg body weight. When adjusted for purity of the test material (11.4%; Evans, 1998; Annex 25), the dose was 576 mg CRY1F per kg body weight. Observations for mortality and/or clinical or behavioural signs of pathology as well as body weights were made during the course of the study, and gross necropsies were conducted at the end of the study. No mortality occurred during the course of the study. Additionally, no adverse clinical signs were observed during the study and no adverse findings were noted at necropsy. The relatively high dose tested in this study did not give rise to any toxicity and therefore the acute LD_{50} for CRY1F protein could not be determined other than estimated to be higher than 576 mg CRY1F per kg body weight.

The safety in terms of toxicity for the PAT protein has already been determined in detail during the assessment of glufosinate-ammonium tolerant maize (EPA, 1995b; EPA, 1997; Canadian Food Inspection Agency, 1998; SCP, 1998; OECD, 1999; Annex 6). The *pat* gene was originally obtained from *Streptomyces viridochromogenes* strain Tü494 which has no known toxic or pathogenic potential. The PAT protein is enzymatically active but it has high substrate specificity to the active ingredient of glufosinate-ammonium (L-PPT), and such a substrate does not occur within the maize plant or within the animal and human diets. A toxicity study consisting of feeding rats with the PAT protein (0, 5000 and 50000 mg/kg body weight) has been carried out (Pfister *et al.*, 1996; Health Canada, 1997) and the results showed that food consumption and body weight were not influenced by the PAT treatment with no occurrence of mortality. Organ weights, gross pathology and histopathology findings did not indicate differences between treated and control animals. Also, no changes were found in haematology or urine analyses. All immunological screening parameters indicated that PAT protein does not induce immunological effects.

In addition, an acute oral toxicity study consisting of feeding mice with 6000 mg test material per kg body weight containing approximately 5000 mg PAT per kg body weight has been carried out (Brooks, 2000). There were no treatment-related clinical observations. All the mice in the study, 5 males and 5 females, gained body weight normally over the two-week observation period with no gross pathologic lesions. Due to the absence of any symptoms of

toxicity in this study, the LD_{50} for PAT protein could not be determined other than estimated to be higher than 5000 mg PAT per kg body weight.

A poultry feeding study over a period of 42 days has also been carried out with grain from 1507 maize and non-GM control maize with comparable genetics (Point **D.8.**). The mortality, body weight gain and feed conversion of the chickens fed with 1507 maize were compared to chickens fed a standard diet containing yellow dent maize. The results have been summarized in Table 17, and the final report is attached at Annex 5. No statistically significant differences were observed on mortality, body weight gain or feed conversion between chickens fed a diet containing grain from 1507 maize or any of the other diets.

A thirteen-week (90-day) oral toxicity feeding study in rats has been carried out with 1507 maize grain in order to confirm the absence of toxicity of the proteins CRY1F and PAT expressed in 1507 maize grain. A summary of the study design, protocols, results and conclusions obtained has been included below, and a complete copy of the report is attached as Annex 39.

As shown in Table 22, the study involved a total of 10 groups of 12 young rats each, which were fed with diets containing 33% or 11% grain from 1507 maize, non-GM maize with comparable genetic background (33P66 maize) or from commercial non-GM maize (33J56 maize) for approximately 90 days. All diets contained a total of 33% maize grain. Diets formulated with 11% of GM or near isogenic non-GM maize (groups VII – X) also contained 22% commercial hybrid maize (33J56) for a final concentration of 33% maize.

Body weights, food consumption, food efficiency and clinical signs were evaluated weekly. Neurobehavioural and ophthalmological evaluations were carried out at the start and near the end of the study. Clinical, gross and microscopic pathological evaluations were also conducted at the end of the study.

No biologically significant, diet-related differences were observed in mean body weight, body weight gain, or food efficiency. Statistically significantly higher food consumption was observed in male rats fed with a diet containing 33% grain from 1507 maize compared to male rats fed with a diet containing 33% grain from 33P66 maize (non-GM maize with a comparable genetic background). However, no statistically significant differences were observed between the male rats fed with a diet containing 33% grain from 1507 maize compared to male rats fed with a diet containing 33% grain from commercial non-GM maize (33J56 maize). In addition, the observed differences were very small in absolute terms and no differences in food consumption were observed among the groups of female rats fed with any of the diets. The differences observed between male rats fed with a diet containing 33% grain from 1507 maize compared to male rats fed with a diet containing 33% grain from 33P66 maize were not considered toxicologically significant as there were no meaningful differences between these groups in either body weight, body weight gain or food efficiency.

The results also confirm that no toxicologically significant diet-related differences were observed among the groups fed with any of the different diets with respect to clinical signs of toxicity, ophthalmological observations, neurobehavioral assessments, clinical pathology (hematology, clinical chemistry, coagulation, or urinalysis parameters), organ weights, and gross or microscopic pathology.

In conclusion, exposure of male and female rats to diets containing grain from 1507 maize produced no toxicologically significant differences, compared to rats fed diets containing grain from non-GM maize with comparable genetic background or grain from commercial non-GM maize.

Allergenicity:

The assessment of the allergenic potential of the CRY1F and PAT proteins has been made following the recommendations from FAO/WHO (2000), and according to the decision-tree of Metcalfe *et al.* (1996) for the assessment of the allergenicity of genetically modified crop plants.

The most important factor to consider in assessing allergenic potential is whether the source of the gene being introduced into plants is known to be allergenic (FDA, 1992). Neither *Bacillus thuringiensis* (the source of the *cry*1F gene) nor *Streptomyces viridochromogenes* (the source of the *pat* gene) have a history of causing allergy. Also, both donor organisms are common soil bacteria. In over 30 years of commercial use, there have been no substantiated reports of allergenicity to *B. thuringiensis,* including occupational allergy associated with manufacture of products containing *B. thuringiensis* (EPA, 1995a; McClintock *et al.*, 1995; EPA, 1996).

The biochemical profile of the CRY1F and PAT proteins also provides a basis for allergenic assessment when compared with known protein allergens. A comparison of the amino acid sequence of an introduced protein with the amino acid sequences of known allergens is a useful indicator of allergenic potential (FAO/WHO, 2000). A database search was compiled by Meyer (1999; Annex 20) using the Wisconsin Genetics Computer Group (GCG) sequence analysis computer program with the keyword "allergen" to search standard DNA and protein sequence databases. A significant homology is defined as a sequence identity of 8 or more contiguous amino acids. Comparison of the 15 most homologous database sequences confirmed that CRY1F protein does not share any significant amino acid sequence homology with known protein allergens.

In a similar way, a comparison of the amino acid sequence of PAT protein to known protein allergens was also carried out (Meyer, 1999; Annex 20). As expected, the results demonstrated that PAT protein shares no significant amino acid homology with known protein allergens. In addition, the PAT protein has been the subject of previous safety assessments for genetically modified plants and found to have no potential for allergenicity (EPA, 1995b; EPA, 1997; Health Canada, 1997; Canadian Food Inspection Agency, 1998; SCP, 1998; OECD, 1999; Annex 6).

Protein allergens are typically stable to the peptic and tryptic digestion, and to the acid conditions of the human digestive system, which allows them to reach and pass through the intestinal mucosa to elicit an allergenic response. Both CRY1F and PAT proteins are readily degradable in simulated digestive juice, minimising any potential for these proteins to be absorbed by the intestinal mucosa when consumed. The CRY1F protein was nearly completely proteolysed in simulated gastric conditions within one minute at a molar ratio of 188:1 (CRY1F:pepsin) (Evans, 1998; Annex 25), whereas the PAT protein has been shown to degrade to non-detectable levels within 5 seconds after introduction to simulated gastric fluid containing pepsin (Glatt, 1999; OECD, 1999; Annexes 21 and 6, respectively). Additional data from a recent study confirms that CRY1F protein is in fact digested within 15 seconds in SGF as demonstrated by SDS-PAGE and Western blot analysis (Figure 30; Annex 22). Any low molecular weight fragments derived from CRY1F protein as a result of the production, purification and degradation processes are also readily digested in less than 15 seconds (Figure 30; Annex 22).

In vitro digestibility studies in a simulated intestinal fluid (SIF) model of microbially-derived CRY1F have also been carried out (Korjagin and Ernest, 2000). Digestibility of CRY1F, the non-allergenic protein acid phophatase and the allergenic proteins ovalbumin and β-lactoglobulin were compared. The CRY1F protein, the non-allergenic acid phosphatase and the allergenic protein ovalbumin remained undigested for the duration of the test (120 minutes), while the allergenic protein β-lactoglobulin was digested after 1 minute in SIF. The results indicate that: *i)* the *in vitro* SIF model is apparently unable to discriminate between known non-allergenic and allergenic proteins using stability to proteolysis as an experimental endpoint; *ii)* the CRY1F protein remained undigested in SIF as expected, based on the characteristics and function of the CRY proteins which are processed to the protease-resistant active core by the alkaline pH and enzymatic conditions of the insect mid-gut; and, *iii)* the CRY1F protein shares comparable proteolytic characteristics in SGF and SIF systems with other CRY proteins (e.g., CRY1A(b) and CRY3A) which are not considered to share the characteristics of known food allergens.

The immunoblot detection technique has demonstrated that CRY1F is not glycosylated, which is an additional indicator of the absence of allergenic potential in the CRY1F protein (Annex 18). Furthermore, CRY1F protein looses immunoreactivity after heat processing (Evans, 1998; Annex 25) and it has a history of safe use in microbial pesticides (McClintock *et al.*, 1995). An additional study on heat lability of CRY1F protein at various temperatures has shown that CRY1F protein looses its biological activity against neonate tobacco budworm, *Heliothis virescens*, after exposure to ≥75 °C for 30 minutes (Herman, 2000; Annex 23).

Therefore, the *cry*1F and *pat* genes introduced into 1507 maize do not encode for known allergens, and neither the CRY1F nor the PAT proteins share immunologically significant amino acid sequences with known allergens. This together with the rapid breakdown of these proteins under digestive conditions supports the conclusion that the CRY1F and PAT proteins, as expressed in 1507 maize, do not pose any significant allergenic risk to humans.

8. Information on the safety of the GMHP to animal health, particularly regarding any toxic, allergenic or other harmful effects arising from the genetic modification, where the GMHP is intended to be used in animal feedstuffs

A detailed safety evaluation concerning all possible feed applications of 1507 maize and feed products derived from 1507 maize (processed and non-processed) has been attached at Annex 1 of this notification.

The conclusions obtained confirm that feed products from 1507 maize are substantially equivalent to, nutritionally equivalent to, and as safe as feed products derived from commercially available (non-GM) maize. This is based on the compositional analyses comprising proximates, minerals, fatty acids, amino acids, vitamins, secondary metabolites and anti-nutrients of forage and grain samples from 1507 maize; nutritional equivalence shown in a poultry feeding study; and, safety evaluation of the CRY1F and PAT proteins expressed as intended by the genetic modification in 1507 maize.

The feed products obtained from 1507 maize are comparable to those obtained from non-GM maize and there are no known toxic, allergenic or other harmful effects arising from the genetic modification.

9. Mechanism of interaction between the genetically modified plant and target organisms

As described in detail in Point C.3., 1507 maize has been genetically modified to express a synthetic version of plant optimized and truncated *cry*1F gene from *Bacillus thuringiensis* sbsp. *aizawai* which codes for the active core of CRY1F protein and corresponds to amino acids 1-605 of the native CRY1F protein from *Bacillus thuringiensis* sbsp. *aizawai*. Expression of CRY1F protein in 1507 maize provides resistance against certain lepidopteran pests that attack maize plants, such as European corn borer and *Sesamia* spp.

The mechanism of interaction between CRY1F protein expressed in 1507 maize and target organisms is similar to the well characterized interactions between *Bacillus thuringiensis* CRY proteins and target organisms. Many natural δ-endotoxins are produced by *Bacillus thuringiensis* strains as insoluble parasporal crystalline inclusions comprising of proteins (protoxins) approximately 120-140 kDa in size (Schnepf *et al.*, 1998). Upon ingestion by susceptible insects, these classes of protoxin crystals dissolve in the alkaline conditions of the insect gut and are processed by proteases to release the active core toxin comprising of the amino-terminal portion of the molecule (e.g. residues 28 to 612 of native CRY1F protein). The activated toxins are typically 65-70 kDa in size. These toxins bind to specific receptors on the apical microvilli of epithelial midgut cells of insect pests. Binding of the activated toxin is followed by a conformational change of the toxin and its insertion into the insect gut membrane. Toxin oligomerization occurs with formation of pores in the cell membrane of the midgut cells causing osmotic cell lysis leading to insect death.

The protease-resistant core of CRY1 δ-endotoxins is comprised of three structural domains. Domain 1, the pore-forming domain, consists of a bundle of seven anti-parallel α-helices in which helix-5 is encircled by the remaining helices. Domain 2 consists of three anti-parallel β-sheets joined in a "Greek-key" topology. Loops extending from domain 2 are involved in receptor binding. Domain 3 is a β-sandwich of two antiparallel β-sheets. This domain is proposed to stabilize the toxin. In addition, recent evidence demonstrates involvement of domain 3 in receptor binding (Schnepf *et al.*, 1998).

10. Potential changes in the interactions of the GMHP with non-target organisms resulting from the genetic modification

There are no potential changes in the interactions of 1507 maize with non-target organisms resulting from the genetic modification. This has been confirmed by the specificity of the biological activities of CRY1F and PAT proteins and by thoroughly assessing the absence of toxicity of CRY1F protein to non-target and beneficial organisms through multiple studies.

Microbially-derived CRY1F protein, produced in *Pseudomonas fluorescens*, was used for some of these toxicity studies based on the equivalency study which showed that microbially-derived CRY1F protein is equivalent to the CRY1F protein expressed in maize plants transformed with insert PHI8999A (Point **D.7.**).

Non-target arthropods:

A dietary toxicity study carried out with green lacewing larvae (*Chrysoperla carnea*) showed no toxicity of the microbially-derived CRY1F protein. Also, no effect on pupation was observed. The dietary LC_{50} could not be established and therefore the value was estimated to be higher than 480 ppm, which represents up to 30 times the concentration of CRY1F protein present in pollen from 1507 maize (Hoxter *et al.*, 1999a; Annex 26).

Similarly, no mortality and no signs of toxicity were observed in a toxicity study with the ladybird beetle (*Hippodamia convergens*). The LC_{50} value for the ladybird beetle could not be established and therefore was estimated to be higher than 480 ppm (Hoxter *et al.*, 1999b; Annex 27).

The microbially-derived CRY1F protein showed no toxicity against the beneficial parasitic Hymenoptera *Nasonia vitripennis*. The dietary LC_{50} value could not be established and therefore was estimated to be higher than 320 ppm, which represents up to 20 times the concentration of CRY1F protein in 1507 maize pollen (Hoxter *et al.*, 1999c; Annex 28).

The level of beneficial arthropods present in field plots of 1507 maize were compared to those in field plots of non-GM maize with comparable genetics (Higgins, 1999; Annex 30). The numbers of adult and larval lady beetles (*Cycloneda munda* and *Coleomegilla maculata*), insidious flower bugs (ssp. *Orius insidiosus*), assassin bugs (Family: Reduviidae), damsel bugs (Family:

Nabidae), brown lacewings (Family: Hemerobiidae), green lacewings (*Chrysoperla plorabunda*), predatory beetles (Family: Carabidae) and parasitic Hymenoptera (Family: Ichneumonidae and Brachonidae), damsel or dragonflies (Order: Odonata), and spiders were assessed either visually and/or with traps. The results demonstrated that expression of CRY1F protein in 1507 maize had no effect on the presence of the beneficial arthropods observed. The field plots were large enough (106 m^2) to minimize plot to plot movement of most species, although the most mobile insects, such as *Orius*, a parasitic Hymenoptera, are likely to have moved among plots.

In addition, the microbially-derived CRY1F protein demonstrates no toxicity to larvae of the monarch butterfly (*Danaus plexippus*) (Bystrak, 2000). The microbially-derived CRY1F protein was incorporated into a modified lepidopteran diet. Neonate larvae of the monarch butterfly were placed on this diet for 7 days, after which mortality and growth inhibition were assessed. The LC_{50} for CRY1F to monarch butterfly neonates could not be determined because there was no mortality at the highest dose tested (10000 ng/ml diet): this was the highest dose that could be physically incorporated into the diet. Further tests using higher concentration of microbially-derived CRY1F protein have confirmed that the LC_{50} for CRY1F to monarch butterfly neonates could not be determined and could only be estimated to be higher than 30 ppm (Blair Siegfried, personal communication).

A faunistic field study has been carried out in 2000 in France to study the complex tritrophic interactions in the 1507 maize ecosystem compared to the non-target effects observed after application of a synthetic insecticide (Karate Xpress, active ingredient lambda-cyhalothrin) commonly used to control European corn borer infestation (Vernier, *et al.*, 2001a; Annex 36). The results clearly showed that while the Karate Xpress treatment significantly reduced the population of non-target arthropods such as thrips, *Orius* sp. and leafhoppers, there were no adverse effects of 1507 maize in the population of non-target arthropods.

Additional field trials in Spain were also used to study the effects of 1507 maize on non-target species associated to maize fields in 2002. The findings from these field trials confirm that 1507 maize showed the expected behaviour and efficacy under the environmental conditions found in Spain, and provides further evidence to support the conclusion for the absence of any significant adverse unexpected effects on the populations of non-target species associated to maize agrosystems. The results obtained have been detailed in Annex 33. In summary, hybrid entries were pooled by type: conventional control or 1507 maize. Means for control hybrids result from pooling estimates from two official check hybrids and four hybrids that are isogenic to the 1507 maize hybrids. Means for 1507 maize result from pooling estimates from four 1507 maize hybrids expressing the CRY1F protein. Means and standard errors for both locations are listed in Table 27.

Abundance of these beneficial arthropod groups followed similar trends in both maize types. Moreover, the trends across four sample periods for both maize types each are highly correlated (> 0.99) with the trend of pooled (control and

1507 maize, Figures 35 to 40) estimates, and this result is consistent across both locations. These results do not highlight any potential differences in how members of these beneficial arthropod groups use conventional and 1507 maize. Although not conclusive, these results on the potential effects of 1507 maize on the Spanish beneficial insect complex do support previous risk assessment studies of non-target organisms in 1507 maize where favorable conclusions have been reached (Higgins 1999, Vernier *et al.* 2001a ; see Annexes 30 and 36, respectively).

In addition, a detailed non-target exposure and risk assessment for the cultivation of 1507 maize in Europe has been carried out by Wolt and Conlan (2001) and is attached as Annex 35. The conclusions obtained from this detailed study confirm that there is no significant risk for any adverse effects on non-target organisms, and in particular there will be no significant adverse effects on potential sensitive non-target lepidopteran species from exposure to cultivated 1507 maize.

Honey bees:

No effects were observed on larval survival nor adult behaviour in honey bees (*Apis mellifera*). A single dose of 2 mg of pollen from 1507 maize or of 5.6 µg of microbially-derived CRY1F protein suspended in a 30% sucrose solution was administered to each cell. The results indicate that the CRY1F protein did not adversely affect either honey bee larval survival nor emergence (Maggi, 1999; Annex 31).

Terrestrial organisms:

The microbially-derived CRY1F protein showed no toxicity to earthworms (*Eisenia foetida*) at a concentration equivalent of up to 100 times the incorporation of senescent 1507 plants into the top 15 cm of soil (at a rate of 62000 plants per hectare). Therefore, the LC_{50} could not be established other than estimated to be higher than 1.7 mg CRY1F per kg dry soil (Hoxter *et al.*, 1999d; Annex 29).

A laboratory study to determine the chronic effects of CRY1F protein on survival and reproduction of the soil dwelling invertebrate collembola (*Folsomia candida*) has also been carried out. Collembola plays a major role in soil ecosystems due to their feeding on decaying plant materials. Microbially-derived CRY1F protein was added to Brewer's yeast (standard food for collembola) to concentrations representing estimated exposure rates that are 1560-, 388- and 79-fold higher than those that would be found in the field. The results indicated that after feeding on these diets for 28 days, there was no mortality and no reduction in the number of progeny as compared to the controls (Halliday, 1998a).

Wildlife birds:

Grain from 1507 maize was ground and fed to juvenile northern bobwhite quail (*Colinus virginianus*) in the diet for 5 days. The results showed that there were

no mortalities, no signs of toxicity, no effects on body weight and no effects on feed consumption related to the treatment. The dietary LC_{50} value for northern bobwhite quail exposed to 1507 maize could not be established and therefore was estimated to be higher than 100000 mg of 1507 maize grain per kg diet (Gallagher *et al.*, 1999).

Aquatic organisms:

A 48-hour static-renewal acute toxicity test with the cladoceran aquatic invertebrate *Daphnia magna* was conducted using the microbially-derived CRY1F protein and pollen from 1507 maize. The results showed that 100 mg/L of pollen from 1507 maize caused no mortality. An EC_{50} could not be established and therefore the value was estimated to be higher than 100 mg pollen/L. In addition, the 48-hour EC_{50} value for *Daphnia magna* exposed to the CRY1F protein could also not be established and therefore the value was estimated to be higher than 100 mg CRY1F per litre (Drottar and Krueger, 1999).

Using the maximum maize content found in fish diets (35 to 40%), comparable diets using either GM maize expressing the CRY1F protein or non-GM maize were formulated and processed involving a heating step (Mayes, 1999; Annex 24). The presence of the CRY1F protein was analysed by ELISA and bioassays with first instar tobacco budworm. The CRY1F protein content of the kernels before processing into feed was 2.2 – 3.5 ng/mg fresh weight. Analysis of the fish diet samples by ELISA demonstrated that CRY1F was not detectable with a detection limit of 0.04 ng/mg fresh weight. In addition, the results of the bioassays demonstrated that there was no statistically significant biological activity associated with the diet containing 1507 maize meal. Therefore, a fish toxicity test was considered not necessary because of the lack of exposure of fish to CRY1F protein in commercially manufactured fish diet prepared from grain of 1507 maize.

11. Potential interactions with the abiotic environment

Expression of CRY1F and PAT proteins in 1507 maize does not alter the natural interactions of maize plants with the abiotic environment. The very limited persistence of the microbially-derived CRY1F protein in the soil environment (DT_{50} = 3.13 days; Halliday, 1998b; Annex 32) coupled with the natural ubiquity of the *cry*1F and *pat* genes in the soil environment and the absence of adverse effects on soil biota (Point **D.10.**) means negligible possibility for adverse interactions with the abiotic environment and no adverse effects on the biogeochemical cycles.

12. Description of detection and identification techniques for the genetically modified plant

The 1507 maize can be detected and identified by placing small amounts of glufosinate-ammonium herbicide on leaves of maize plants. Maize plants with expression of PAT protein will be those with leaves that do not show any necrosis at point of herbicide application. Alternatively, maize plants can be

sprayed with glufosinate-ammonium herbicide, and those that survive will be expressing PAT protein.

Plant parts of 1507 maize can also be analysed by ELISA to detect the proteins expressed by the *cry*1F and *pat* genes. Additionally, an insect bioassay with sensitive lepidopteran insect species such as European corn borer (*Ostrinia nubilalis)* can be used to identify maize plants expressing the CRY1F protein.

A PCR detection method to confirm the molecular identity of 1507 maize has been developed by GeneScan Analytics GmbH and is attached as Annex 15. The PCR method can also be used to confirm presence of 1507 maize for the purposes of labelling products containing or consisting of 1507 maize. The PCR detection method is based on the border sequences between the 3' end of the full-length insert and the adjacent sequence showing homology to the inverted ORF25PolyA terminator. The product of the amplification consists of a single 194 bp band unique to 1507 maize, as confirmed by the validation of the method (Annex 15).

13. **Information about previous releases of the genetically modified plant**

i. History of previous releases notified under Part B of the Directive

1. Notification number:

B/ES/02/11

2. Release site:

Five sites in Spain

3. Aim of the release:

To evaluate agronomic performance of 1507 maize

4. Duration of the release:

One year (2002)

5. Duration of post-release monitoring:

One season

6. Aim of post-release monitoring:

Control of potential volunteers

7. Conclusions of post-release monitoring:

The 1507 maize plants performed as expected, with no evidence of any unintentional morphological or phenotypical characteristics. In particular, there was no evidence of enhanced weediness of 1507 maize.

8. Results of the release in respect to any risk to human health and the environment (submitted to the competent authority according to Article 8 of Directive 90/220/EEC):

No adverse effects on human health and the environment observed from the release of 1507 maize

1. Notification number:

B/IT/98/19

2. Release site:

Three sites in Italy

3. Aim of the release:

To evaluate agronomic performance of 1507 maize

4. Duration of the release:

Five years (1998-2002)

5. Duration of post-release monitoring:

One season

6. Aim of post-release monitoring:

Control of potential volunteers

7. Conclusions of post-release monitoring:

The 1507 maize plants performed as expected, with no evidence of any unintentional morphological or phenotypical characteristics. In particular, there was no evidence of enhanced weediness of 1507 maize.

8. Results of the release in respect to any risk to human health and the environment (submitted to the competent authority according to Article 8 of Directive 90/220/EEC):

No adverse effects on human health and the environment observed from the release of 1507 maize

1. Notification number:

B/FR/99.03.09

2. Release site:

Three sites in France

3. Aim of the release:

To evaluate agronomic performance of 1507 maize

4. Duration of the release:

Two years (1999-2000)

5. Duration of post-release monitoring:

One season

6. Aim of post-release monitoring:

Control of potential volunteers

7. Conclusions of post-release monitoring:

The 1507 maize plants performed as expected, with no evidence of any unintentional morphological or phenotypical characteristics. In particular, there was no evidence of enhanced weediness of 1507 maize.

8. Results of the release in respect to any risk to human health and the environment (submitted to the competent authority according to Article 8 of Directive 90/220/EEC):

No adverse effects on human health and the environment observed from the release of 1507 maize

ii. History of previous releases carried out outside the Community

1. Release country:

Argentina

2. Authority overseeing the release:

Secretary of Agriculture

3. Release site:

Pergamino area, 3 sites. Buenos Aires Province

4. Aim of the release:

Efficacy trials and hybrid registration

5. Duration of post-release monitoring:

One season

6. Aim of post-release monitoring:

Control of potential volunteers

7. Conclusions of post-release monitoring:

The 1507 maize plants performed as expected, with no evidence of any unintentional morphological or phenotypical characteristics. In particular, there was no evidence of enhanced weediness of 1507 maize.

8. Results of the release in respect to any risk to human health and the environment:

No adverse effects on human health and the environment observed from the release of 1507 maize

1. Release country:

Brazil

2. Authority overseeing the release:

CTNBio

3. Release site:

One site

4. Aim of the release:

Research

5. Duration of post-release monitoring:

One season

6. Aim of post-release monitoring:

Control of potential volunteers

7. Conclusions of post-release monitoring:

The 1507 maize plants performed as expected, with no evidence of any unintentional morphological or phenotypical characteristics. In particular, there was no evidence of enhanced weediness of 1507 maize.

8. Results of the release in respect to any risk to human health and the environment:

No adverse effects on human health and the environment observed from the release of 1507 maize

1. Release country:

Bulgaria

2. Authority overseeing the release:

Biotechnology Committee

3. Release site:

One site

4. Aim of the release:

Research

5. Duration of post-release monitoring:

One season

6. Aim of post-release monitoring:

Control of potential volunteers

7. Conclusions of post-release monitoring:

The 1507 maize plants performed as expected, with no evidence of any unintentional morphological or phenotypical characteristics. In particular, there was no evidence of enhanced weediness of 1507 maize.

8. Results of the release in respect to any risk to human health and the environment:

No adverse effects on human health and the environment observed from the release of 1507 maize

1. Release country:

Chile

2. Authority overseeing the release:

Ministry of Agriculture

3. Release site:

Four sites

4. Aim of the release:

Research

5. Duration of post-release monitoring:

One season

6. Aim of post-release monitoring:

Control of potential volunteers

7. Conclusions of post-release monitoring:

The 1507 maize plants performed as expected, with no evidence of any unintentional morphological or phenotypical characteristics. In particular, there was no evidence of enhanced weediness of 1507 maize.

8. Results of the release in respect to any risk to human health and the environment:

No adverse effects on human health and the environment observed from the release of 1507 maize

1. Release country:

South Africa

2. Authority overseeing the release:

Ministry of Agriculture

3. Release site:

One site

4. Aim of the release:

Research

5. Duration of post-release monitoring:

One season

6. Aim of post-release monitoring:

Control of potential volunteers

7. Conclusions of post-release monitoring:

The 1507 maize plants performed as expected, with no evidence of any unintentional morphological or phenotypical characteristics. In particular, there was no evidence of enhanced weediness of 1507 maize.

8. Results of the release in respect to any risk to human health and the environment:

No adverse effects on human health and the environment observed from the release of 1507 maize