Vitenskapskomiteen for mattrygghet
Norwegian Scientific Committee for Food Safety

Food/feed and environmental risk assessment of application (Reference EFSA/GMO/UK/2008/56) for authorization of insect resistant and herbicide tolerant genetically modified maize Bt11 x MIR604 x GA21 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Syngenta Seeds

Opinion of the Panel on Genetically Modified Organisms of the Norwegian Scientific Committee for Food Safety

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Persons working for VKM, either as appointed members of the Committee or as ad hoc experts, do this by virtue of their scientific expertise, not as representatives for their employers. The Civil Services Act instructions on legal competence apply for all work prepared by VKM.

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Summary

In preparation for a legal implementation of EU-regulation 1829/2003, the Norwegian Environment Agency has requested the Norwegian Food Safety Authority to give final opinions on all genetically modified organisms (GMOs) and products containing or consisting of GMOs that are authorized in the European Union under Directive 2001/18/EC or Regulation 1829/2003/EC within the Authority's sectorial responsibility. The Norwegian Food Safety Authority has therefore, by letter dated 13 February 2013 (ref. 2012/150202), requested the Norwegian Scientific Committee for Food Safety (VKM) to carry out scientific risk assessments of 39 GMOs and products containing or consisting of GMOs that are authorized in the European Union. The request covers scope(s) relevant to the Gene Technology Act. The request does not cover GMOs that VKM already has conducted its final risk assessments on. However, the Agency requests VKM to consider whether updates or other changes to earlier submitted assessments are necessary.

The insect-resistant and herbicide-tolerant genetically modified maize Bt11 x MIR604 x GA21 (Unique Identifier SYN-BTØ11-1 x SYN-IR6Ø4-5 x MON-ØØØ21-9) from Syngenta Seeds is approved under Regulation (EC) No 1829/2003 for food and feed uses, import and processing since 22 December 2011 (Commission Decision 2011/893/EC).

Genetically modified maize Bt11 x MIR604 x GA21 has previously been risk assessed by the VKM Panel on Genetically Modified Organisms (GMO), commissioned by the Norwegian Food Safety Authority and the Norwegian Environment Agency related to the EFSAs public hearing of the application EFSA/GMO/UK/2008/56 in 2008 (VKM 2008a). In addition, Bt11, MIR604 and GA21 has been evaluated by the VKM GMO Panel as single events and as a component of several stacked GM maize events (VKM 2005a,b,c, 2007, 2009a,b,c,d, 2010, 2011, 2012a,b,).

The food/feed and environmental risk assessment of the maize Bt11 x MIR604 x GA21 is based on information provided by the applicant in the application EFSA/GMO/UK/2008/56 and scientific comments from EFSA and other member states made available on the EFSA website GMO Extranet. The risk assessment also considered other peer-reviewed scientific literature as relevant.

The VKM GMO Panel has evaluated Bt11 x MIR604 x GA21 with reference to its intended uses in the European Economic Area (EEA), and according to the principles described in the Norwegian Food Act, the Norwegian Gene Technology Act and regulations relating to impact assessment pursuant to the Gene Technology Act, Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms, and Regulation (EC) No 1829/2003 on genetically modified food and feed. The Norwegian Scientific Committee for Food Safety has also decided to take account of the appropriate principles described in the EFSA guidelines for the risk assessment of GM plants and derived food and feed (EFSA 2011a), the environmental risk assessment of GM plants (EFSA 2010) and selection of comparators for the risk assessment of GM plants (EFSA 2011b).

The scientific risk assessment of maize Bt11 x MIR604 x GA21 include molecular characterisation of the inserted DNA and expression of novel proteins, comparative assessment of agronomic and phenotypic characteristics, nutritional assessments, toxicology and allergenicity, unintended effects on plant fitness, potential for gene transfer, interactions between the GM plant and target and non-target organisms and effects on biogeochemical processes.

It is emphasized that the VKM mandate does not include assessments of contribution to sustainable development, societal utility and ethical considerations, according to the Norwegian Gene Technology Act and Regulations relating to impact assessment pursuant to the Gene Technology Act. These

considerations are therefore not part of the risk assessment provided by the VKM Panel on Genetically Modified Organisms.

The genetically modified maize stack Bt11 x MIR604 x GA21 has been produced by conventional crossing between inbred lines of maize containing the single events Bt11, MIR604 and GA21. The F_1 hybrid was developed to provide protection against certain lepidopteran and coleopteran target pests, and to confer tolerance to glufosinate-ammonium glyphosate-based herbicides.

Molecular characterisation

Southern blot and PCR analyses have indicated that the recombinant inserts in the parental maize lines Bt11, MIR604 and GA21 are retained in the stacked maize Bt11 x MIR604 x GA21. Genetic stability of the inserts has previously been demonstrated in the parental maize lines. Protein levels measured by ELISA show comparable levels of the Cry1Ab, PAT, mCry3A, PMI and mEPSPS proteins between the stacked and single maize lines. Phenotypic analyses also indicate stability of the insect resistance and herbicide tolerance traits in the stacked maize. The VKM Panel on GMO considers the molecular characterisation of maize Bt11 x MIR604 x GA21 and its parental events Bt11, MIR604 and GA21 as adequate.

Comparative assessment

Comparative analyses of data from field trials located at representative sites and environments in North America during the 2006 growing season indicate that maize stack Bt11 x MIR604 x GA21 is compositionally, agronomically and phenotypically equivalent to its conventional counterpart, with the exception of the insect resistance and the herbicide tolerance, conferred by the expression of Cry1Ab, mCry3A, PAT, PMI and mEPSPS proteins.

Based on the assessment of available data, the VKM GMO Panel is of the opinion that conventional crossing of maize Bt11, MIR604 and GA21 to produce the hybrid Bt11 x MIR604 x GA21 does not result in interactions between the newly expressed proteins affecting composition and agronomic characteristics.

Food and feed risk assessment

A whole food feeding study on broilers has not indicated any adverse health effects of maize Bt11 x MIR604 x GA21, and shows that maize Bt11 x MIR604 x GA21 is nutritionally equivalent to conventional maize. The Cry1Ab, PAT, mEPSPS, mCry3A or PMI proteins do not show sequence resemblance to other known toxins or IgE allergens, nor have they been reported to cause IgE mediated allergic reactions. Some studies have however indicated a potential role of Cry-proteins as adjuvants in allergic reactions.

Based on current knowledge, the VKM GMO Panel concludes that maize Bt11 x MIR604 x GA21 is nutritionally equivalent to conventional maize varieties. It is unlikely that the Cry1Ab, PAT, mEPSPS, mCry3A or PMI proteins will introduce a toxic or allergenic potential in food or feed based on maize Bt11 x MIR604 x GA21 compared to conventional maize.

Environmental risk assessment

The scope of the application EFSA/GMO/UK/2008/56 includes import and processing of maize stack Bt11 x MIR604 x GA21 for food and feed uses. Considering the intended uses of maize Bt11 x MIR604 x GA21, excluding cultivation, the environmental risk assessment is concerned with accidental release into the environment of viable grains during transportation and processing, and indirect exposure, mainly through manure and faeces from animals fed grains from maize Bt11 x MIR604 x GA21.

Maize Bt11 x MIR604 x GA21 has no altered survival, multiplication or dissemination characteristics, and there are no indications of an increased likelihood of spread and establishment of feral maize plants in the case of accidental release into the environment of seeds from maize GA21. Maize is the only representative of the genus Zea in Europe, and there are no cross-compatible wild or weedy relatives outside cultivation. The VKM GMO Panel considers the risk of gene flow from occasional feral GM maize plants to conventional maize varieties to be negligible in Norway. Considering the intended use as food and feed, interactions with the biotic and abiotic environment are not considered by the GMO Panel to be an issue.

Overall conclusion
Based on current knowledge, the VKM GMO Panel concludes that maize Bt11 x MIR604 x GA21 is nutritionally equivalent to conventional maize varieties. It is unlikely that the Cry1Ab, PAT, mEPSPS, mCry3A or PMI proteins will introduce a toxic or allergenic potential in food or feed based on maize Bt11 x MIR604 x GA21 compared to conventional maize.

The VKM GMO Panel likewise concludes that maize Bt11 x MIR604 x GA21, based on current knowledge, is comparable to conventional maize varieties concerning environmental risk in Norway with the intended usage.

Keywords

Maize, *Zea mays* L., genetically modified maize Bt11 x MIR604 x GA21, EFSA/GMO/UK/2008/56, insect- resistance, herbicide-tolerance, Cry proteins, Cry1Ab, PAT, mEPSPS, mCry3A, MIR604 PMI, glufosinate-ammonium, glyphosate, food and feed risk assessment, environmental risk assessment, Regulation (EC) No 1829/2003

Norsk sammendrag

I forbindelse med forberedelse til implementering av EU-forordning 1829/2003 i norsk rett har Miljødirektoratet (tidligere Direktoratet for Naturforvalting) bedt Mattilsynet om vurderinger av alle genmodifiserte organismer (GMOer) og avledete produkter som inneholder eller består av GMOer som er godkjent under forordning 1829/2003 eller direktiv 2001/18, og som er godkjent for ett eller flere bruksområder som omfattes av genteknologiloven. På den bakgrunnen har Mattilsynet, i brev av 13. februar 2013 (ref. 2012/150202), bedt Vitenskapskomiteen for mattrygghet (VKM) om å utarbeide endelige vitenskapelige risikovurderinger av 39 GMOer og avledete produkter som inneholder eller består av genmodifiserte organismer, innen Mattilsynets sektoransvar. VKM er bedt om å ferdigstille endelige risikovurderinger for de EU-godkjente søknader hvor VKM ikke har avgitt endelig risikovurdering. I tillegg er VKM bedt om å vurdere hvorvidt det er nødvendig med oppdatering eller annen endring av de endelige risikovurderingene som VKM tidligere har levert.

Den genmodifiserte maishybriden Bt11 x MIR604 x GA21 (Unik kode SYN-BTØ11-1 x SYN-IR6Ø4-5 x MON-ØØØ21-9) fra Syngenta Seeds Inc. (søknad EFSA/GMO/UK/2008/56).) ble godkjent til import, videreforedling og bruk som mat og fôr under EU-forordning 1829/2003 22. desember 2011 (Kommisjonsbeslutning 2011/893/EU).

Maishybrid Bt11 x MIR604 x GA21 er tidligere vurdert av VKMs faggruppe for genmodifiserte organismer med hensyn på mulig helse- og miljørisiko i forbindelse med EFSAs offentlige høring av søknaden i 2008 (VKM 2008). Foreldrelinjene Bt11, MIR604 og GA21 er også tidligere risikovurdert av VKM, både som enkelt-eventer og i en rekke andre hybrider (VKM 2005a,b,c, 2007, 2009a,b,c,d, 2010, 2011, 2012a,b).

Risikovurderingen av den genmodifiserte maislinjen er basert på uavhengige vitenskapelige publikasjoner og dokumentasjon som er gjort tilgjengelig på EFSAs nettside EFSA GMO Extranet. Vurderingen er gjort i henhold til tiltenkt bruk i EU/EØS-området, og i overensstemmelse med miljøkravene i genteknologiloven med forskrifter, først og fremst forskrift om konsekvensutredning etter genteknologiloven. Videre er kravene i EU-forordning 1829/2003/EF, utsettingsdirektiv 2001/18/EF (vedlegg 2,3 og 3B) og veiledende notat til Annex II (2002/623/EF), samt prinsippene i EFSAs retningslinjer for risikovurdering av genmodifiserte planter og avledete næringsmidler (EFSA 2006, 2010, 2011a,b,c) lagt til grunn for vurderingen.

Den vitenskapelige vurderingen omfatter transformeringsprosess og vektorkonstruksjon, karakterisering og nedarving av genkonstruksjonen, komparativ analyse av ernæringsmessig kvalitet, mineraler, kritiske toksiner, metabolitter, antinæringsstoffer, allergener og nye proteiner. Videre er agronomiske egenskaper, potensiale for utilsiktede effekter på fitness, genoverføring og effekter på ikke-målorganismer vurdert.

Det presiseres at VKMs mandat ikke omfatter vurderinger av etikk, bærekraft og samfunnsnytte, i henhold til kravene i den norske genteknologiloven og dens konsekvensutredningsforskrift. Disse aspektene blir derfor ikke vurdert av VKMs faggruppe for genmodifiserte organismer.

F_1-hybriden Bt11 x MIR604 x GA21 er resultat av konvensjonelle kryssinger mellom innavlede maislinjer med eventene Bt11, MIR604 og GA21. Kryssingene er utført for å utvikle en maishybrid med resistens mot visse skadegjørere i sommerfuglordenen Lepidoptera og billeslekten *Diabroticia,* samt toleranse mot herbicider med virkestoff glufosinat-ammonium og glyfosat.

Foreldrelinjen Bt11 inneholder de bakterielle genene *cry1Ab* og *pat*, fra henholdsvis *Bacillius thuringiensis* subsp. *kurstaki* og *Streptomyces viridochromogenes* strain Tu494. *Cry1Ab*-genet koder for et δ-endotoksin, som gir plantene toleranse mot enkelte arter i ordenen Lepidoptera. *Pat*-genet

koder for enzymet phosphinothricin acetyl transferase (PAT), som acetylerer og inaktiverer glufosinat-ammonium, virkestoffet i fosfinotricin-herbicider av typen Finale. Fosfinotricin er et ikke-selektivt kontaktherbicid som hemmer glutaminsyntetase. Enzymet deltar i assimilasjonen av nitrogen og katalyserer omdanning av glutamat og ammonium til aminosyren glutamin. Hemming av glutaminsyntetase fører til akkumulasjon av ammoniakk, og til celledød i planten. Bt11-plantene vil derfor tolerere høyere doser av sprøytemiddelet glufosinat sammenlignet med konkurrerende ugras.

Foreldrelinjen MIR604 har fått innsatt et modifisert *cry3A*-gen (*mcry3A*) fra *Bacillius thuringiensis* subsp. *tenebrionis* og genet *pmi* fra *E. coli*. *mCry3A* genet uttrykker δ-endotoksinet mCry3A, som gir plantene toleranse mot angrep fra bladbiller i slekten *Diabrotica*. *Pmi* genet uttrykker enzymet fosfomannose isomerase, som gir toleranse overfor sukkerarten mannose.

Foreldrelinjen GA21 er fremkommet ved biolistisk transformasjon av embryonale maisceller fra en ikke navngitt maislinje. Den innsatte genkonstruksjonen inneholder et endogent 5-enolpyruvylsikimat-3-fosfatsyntetase (*mepsps*)-gen, som er modifisert ved hjelp av *in vitro*-mutagenese. *Mepsps*-genet koder for enzymet 5-enolpyruvylsikimat-3-fosfatsyntetase (mEPSPS), som omdanner fosfoenolpyruvat og sikimat-3-fosfat til 5-enolpyruvylsikimat-3-fosfat, viktige metabolitter i syntesen av aromatiske aminosyrer. N-fosfonometylglycin er et systemisk, ikke selektivt herbicid som hemmer EPSPS-enzymer og blokkerer biosyntesen av aromatiske aminosyrer i planter. I motsetning til plantens EPSPS-enzym er det modifiserte mEPSPS-enzymet fra mais også aktivt ved nærvær av glyfosat. De transgene plantene vil derfor tolerere høyere doser av herbicider med virkestoff glyfosat sammenlignet med konkurrerende ugras.

Molekylær karakterisering
Maishybriden Bt11 x MIR604 x GA21 er dannet ved konvensjonelle kryssinger mellom maislinjene Bt11, MIR604 og GA21. Spaltingsdata, Southern blot og PCR-analyser indikerer at de rekombinante innskuddene fra mais Bt11, MIR604 og GA21 er stabilt nedarvet i mais Bt11 x MIR604 x GA21, og at antall innsatte gener, struktur og organiseringen av disse er ekvivalent med de som finnes i mais Bt11, MIR604 og GA21. Nivåene av Cry1Ab-, PAT-, mCry3A-, PMI- og mEPSPS-proteiner i vegetativt vev og korn fra mais Bt11 x GA21 er også sammenlignbare med nivåene i henholdsvis mais Bt11, MIR604 og GA21.

Komparative analyser
Data fra feltforsøk i Nord Amerika vekstsesongen 2006 indikerer, med unntak av insektsresistens og herbicidtoleranse, ekvivalens mellom maishybrid Bt11 x MIR604 x GA21 og korresponderende, nær-isogen kontrollhybrid med hensyn på ernæringsmessige, agronomiske og fenotypiske karakterer.

Basert på tilgjengelig dokumentasjon, konkluderer VKMs GMO-panel med at konvensjonelle kryssinger mellom de genmodifiserte maislinjene Bt11, MIR604 og GA21 ikke resulterer i nye interaksjoner mellom genproduktene fra de genmodifiserte foreldrelinjene som påvirker ernæringsmessige og agronomiske karakterer i hybriden Bt11 x MIR604 x GA21.

Helserisiko
I en fôringsstudie utført på broilere ble det vist at mais Bt11 x MIR604 x GA21 ikke førte til negative helseeffekter blant dyrene, og at maisen var ernæringsmessig ekvivalent konvensjonell mais. De introduserte proteinene Cry1Ab, PAT, mEPSPS, mCry3A og PMI viser ingen sekvenslikhet til kjente toksiner eller IgE-allergener. Det er heller ikke dokumentert at noen av disse proteinene kan utløse IgE-medierte allergiske reaksjoner. Enkelte studier har derimot indikert at noen typer Cry-proteiner potensielt kan forsterke andre allergiske reaksjoner (virke som adjuvans).

Ut i fra dagens kunnskap konkluderer VKMs faggruppe for GMO at mais Bt11 x MIR604 x GA21 er ernæringsmessig ekvivalent med konvensjonell mais. Det er lite sannsynlig at proteinene Cry1Ab,

PAT, mEPSPS, mCry3A eller PMI vil introdusere et toksisk eller allergent potensiale i mat eller fôr basert på mais Bt11 x MIR604 x GA21 sammenliknet med konvensjonelle maissorter.

Miljørisiko
Søknaden EFSA/GMO/UK/2008/56 gjelder godkjenning av maislinje Bt11 x MIR604 x GA21 for import, prosessering og til bruk i næringsmidler og fôrvarer, og omfatter ikke dyrking. Med bakgrunn i tiltenkt bruksområde er miljørisikovurderingen avgrenset til mulige effekter av utilsiktet frøspredning i forbindelse med transport og prosessering, samt indirekte eksponering gjennom gjødsel fra husdyr fôret med genmodifisert mais.

Det er ingen indikasjoner på økt sannsynlighet for spredning, etablering og invasjon av maislinjen i naturlige habitater eller andre arealer utenfor jordbruksområder som resultat av frøspill i forbindelse med transport og prosessering. Risiko for utkryssing med dyrkede sorter vurderes av GMO panelet til å være ubetydelig. Ved foreskreven bruk av maislinjen Bt11 x MIR604 x GA21 antas det ikke å være risiko for utilsiktede effekter på målorganismer, ikke-målorganismer eller på abiotisk miljø i Norge.

Samlet vurdering
Ut i fra dagens kunnskap konkluderer VKMs faggruppe for GMO at mais Bt11 x MIR604 x GA21 er ernæringsmessig ekvivalent konvensjonell mais. Det er lite sannsynlig at proteinene Cry1Ab, PAT, mCry3A, PMI eller mEPSPS vil introdusere et toksisk eller allergent potensiale i mat eller fôr basert på mais Bt11 x MIR604 x GA21 sammenliknet med konvensjonelle maissorter.

Faggruppen finner at maishybrid Bt11 x MIR604 x GA21, ut fra dagens kunnskap og omsøkt bruk, er sammenlignbar med konvensjonell mais når det gjelder mulig miljørisiko i Norge.

Abbreviations and explanations

ALS	Acetolactate synthase, an enzyme that catalyses the first step in the synthesis of the branched-chain amino acids, valine, leucine, and isoleucine
AMPA	Aminomethylphosphonic acid, one of the primary degradation products of glyphosate
ARMG	Antibiotic resistance marker gene
BC	Backcross. Backcross breeding in maize is extensively used to move a single trait of interest (e.g. disease resistance gene) from a donor line into the genome of a preferred or "elite" line without losing any part of the preferred lines existing genome. The plant with the gene of interest is the donor parent, while the elite line is the recurrent parent. BC_1, BC_2 etc. designates the backcross generation number.
BLAST	Basic Local Alignment Search Tool. Software that is used to compare nucleotide (BLASTn) or protein (BLASTp) sequences to sequence databases and calculate the statistical significance of matches, or to find potential translations of an unknown nucleotide sequence (BLASTx). BLAST can be used to understand functional and evolutionary relationships between sequences and help identify members of gene families.
bp	Basepair
Bt	*Bacillus thuringiensis*
CaMV	Cauliflower mosaic virus
Codex	Set by The Codex Alimentarius Commission (CAC), an intergovernmental body to implement the Joint FAO/WHO Food Standards Programme. Its principle objective is to protect the health of consumers and to facilitate the trade of food by setting international standards on foods (i.e. Codex Standards).
Cry	Any of several proteins that comprise the crystal found in spores of *Bacillus thuringiensis*. Activated by enzymes in the insects midgut, these proteins attack the cells lining the gut, and subsequently kill the insect.
Cry1Ab	Cry1 class crystal protein from *Bacillus thuringiensis* subsp. *kurstaki.* Provide protection against certain lepidopteran target pests, such as the European maize borer (*Ostrinia nubilalis*), and species belonging to the genus *Sesamia*
Cry3A	Cry3 class crystal protein from *Bacillus thuringiensis* subsp. *tenebrionis.* Provide protection against certain coleopteran target pests.
mCry3A	Modified Cry3A protein optimized for maize
CTP	Chloroplast transit peptide
DAP	Days after planting
DNA	Deoxyribonucleic acid
DT50	Time to 50% dissipation of a protein in soil

DT90	Time to 90% dissipation of a protein in soil
dw	Dry weight
dwt	Dry weight tissue
EC	European Commission
ECB	European corn borer, *Ostrinia nubilalis*
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
ERA	Environmental risk assessment
E-score	Expectation score
EU	European Union
fa	Fatty acid
FAO	Food and Agriculture Organisation
FIFRA	US EPA Federal Insecticide, Fungicide and Rodenticide Act
Fitness	Describes an individual's ability to reproduce successfully relative to that of other members of its population.
fw	Fresh weight
fwt	Fresh weight tissue
GAT	Glyphosate N-acetyltransferase
GLP	Good Laboratory Practice
Glufosinate-ammonium	Broad-spectrum systemic herbicide
Glyphosate	Broad-spectrum systemic herbicide
GM	Genetically Modified
GMO	Genetically Modified Organism
GMP	Genetically Modified Plant
H	Hybrid
ha	Hectare
ILSI	International Life Sciences Institute
IPM	Integrated Pest Management
IRM	Insect Resistance Management
Locus	The position/area that a given gene occupies on a chromosome
LOD	Limit of detection
LOQ	Limit of quantification
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization-Time Of Flight. A mass spectrometry method used for detection and characterisation of biomolecules, such as proteins, peptides, oligosaccharides and oligonucleotides, with molecular masses between 400 and 350,000 Da.

MCB	Mediterranean corn borer, *Sesamia nonagrioides*
mEPSPS	Modified 5-enolpyruvylshikimate-3-phosphate synthase
mRNA	Messenger RNA
MT	Norwegian Food Safety Authority (Mattilsynet)
NDF	Neutral detergent fibre, measure of fibre used for animal feed analysis. NDF measures most of the structural components in plant cells (i.e. lignin, hemicellulose and cellulose), but not pectin.
Northern blot	Northern blot is a technique used to study gene expression by detection of RNA or mRNA separated in a gel according to size.
NTO	Non-target organism
Nicosulfuron	Herbicide for maize that inhibits the activity of acetolactate synthase
Near-isogenic lines	Term used in genetics/plant breeding, and defined genetic lines that are identical except for differences at a few specific locations or genetic loci.
OECD	Organisation for Economic Co-operation and Development
ORF	Open Reading Frame, in molecular genetics defined as a reading frame that can code for amino acids between two stop codons (without stop codons).
OSL	Over season leaf
OSR	Over season root
OSWP	Over season whole plant
pat	*Phosphinothricin-Acetyl-Transferase* gene
PAT	Phosphinothricin-Acetyl-Transferase protein
PCR	Polymerase chain reaction, a technique to amplify DNA by copying it
PMI	Phosphomannose Isomerase enzyme. Metabolizes mannose and allows positive selection for recovery of transformed plants.
R0	First transformed generation, parent
Rimsulferon	Herbicide, inhibits acetolactate synthase
RNA	Ribonucleic acid
RP	Recurrent parent
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis. Technique to separate proteins according to their approximate size
SAS	Statistical Analysis System
SD	Standard deviation
Southern blot	Method used for transfer of electrophoresis-separated DNA fragments to a filter membrane and possible subsequent fragment detection by probe hybridisation
T-DNA	Transfer DNA, the transferred DNA of the tumour-inducing (Ti) plasmid of some species of bacteria such as *Agrobacterium tumefaciens* and *A. rhizogenes*, into plant's nuclear genome. The T-DNA is bordered by 25-base-pair repeats on each end. Transfer is initiated at the left border and

	terminated at the right border and requires the *vir* genes of the Ti plasmid.
TI	Trait integrated
TMDI	Theoretical Maximum Daily Intake
U.S. EPA	United States Environmental Protection Agency.
Maize growth stages	*Vegetative*
	VE: emergence from soil surface
	V1: collar of the first leaf is visible
	V2: collar of the second leaf is visible
	Vn: collar of the leaf number 'n' is visible
	VT: last branch of the tassel is completely visible
	Reproductive
	R0: Anthesis or male flowering. Pollen shed begins
	R1: Silks are visible
	R2: Blister stage. The kernels are filled with a clear nourishing endosperm fluid and the embryo can be seen
	R3: Milk stage. The kernels endosperm is milky white.
	R4: Dough stage. The kernels endosperm has developed to a white paste
	R5: Dent stage. If the genotype is a dent type, the grains are dented
	R6: Physiological maturity
Western blot	Technique used to transfer proteins separated by gel electrophoresis by 3-D structure or denatured proteins by the length of the polypeptide to a membrane, where they might be identified by antibody labelling.
WHO	World Health Organisation
ZM	*Zea maize* L.
ZM-HRA	A modified version of the native acetolactate synthase protein from maize. Confers tolerance to the ALS-inhibiting class of herbicides

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Background

On 28 May 2008, the European Food Safety Authority (EFSA) received from the Competent Authority of the United Kingdom an application (Reference EFSA/GMO/UK/2008/56) for authorisation of the insect-resistant and herbicide-tolerant genetically modified (GM) maize Bt11 x MIR604 x GA21 (Unique Identifier SYNBTØ11-1xSYN-IR6Ø4-5xMON-ØØØ21-9), submitted by Syngenta Seeds S.A.S. within the framework of Regulation (EC) No 1829/2003.

The scope of the application covers:

- Import and processing of maize Bt11 x MIR604 x GA21
- GM plants for food and feed use
- Food and feed, containing or consisting of maize Bt11 x MIR604 x GA21
- Food and feed produced from maize Bt11 x MIR604 x GA21
- Food containing ingredients produced from maize Bt11 x MIR604 x GA21

After receiving the application EFSA/GMO/UK/2008/56 and in accordance with Articles 5(2)(b) and 17(2)b of Regulation (EC) No 1829/2003, EFSA informed the EU- and EFTA Member States (MS) and the European Commission and made the summary of the dossier publicity available on the EFSA website. EFSA initiated a formal review of the application to check compliance with the requirements laid down in Articles 5(3) and 17(3) of regulation (EC) No 1829/2003. On 24 July 2008, EFSA declared the application as valid in accordance with Articles 6(1) and 18(1) of Regulation (EC) No 1829/2003.

EFSA made the valid application available to Member States and the EC and consulted nominated risk assessment bodies of the MS, including the Competent Authorities within the meaning of Directive 2001/18/EC (EC 2001), following the requirements of Articles 6(4) and 18(4) of Regulation (EC) No 1929/2003, to request their scientific opinion. Within three months following the date of validity, all MS could submit via the EFSA GMO Extranet to EFSA comments or questions on the valid application under assessment.

The EFSA GMO Panel its scientific opinion in May 2010 (EFSA 2010b). The Commission Decision 2011/893/EC authorised the placing on the market of products containing, consisting of, or produced from maize Bt11 x MIR604 x GA21 pursuant to Regulation (EC) No 1829/2003 (EC 2008) on 22 December 2011.

An application for authorisation of seeds and plant propagation materials for cultivation of maize Bt11 x MIR604 x GA21 in the EU was submitted by Syngenta Seeds in July 2010 (EFSA/GMO/UK/2010/84). EFSA stopped the application process in October 2011, pending the finalisation of the risk assessment of the applications EFSA/GMO/UK/2008/60 (maize GA21) and EFSA/GMO/UK/2010/83 (maize MIR604). The EFSA GMO Panel adopted its scientific opinion on maize GA21 in December 2011 (EFSA 2011d). EFSA has however requested additional information from Syngenta regarding maize MIR604 and the clock for application EFSA/GMO/UK/2010/84 remains stopped by EFSA.

Genetically modified maize Bt11 x MIR604 x GA21 has previously been risk assessed by the VKM Panel on Genetically Modified Organisms (GMO), commissioned by the Norwegian Food Safety Authority and the Norwegian Environment Agency related to the EFSAs public hearing of the application EFSA/GMO/UK/2008/56 in 2008 (VKM 2008a). In addition, Bt11, MIR604 and GA21 has been evaluated by the VKM GMO Panel as single events and as a component of several stacked GM maize events (VKM 2005a,b,c, 2007, 2009a,b,c,d, 2010, 2011, 2012a,b,).

Exemption of the authorisation requirements of 19 existing products in Norway
Through the Agreement of the European Economic Area (EEA), Norway is obliged to implement the EU regulations on GM food and feed (regulations 1829/2003, 1830/2003 et al). Until implementation of these regulations, Norway has a national legislation concerning processed GM food and feed products that are harmonised with the EU legislation. These national regulations entered into force 15 September 2005. For genetically modified feed and some categories of genetically modified food, no requirements of authorisation were required before this date. Such products that were lawfully placed on the Norwegian marked before the GM regulations entered into force, the so-called existing products, could be sold in a transitional period of three years when specific notifications were sent to the Norwegian Food Safety Authority. Within three years after 15. September 2005, applications for authorisation should be sent to the Authority before further marketing. Four fish feed producing companies have once a year since 2008, applied for an exemption of the authorisation requirements of 19 existing products, including maize Bt11 and GA21. These 19 GM events are all authorised in the EU, and the Norwegian Food Safety Authority has granted exemption for a period of one year each time.
http://www.mattilsynet.no/planter_og_dyrking/genmodifisering/fire_virksomheter_har_faatt_dispensasjon_fra_kravet_om_godkjenning_av_genmodifisert_fiskefor.10951

Terms of reference

The Norwegian Environment Agency has the overall responsibility for processing applications for the deliberate release of genetically modified organisms (GMOs). This entails inter alia coordinating the approval process, and to make a holistic assessment and recommendation to the Ministry of the Environment regarding the final authorization process in Norway. The Directorate is responsible for assessing environmental risks on the deliberate release of GMOs, and to assess the product's impact on sustainability, benefit to society and ethics under the Gene Technology Act.

The Norwegian Food Safety Authority (NFSA) is responsible for assessing risks to human and animal health on deliberate release of GMOs pursuant to the Gene Technology Act and the Food Safety Act. In addition, the NFSA administers the legislation for processed products derived from GMO and the impact assessment on Norwegian agriculture according to sector legislation.

In preparation for a legal implementation of EU-regulation 1829/2003, the Norwegian Environment Agency has requested the Norwegian Food Safety Authority to give final opinions on all genetically modified organisms (GMOs) and products containing or consisting of GMOs that are authorized in the European Union under Directive 2001/18/EC or Regulation 1829/2003/EC within the Authority's sectoral responsibility. The request covers scope(s) relevant to the Gene Technology Act.

The Norwegian Food Safety Authority has therefore, by letter dated 13 February 2013 (ref. 2012/150202), requested the Norwegian Scientific Committee for Food Safety (VKM) to carry out final scientific risk assessments of 39 GMOs and products containing or consisting of GMOs that are authorized in the European Union.

The assignment from NFSA includes food and feed safety assessments of genetically modified organisms and their derivatives, including processed non-germinating products, intended for use as or in food or feed.

In the case of submissions regarding genetically modified plants (GMPs) that are relevant for cultivation in Norway, VKM is also requested to evaluate the potential risks of GMPs to the Norwegian agriculture and/or environment. Depending on the intended use of the GMP(s), the environmental risk assessment should be related to import, transport, refinement, processing and cultivation. If the submission seeks to approve the GMP(s) for cultivation, VKM is requested to evaluate the potential environmental risks of implementing the plant(s) in Norwegian agriculture compared to existing varieties (e.g. consequences of new genetic traits, altered use of pesticides and tillage). The assignment covers both direct and secondary effects of altered cultivating practices.

VKM is further requested to assess risks concerning coexistence of cultivars. The assessment should cover potential gene flow from the GMP(s) to conventional and organic crops as well as to compatible wild relatives in semi-natural or natural habitats. The potential for establishment of volunteer populations within the agricultural production systems should also be considered. VKM is also requested to evaluate relevant segregation measures to secure coexistence during agricultural operations up to harvesting. Post-harvest operations, transport, storage are not included in the assignment.

Evaluations of suggested measures for post-market environmental monitoring provided by the applicant, case-specific monitoring and general surveillance, are not covered by the assignment from the Norwegian Food Safety Authority.

Assessment

1 Introduction

The genetically modified maize stack Bt11 x MIR604 x GA21 has been produced by conventional crossing between inbred lines of maize containing the single events Bt11, MIR604 and GA21. The F_1 hybrid was developed to provide protection against certain lepidopteran and coleopteran target pests, and to confer tolerance to glufosinate-ammonium and glyphosate-based herbicides.

None of the target pests for maize Bt11 x MIR604 x GA21 are present in the Norwegian agriculture. The PAT protein expressed in maize Bt11 has been used as selectable markers to facilitate the selection process of transformed plant cells and is not intended for weed management purposes.

Maize stack Bt11 x MIR604 x GA21 has been evaluated with reference to its intended uses in the European Economic Area (EEA), and according to the principles described in the Norwegian Food Act, the Norwegian Gene Technology Act and regulations relating to impact assessment pursuant to the Gene Technology Act, Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms, and Regulation (EC) No 1829/2003 on genetically modified food and feed.

The Norwegian Scientific Committee for Food Safety has also decided to take into account the appropriate principles described in the EFSA guidelines for the risk assessment of GM plants and derived food and feed (EFSA 2011a), the environmental risk assessment of GM plants (EFSA 2010), the selection of comparators for the risk assessment of GM plants (EFSA 2011b), and for the post-market environmental monitoring of GM plants (EFSA 2011c).

The food/feed and environmental risk assessment of the genetically modified maize Bt11 x MIR604 x GA21 is based on information provided by the applicant in the applications EFSA/GMO/UK/2005/20, and scientific opinions and comments from EFSA and other member states made available on the EFSA website GMO Extranet. The risk assessment is also based on a review and assessment of relevant peer-reviewed scientific literature.

It is emphasized that the VKM mandate does not include assessments of contribution to sustainable development, societal utility and ethical considerations, according to the Norwegian Gene Technology Act and Regulations relating to impact assessment pursuant to the Gene Technology Act. These considerations are therefore not part of the risk assessment provided by the VKM Panel on Genetically Modified Organisms.

2 Molecular characterisation

2.1 Evaluation of relevant scientific data

2.1.1 Method of production of maize Bt11 x MIR604 x GA21

The Bt11 x MIR604 x GA21 maize has been produced by crossing genetically modified insect-resistant Bt11 and MIR604 maize and herbicide tolerant GA21 maize through conventional breeding techniques.

Bt11 x MIR604 x GA21 maize plants contain the five traits present in Bt11, MIR604 and GA21 maize plants through the production of:

1. A truncated Cry1Ab protein for control of certain lepidopteran pests like the common European maize pests: *Ostrinia nubilalis* (European corn borer; ECB) and *Sesamia nonagrioides* (Mediterranean corn borer; MCB).

2. A phosphinothricin acetyltransferase (PAT) protein that confers tolerance to herbicide products containing glufosinate ammonium.

3. A modified Cry3A (mCry3A) protein for control of certain coleopteran pests like *Diabrotica virgifera virgifera* (Western corn rootworm; WCRW) a maize pest recently introduced and rapidly expanding in the EU.

4. A phosphomannose isomerase (MIR604 PMI) protein as a selectable marker. PMI allows transformed corn cells to grow on a minimal medium during tissue culture, while non-transformed cells fail to and thereby are selected against (removed). GM cells utilize mannose as a sole carbon source, and this selection replaces previously controversial commonly used antibiotic resistance and as such meets demands to avoid using antibiotics.

5. A modified maize 5-enolpyruvylshikimate-3-phosphate synthase enzyme (mEPSPS) that confers tolerance to herbicide products containing glyphosate.

2.1.2 Summary of evaluation of the single events

2.1.2.1 Maize Bt11

Maize Bt11 was generated by transformation of a proprietary inbred maize line, H8540, using a DNA fragment obtained by a restriction digest of the plasmid pZO1502 with the enzyme *Not*I by biolistics. Regenerated plants were backcrossed to a selected line resulting in maize Bt11. The DNA fragment used for transformation carried two expression cassettes; a selectable marker gene *pat,* encoding phosphinothricin-N-acetyl transferase and a trait gene encoding a variant *Bacillus thuringiensis cry1Ab* gene encoding Bt endotoxin. Both the *cry1Ab* and *pat* gene cassette are controlled by the 35S promoter from the *Cauliflower mosaic virus* (CaMV), supplemented with the intron sequences to enhance gene expression. The polyadenylation signals are derived from the nopaline synthase (*nos*) gene from *Agrobacterium tumefaciens* (Fig.1).

Southern analyses of the single maize event Bt11 used a variety of DNA probes that included the *pat* and *cry1Ab* genes as probes for the genes intended to be inserted and the *amp* gene and the entire

plasmid as probes to detect genome wide unintended insertions. The data obtained indicated that maize Bt11 contains a single DNA insertion with one copy of both the *cry1Ab* and the *pat* cassettes.

The entire Bt11 maize insert including the flanking regions was sequenced. The maize sequences flanking the Bt11 maize insert were also identified. A BLAST analysis of the sequences flanking the Bt11 maize insert was carried out against publicly available nucleotide databases. DNA sequences at the junctions between the insert and the parent genome were determined. At the 5' flank, approximately 350 bp of the plant DNA adjacent to the insert was sequenced. At the 3' flank, approximately 540 bp of the plant DNA adjacent to the insert was sequenced. The 5' and 3' flanking sequences were screened for homologies with sequences found in public databases. BLAST analysis of both the 5' and 3' regions of the Bt11 maize insert revealed homology primarily to the *Zea mays* 180 bp knob-associated tandem repeat. The data do not indicate any safety concern with regard to the interruption of known genes or from the potential production of new toxins or allergens.

The range of expression of Cry1Ab and PAT proteins in Bt11 maize plants were determined by ELISA in several plant tissues and whole plants at various growth stages from different hybrids.

Levels in pollen were below the lower limit of quantification, < 0.08 µg/g fresh wt. pollen and < 0.15 µg/g dry wt. pollen. Across all plant stages, mean Cry1Ab levels measured in leaves, roots and whole plants ranged from *ca.* 10 - 22 µg/g fresh wt. (12 – 154 µg/g dry wt.), 2 – 4 µg/g fresh wt. (9 – 22 µg/g dry wt.), and 4 – 9 µg/g fresh wt. (6 – 70 µg/g dry wt.), respectively. Mean Cry1Ab levels measured in grain at seed maturity and senescence were 1 – 2 µg/g fresh wt (2 µg/g dry wt.).

The level of the Cry1Ab protein was present at low levels in Bt11 sweet maize hybrids. Cry1Ab protein was not detectable in any of the canned maize samples tested. The level of the PAT protein was determined using Bt11 field maize plants; measurable levels (ng/g) were only found in leaves, silk and tassel. For grain, pollen, root and stalk concentrations were below the limits of detection. The PAT protein is present at less than 0.000008% fresh weight and 0.00016% of the total maize grain protein.

The genetic stability of the inserted DNA in maize Bt11 was demonstrated over several generations by Southern analysis. Segregation data for PAT and Cry1Ab (glufosinate-ammonium tolerance and insect resistance) also demonstrated the traits are stable and inherited according to Mendelian segregation of a single genetic locus.

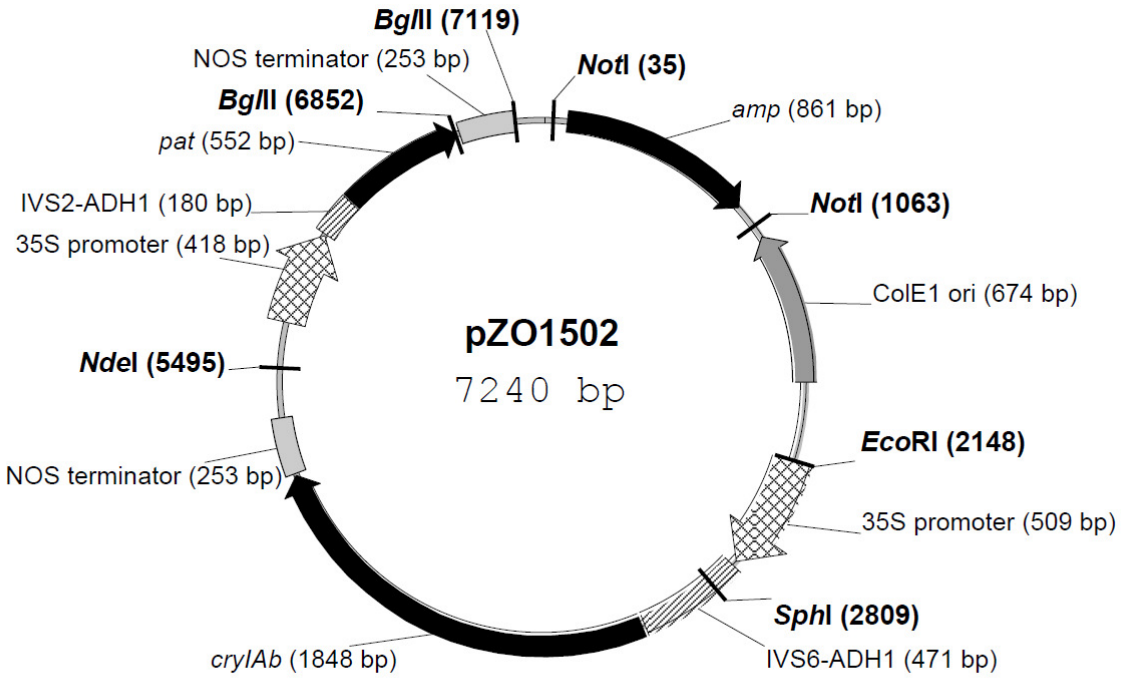

Figure 1. Various gene elements of transformation vector pZO1502 used for generation of the maize strain Bt11.

2.1.2.2. Maize MIR604

Maize MIR604 was developed by transforming immature maize embryos derived from a proprietary *Zea mays* line (A188) via *Agrobacterium*-mediated transformation, using the binary transformation vector pZM26. The T-DNA genetic elements transferred to produce maize MIR604 are shown in Figure 2.

Maize MIR604 expresses the *mcry3A* gene, which is a modified version of the *cry3A* gene from *Bacillus thuringiensis* subsp. *tenebrionis.* The *mcry3A* gene encodes the mCry3A protein that confers resistance to the Western Corn rootworm (*Diabrotica virgifera virgifera*) and other related coleopteran pests of maize. The native *cry3A* gene was modified to incorporate a cathepsin-G serine protease recognition site within the expressed protein. The original N-terminal region of this protein has been removed and the mCry3A protein commences at a methionine residue in position 48 of the native protein. The *mcry3A* gene is regulated by the promoter from the metallothionein-like gene from *Zea mays*, which is preferentially expressed in root tissue, and the nopaline synthase (NOS) terminator from *Agrobacterium tumefaciens.*

MIR604 also expresses the *pmi* (*manA*) gene from *Escherichia coli*, which encodes the enzyme phosphomannose isomerase (PMI). The gene was introduced as a selectable marker for the development of maize MIR604. Mannose is taken up by plants and converted to mannose-6-phosphate by hexokinase. Usually this product cannot be further utilised in maize plants as they lack the PMI enzyme. The accumulation of mannose-6-phosphate inhibits phosphoglucose isomerase, causing a block in glycolysis. It also depletes cells of orthophosphate required for the production of ATP. Therefore, while mannose has no direct toxicity on plant cells, it causes growth inhibition. This does

not occur in plants transformed with the *pmi* gene as they can utilise mannose as a source of carbon. The *pmi* gene is regulated by the polyubiquitin promoter (ZmUbilnt) from Zea mays and the NOS terminator from *A. tumefaciens*.

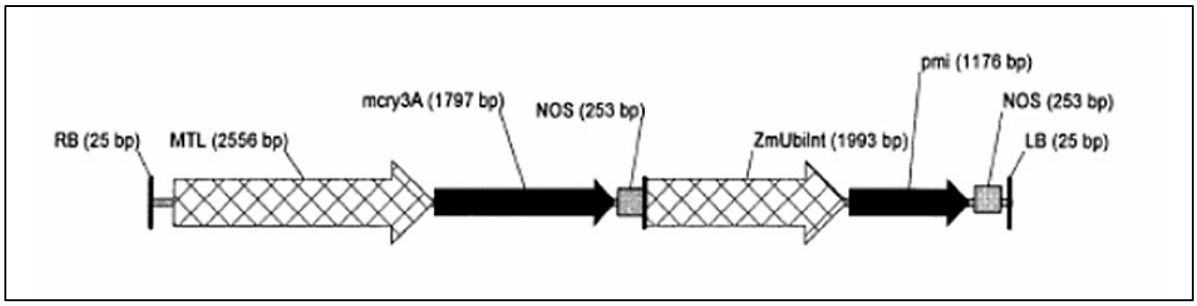

Figure 2. Genes and regulatory elements inserted in MIR604

Southern blot analyses have indicated that the maize event MIR604 contains a single intact T-DNA from plasmid pZM26, without the plasmid backbone.

Sequence analyses of the T-DNA including the flanking regions have shown that a 8416 bp T-DNA was inserted in the maize genome, and that a 44bp segment was missing from the Right border region, and 43bp at the Left border region. Three base pair changes were found within the insert in MIR604: one within the *MTL* promoter, and two within the *pmi* gene. These modifications have resulted in two amino acid substitutions, however without affecting the functions of the inserted elements in MIR604. The sequence analyses indicated that the overall integrity of the insert and the contiguousness of the functional elements from pZM26 are maintained.

BLAST analyses show that the insertion of the T-DNA in MIR604 occurred in a region of the *Zea mays* genome that was not well annotated and that the insert further did not appear to disrupt any identified endogenous *Zea mays* genes. Analyses of the six potential reading frames covering the T-DNA and genome junctions, did not show the presence of any novel ORF.

The levels of mCry3A and PMI proteins in maize MIR604 were determined by ELISA at the four growth stages: whorl, anthesis, seed maturity and senescence.
Across all growth stages, mean mCry3A levels measured ranged from 4 – 94 µg/g dry weight (dw) in leaves, 7 – 62 µg/g dw in roots, and 3 - 28 µg/g dw in whole plants. Mean mCry3A levels measured in grain at seed maturity and senescence ranged from 0.8 – 2.0 µg/g dw. Mean mCry3A levels measured in silk tissue at anthesis were below the lower limit of quantification (LOQ), <1.0 µg/g dw. Mean mCry3A levels measured in silk tissue at seed maturity ranged from 1 – 3 µg/g dw. No mCry3A protein was detectable in pollen.
PMI protein was detected in most maize MIR604 plant tissues, although at low levels. Across all plant stages, mean PMI levels ranged from not detectable (ND) to 2.1 µg/g dw in leaves, below the LOQ (<0.04 µg/g dw) to 2 µg/g dw in roots, and below the LOQ (<0.1 µg/g dw) to 1.0 µg/g dw in whole plants. Mean PMI levels measured in grain at seed maturity and senescence ranged from below the LOQ (<0.07 µg/g dw) to 0.5 µg/g dw. Mean PMI levels measured in silk tissue at anthesis and seed maturity ranged from below the LOQ (<0.2 µg/g dw.) to 6.8 µg/g dw. PMI in pollen ranged from 3.9 – 5.2 µg/g dw.

Overall levels of mCry3a protein were similar across four generations analysed without any significant trend either up or down, indicating that the expression of *mcry3A* in MIR604 is stable. A similar result was obtained for the PMI protein. Since no novel ORF were identified that spanned either the 5' or 3'

junctions between the MIR604 T-DNA and *Zea mays* genomic sequence, no fusion protein is expected.

Segregation analyses of both trait negative and trait positive plants, determined by ELISA and PCR, from a selected generation of maize (T5), have shown that the introduced traits in MIR604 are stably inherited in a Mendelian fashion, by Chi square analysis.

2.1.2.3. Maize GA21

Maize GA21 was generated by microprojectile bombardment transformation with a 3.49 kb *Not*I restriction fragment of the plasmid pDPG434 (derived from pUC19 via cloning into a commonly used pSK-vector). The DNA fragment used for transformation consisted of the following m*epsps* cassette: the rice actin promoter (5' region of the rice actin 1 gene containing the promoter and first non-coding exon and intron), an optimised transit peptide containing sequences from maize and sunflower, a modified maize *epsps* coding sequence (m*epsps*), and the 3‟ nos terminator from *Agrobacterium tumefaciens*. The mutations in the coding sequence of the maize *epsps* gene led to amino acid changes at positions 102 (threonine to isoleucine) and 106 (proline to serine). As a result of these mutations, the m*epsps* containing maize line GA21 is tolerant to glyphosate-based herbicides. The vector backbone contained the origin of replication (*ori* ColE1), the *lac* sequence as present in pUC19, and the bacterial *bla* gene conferring resistance to ampicillin in bacteria (Fig. 3). The mEPSPS is only different from the naturally present EPSPS protein by two amino acids.

Southern analyses showed that the insert in maize GA21 consists of six contiguous complete and truncated versions (fragments 1 to 6) of the 3.49 kb *Not*I restriction fragment. The insertions are located as a single locus. The absence of vector backbone sequences in GA21 plants has been demonstrated using a probe specific for the pDPG434 vector backbone. Therefore, the *bla* gene has not been transferred to maize GA21.

The nucleotide sequence of the insert introduced into maize GA21 has been determined in its entirety and even though regarded as a single locus consisting of six fragments or copies of the transgene construct as specified below. Fragment 1 contains the rice actin promoter with a deletion of 696 bp at the 5' end, the actin first exon and intron, the optimized transit peptide, the m*epsps* gene and *nos* terminator. Fragments 2, 3 and 4 are complete versions of the 3.49 kb NotI fragment. Fragment 5 contains the complete rice actin promoter, the actin first exon and intron, the optimized transit peptide, and 288 bp of the m*epsps* gene which ends in a stop codon. Fragment 6 only contains the rice actin promoter and a truncated actin first exon. A single base pair change was observed in the *nos* terminator in fragments 1 and 2 (nucleotide C instead of G). In addition, a single base pair deletion is observed in the actin promoter of fragment 6. The observed mutations do not have any impact on the amino acid sequence of the newly expressed protein.

The sequences of 1 kb of the plant genome adjacent to the 3' and 4.2 kb at the 5' end were also determined and bioinformatic analysis gave no indication that the sequence was inserted in a functional maize gene. The 3‟ sequence shows homology to repetitive sequences in the maize genome. The 5‟ flanking sequence was shown to be of chloroplast origin. The five putative ORFs found at the junction between the insert and the plant DNA show no significant sequence homology to any known toxic proteins or allergens. One potential new ORF was apparently created at the junction between fragment 5 and 6 but lacked the necessary components to be likely to be transcribed. This ORF does not show homology to known or putative allergens or toxic proteins. Updated (2008) bioinformatic analysis of the 5‟ and 3‟ flanking regions of the GA21 insert provided data which were similar to that previously reported and do not indicate any safety concerns with regard to the interruption of known genes or from the potential production of new toxins or allergens.

The concentrations of the mEPSPS protein in maize plants derived from GA21 were examined by ELISA in several plant tissues and whole plants at four growth stages (whorl, anthesis, seed maturity and senescence) in two maize hybrids. Across all growth stages, mean mEPSPS concentrations measured in leaves, roots and whole plants ranged from below the limit of quantification (<0.2 µg/g fw) to 15 µg/gfw (<0.4—71 µg/g dw). Mean mEPSPS concentrations measured in grain ranged from 4—7 µg/g fw (5—10 µg/gdw) and in pollen averaged 168 µg/g fw.

The inheritance of the introduced glyphosate tolerant phenotype follows a Mendelian segregation pattern of a single functional locus and the mEPSPS protein is stably expressed in maize GA21 across multiple generations. Southern analysis demonstrated that the insert in maize GA21 is stably inherited over three backcross generations.

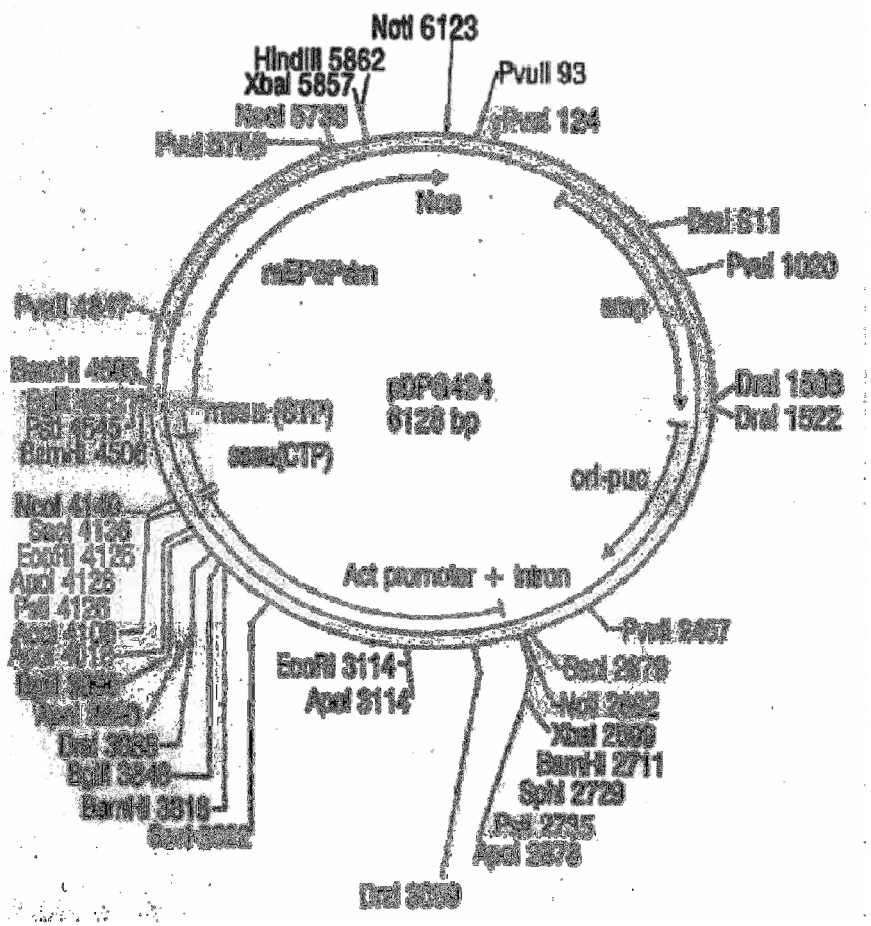

Figure 3. Various gene elements of t transformation vector pDPG434 used for generation of the maize strain GA21.

2.1.3 Transgene constructs in Bt11 x MIR604 x GA21 maize

The integrity of the individual inserts present in Bt11 x MIR604 x GA21 maize was investigated using Southern analyses. This involved the use of DNA probes specific for the Bt11, MIR604 and GA21 inserts and enzymatic digestions informative of the structure of the three events, including the junctions with the host genomic DNA. According to the applicant, the predicted DNA hybridisation patterns from each single maize event were retained in the stacked maize events Bt11 x MIR604 x GA21.

2.1.3.1 Information on the expression of insert

The levels of newly expressed proteins Cry1Ab, PAT, mCry3A, PMI and mEPSPS in forage and grain of maize Bt11 x MIR604 x GA21 were assessed by enzyme-linked immunosorbent assay (ELISA). According to the applicant, the plants used in this study were grown in 2006 at a Syngenta Seeds research station in USA, according to standard local agronomic practices. A set of four maize plants were collected at anthesis (stage) from each of five replicate blocks and a set of two plants were collected at maturity (physiological stage), also from five replicate blocks. From these plants, leaves, roots, and whole plants at the same stages were analyzed to compare the concentrations of transgenic proteins in the hybrids listed above. Five replicated pollen samples per hybrid were collected in the field, and analyzed by ELISA in the same manner.

The scope of the application covers food and feed uses, import and processing, therefore protein expression data related to the grains is considered the most relevant. These data are summarised in Table 1. The results indicate that for Cry1Ab and PAT, the overall concentrations were generally comparable between the Bt11 x MIR604 x GA21 hybrid and the Bt11 hybrid. For mCry3A and MIR604 PMI, the overall concentrations were, generally comparable between the Bt11 x MIR604 x GA21 hybrid and the MIR604 hybrid. Similarly, the overall concentrations of mEPSPS were comparable between the Bt11 x MIR604 x GA21 hybrid and the GA21 hybrid (data not shown).

Cry1Ab, PAT, mCry3A, MIR604 PMI and mEPSPS concentrations in the near-isogenic, non-transgenic control samples were below the limit of detection. Some statistically significant differences were seen, but these differences were small or not consistent across the growing season.

2.1.3.2 Parts of the plant where the insert is expressed

To characterize the range of expression of Cry1Ab, PAT, mCry3A, MIR604 PMI and mEPSPS proteins in Bt11 x MIR604 x GA21 maize plants, the concentrations of these proteins were determined by ELISA in several plant tissues (leaves, roots, grain and pollen).

According to the applicant, the concentrations of Cry1Ab, PAT, mCry3A, MIR604 PMI, and mEPSPS proteins were generally similar between the stacked Bt11 × MIR604 × GA21 maize hybrid and the corresponding single-events Bt11, MIR604, and GA21. Out of 20 statistical comparisons of all the transgenic protein concentrations between the single event hybrids and the stacked hybrid, only two significant differences (significant difference established as an F-Test value less than the customary 5% level) were observed. The two significant differences observed were in root tissue, for which the Cry1Ab protein concentrations in the Bt11 hybrid and the mEPSPS protein concentrations in the GA21 hybrid were higher than those of the Bt11 × MIR604 × GA21 hybrid. Although the measured concentrations of these two proteins in Bt11 × MIR604 × GA21 hybrid root tissue were lower than the concentrations measured in root tissue of the two single-event hybrids, the ranges of all protein concentrations from individual replicate samples of the single-event hybrids overlapped considerably with those of the stacked hybrid. Furthermore, the concentrations of both mEPSPS and Cry1Ab proteins in whole-plant samples of the Bt11 × MIR604 × GA21 hybrid were not significantly different

from those of the two single-event hybrids. The VKM GMO panel do not believe this difference pose a safety concern and that the difference is not there at the whole plant level which is more relevant for farming practice and the harvested plant parts.

Quantifiable concentrations of Cry1Ab protein were detected in leaves, roots and grains derived from Bt11 and Bt11 x MIR604 x GA21 maize. Very low levels of Cry1Ab expression were detected in pollen for both hybrids.

Quantifiable concentrations of PAT protein were detected in leaves and roots derived from Bt11 and Bt11 x MIR604 x GA21 maize at most stages of development, however no quantifiable levels could be detected in grains or pollen.

Quantifiable concentrations of mCry3A protein were detected in leaves, roots and grains derived from MIR604 and Bt11 x MIR604 x GA21 maize. Very low levels of mCry3A expression were detected in the pollen of MIR604 and Bt11 x MIR604 x GA21.

Quantifiable concentrations of MIR604PMI and mEPSPS protein were detected in all MIR604 and Bt11 x MIR604 x GA21 maize plant tissues analysed.

2.1.3.3 Potential fusion proteins

Bt11 x MIR604 x GA21 maize was produced by combining Bt11, MIR604 and GA21 maize through conventional breeding. An Open Reading Frame (ORF) analysis was performed for each of the single events.

2.1.3.4 Inheritance and genetic stability of inserted DNA

Molecular analyses indicate that the insert has been stably integrated into the plant genome in each event.

2.2 Conclusion

Southern blot and PCR analyses have indicated that the recombinant inserts in the parental maize lines Bt11, MIR604 and GA21 are retained in the stacked maize Bt11 x MIR604 x GA21. Genetic stability of the inserts has previously been demonstrated in the parental maize lines. Protein levels measured by ELISA show comparable levels of the Cry1Ab, PAT, mCry3A, PMI and mEPSPS proteins between the stacked and single maize lines. Phenotypic analyses also indicate stability of the insect resistance and herbicide tolerance traits in the stacked maize. The VKM Panel on GMO considers the molecular characterisation of maize Bt11 x MIR604 x GA21 and its parental events Bt11, MIR604 and GA21 as adequate.

3 Comparative assessment

3.1 Choice of comparator and production of material for the compositional assessment

3.1.1 Summary of the previous evaluation of the single events

3.1.1.1 Maize Bt11

Maize Bt11 was compared to non-transgenic maize with a comparable genetic background. Forage and grain samples were collected for compositional analysis from field trials conducted in USA (studies involving 3-6 sites in 1995) and Europe (two locations in 1998). No consistent compositional differences were observed between maize Bt11 and non-transgenic maize. In addition, field trials over several seasons at different locations in Europe did not indicate significant differences between maize Bt11 and its comparators with respect to agronomical and phenotypical characteristics, except for herbicide tolerance and insect resistance.

Maize Bt11 has a long history of use and has been evaluated extensively by The VKM GMO Panel. In the latest risk assessment, it was concluded that maize Bt11 is compositionally, agronomically and phenotypically equivalent to conventional maize varieties, except for the herbicide tolerance and insect resistance traits conferred by the transgenic proteins Cry1Ab and PAT (VKM 2014a).

3.1.1.2 Maize MIR604

Maize MIR604 was compared to non-transgenic maize with comparable genetic background (near-isogenic control) during field trials at multiple locations in USA in 2002 and 2003. The composition of forage and grain samples were analysed in line with recommendations from the OECD consensus document on key nutrients, anti-nutrients, and secondary plant metabolites of maize (OECD 2002). No consistent compositional differences were observed between maize MIR604 and non-transgenic maize. Agronomic traits were assessed during field trials (and greenhouse trials) at 22 locations in 8 states in USA in 2002 and 2003. The results did not indicate consistent differences between maize MIR604 and its comparators with respect to agronomical and phenotypical characteristics, except for insect resistance.

Analyses of mono- and disaccharides, including phosphorylated forms of these saccharides, in maize MIR604 and near-isogenic control, were performed by the applicant at six locations in USA in 2006 at the request of the EFSA GMO Panel. In compounds that could theoretically be linked to PMI activity (e.g., starch and other carbohydrates), no consistent compositional differences were observed in the comparison between maize MIR604 and control.

In the latest risk assessment of maize MIR604 the VKM GMO Panel concludes that maize MIR604 is compositionally, agronomically and phenotypically equivalent to conventional maize varieties, except for the presence of the transgenic proteins and the insect resistance traits conferred by the mCry3A protein (VKM 2014b).

3.1.1.3 Maize GA21

Maize GA21 was compared to non-transgenic maize with a comparable genetic background (near-isogenic control) during field trials at multiple locations and over several seasons: five locations in USA in 1996, seven locations in USA in 1997, four locations in Europe in 1997 and six locations during two seasons in USA in 2004 and 2005. Maize GA21 plants treated with glyphosate-based herbicides as well as plants untreated with the target herbicides were included in these field trials. No consistent compositional differences were observed between maize GA21 and non-transgenic maize.

Agronomic traits were assessed during multiple field trials and seasons in USA in 2004, Brazil in 2003 and Europe in 2007 and 2008. Results from these field trials did not indicate consistent differences between maize GA21 and its comparators with respect to agronomical and phenotypical characteristics, except herbicide tolerance.

In the latest risk assessment of maize GA21 the VKM GMO Panel concludes that maize GA21 is compositionally, agronomically and phenotypically equivalent to conventional maize varieties, except for the herbicide tolerance conferred by the mEPSPS protein (VKM 2014c).

3.1.2 Experimental design & statistical analysis

Bt11 x MIR604 x GA21 maize was produced by combining Bt11, MIR604 and GA21 maize through conventional breeding techniques. All three single GM maize events have previously been compared to their respective near-isogenic conventional maize lines for their nutritional effects on humans and animal health. Data on comparisons of agronomic characteristics and compositional analysis between Bt11 maize and near-isogenic non-GM conventional maize, MIR604 maize and near-isogenic maize as well as GA21 maize and near-isogenic maize were generated from field trials conducted at several locations representative of environments where these maize lines will be grown, over more than one season. The results of these evaluations indicate that nutritional contents of Bt11, MIR604 and GA21 maize are not different to those for conventional maize.

Additional studies were conducted to compare the composition of this stacked maize product containing Bt11, MIR604 and GA21 with relevant non-GM control maize lines. Commercial varieties were included in the comparison whenever possible.

According to the updated EFSA guidance on risk assessment of food and feed from genetically modified plants (EFSA 2011a), there should be at least three appropriate non-GM reference varieties of the crop that have a known history of safe use at each site. The test of equivalence is used to verify whether the agronomic, phenotypic and compositional characteristics of the GM plant fall within the normal range of natural variation. Such a range of natural variation is estimated from a set of non-GM reference varieties with a history of safe use (EFSA 2011b) and therefore allows comparisons of the GM plant with a similar food or feed produced without the help of genetic modification and for which there is a well-established history of safe use. These requirements were however not in place at the time of submission.

Bt11 x MIR604 x GA21 maize plants and the corresponding near-isogenic controls were grown at six locations in the North America for grain and forage analysis during the 2006 field trial season. At each location, the hybrids were grown in a randomized complete block design, with three replicates for each genotype. Sixty-five key food and feed nutrients, anti-nutrients and secondary plant metabolites (9 in forage, 56 in grain) were measured in the study, based on recommendations of the Organisation for Economic Co-operation and Development (OECD) for compositional considerations for new varieties of maize (OECD,2002). The data were subjected to analysis of variance across locations. Average levels of nutritional components were compared with the ranges of natural variation, as reported in the International Life Sciences Crop Composition database (ILSI 2006).

All field trials were designed following a randomized complete block design with three replicate plots of each genotype. Data for each genotype were subjected to analysis of variance across locations. For each analyte the statistical significance of the genotype effect was determined using a standard F-test at the 5% probability. The significance of the location x genotype interaction was also assessed using an F-test. For some analytes location x genotype interactions were detected, suggesting that a certain degree of inconsistency existed across sites, therefore an analysis “per location” was also conducted. The results were compared to compositional analysis data for grain and forage published in the

literature and in compositional analysis databases. Moisture content of grain was not statistically analysed, because the grain had been mechanically dried.

Details on the compositional analyses conducted with Bt11 x MIR604 x GA21 maize can be found in the technical dossier from the applicant (Appendix 4), considered confidential by Syngenta.

3.2 Compositional Analysis

The compositional parameters analysed for forage and grain of maize Bt11 x MIR604 x GA21 and its conventional counterpart are in line with those recommended by the OECD consensus document on key compositional parameters of maize (OECD, 2002). For some analytes, significant location-by-genotype interactions suggested some degree of inconsistency among locations. In these cases genotype comparisons across locations may not be valid and no further conclusions were drawn regarding the overall genotype effect. However, the data were compared with the ILSI database to establish whether or not the ranges were within natural variation, as an indication of whether the results were likely to be of biological significance.

3.2.1 Forage composition

Forage from Bt11 x MIR604 x GA21 maize plants and the corresponding non-transgenic maize plants were analyzed for proximates (including acid detergent fiber (ADF) and neutral detergent fiber (NDF)), calcium and phosphorus. No statistically significant differences between genotypes were observed for any of the forage analytes measured. Average levels of all analytes measured in forage were within the ranges for conventional maize hybrids published by ILSI (2006).

Proximates
No statistically significant differences in moisture, protein, ash, carbohydrates, ADF and NDF were observed (Table 1 – appendix). A significant location-by genotype interaction was observed for fat. The mean levels of all proximates across locations and for each location were within the ranges reported in the ILSI database.

Phosphorus and calcium
The levels of phosphorus did not differ significantly between the two genotypes. A significant location-by-genotype interaction was observed for calcium. The mean levels of both minerals across locations and for each location were within the ranges reported in the ILSI database.

3.2.2 Grain composition

Grain was harvested after physiological maturity from Bt11 x MIR604 x GA21 maize and near-isogenic control plants and analyzed for; proximates (ADF, NDF, TDF, starch, carbohydrates, protein, moisture, fat and ash), minerals (calcium, phosphorous, potassium, sodium, iron, copper, magnesium, manganese, selenium and zinc), vitamins (Vitamins E, B1, B2, B3, B6, Folic Acid) and β–carotene, amino acids (eighteen amino acids were analysed), fatty acids (linoleic, oleic, palmitic, stearic and linolenic acids) and secondary metabolites and anti-nutrients (furfural, phytic acid, inositol, trypsin inhibitor, raffinose, ferulic acid and p-coumaric acid).

Of the 56 analytes measured in grain, statistically significant differences were noted for levels of protein, zinc and vitamin B1 and many of the amino acids (as expected from the difference in protein levels). For all the components measured in grain, the mean levels (across locations and at each location) were within the ranges of variation for conventional maize hybrids published in the ILSI database, except for vitamins B2 and E at some locations. Mean levels of vitamin B2 were slightly

higher than the published ranges at one of the six locations for the transgenic grain and one of the six locations for the non-transgenic grain. Vitamin E levels below the limit of quantitation occurred in samples of both the transgenic and non-transgenic grain at some locations.

Proximates and fibers
No statistically significant differences were observed in fat, carbohydrates, ADF, NDF, TDF and starch. However, a statistically significant difference in protein levels between the genotypes was observed. Further, a significant location-by genotype interaction were observed for ash. The mean levels of all proximates across locations and for each location were within the ranges reported in the ILSI database.

Minerals
The levels of copper, iron, magnesium, manganese, phosphorus, potassium, and selenium did not differ significantly between the two genotypes. Zinc levels were statistically significant different and a significant location by-genotype interaction was observed for calcium. For sodium, levels below the limit of quantitation (LOQ) precluded statistical analysis. Mean levels of all minerals across locations and for each location were within the ranges reported in the ILSI database.

Amino acids
Most of the amino acid levels differed significantly between the genotypes, a result consistent with the difference in protein levels . All mean amino acid levels across locations and for each location were within the ranges reported in the ILSI database.

Fatty acids
The proportion of the five most abundant fatty acids, as a fraction of total fatty acids did not differ significantly between the genotypes. All mean levels across locations and for each location were within the ranges reported in the ILSI database.

Vitamins
Levels of vitamins A, riboflavin (B2), niacin (B3), pyridoxine (B6), and folic acid (B9) did not differ significantly between the genotypes . A statistically significant difference was observed for thiamine (B1). For vitamin E, levels below the limit of quantitation (LOQ) precluded statistical analysis. All mean levels across locations and for each location were within the ranges reported in the ILSI database except for riboflavin (B2) levels, which were slightly higher in the transgenic grain at location 8 and in the non-transgenic grain at location 1, and the vitamin E levels that were <LOQ in both transgenic and non-transgenic grain at some locations. Below LOQ values for vitamin E are not represented in the ILSI database.

Secondary metabolites and anti-nutrients
There are no generally recognised anti-nutrients in maize at levels considered to be harmful, but for the purposes of assessment of substantial equivalence, the OECD has asked for analytical data for the following secondary metabolites in maize: ferulic acid, p-coumaric acid, furfural, inositol, phytic acid, raffinose and trypsin inhibitor.

Levels of ferulic acid, *p*-coumaric acid, inositol, phytic acid, and trypsin inhibitor did not differ significantly between the genotypes. Levels of raffinose and furfural below the LOQ precluded statistical analysis. All mean levels across locations and for each location were within the ranges reported in the ILSI database.

For most components measured in forage and grain, mean levels did not differ significantly between Bt11 x MIR604 x GA21 maize and non-transgenic maize. For all components except vitamin B2 and

vitamin E, mean levels across locations were within the ranges for conventional maize hybrids published in the ILSI Crop Composition Database.

3.3 Agronomic and phenotypic characters

During field trials over at ten locations in the USA in the 2006 growing season, data on phenotypic characteristics, agronomic performance (e.g., grain yield, number of emerged plants, plant population at harvest, plant height, ear height, root lodging) and disease susceptibility were collected for the maize stack Bt11 x MIR604 x GA21 and its conventional counterpart (near-isogenic conventional maize). Up to eighteen separate agronomic parameters and one disease trait were assessed at each location, although not all parameters were assessed at all locations. A list of the agronomic characteristics assessed and their descriptions are found in the Appendix 3 in the Technical Dossier.

According to the applicant, the test locations were selected to be representative of the range of environmental conditions under which the tested hybrid varieties would typically be grown. Each of the agronomic trials was conducted as a randomized complete block design with five replications per location. For each agronomic or disease trait suitable for formal analysis, data were subjected to analysis of variance across locations. The statistical significance of the genotype effect (Bt11 x MIR604 x GA21 vs. the near-isogenic control) was determined using a standard F-test at the 5% probability.

A statistical analysis on agronomic and phenotypic characteristics on a per-location basis was provided by the applicant at the EFSA GMO Panel's request, complementing the across-location analysis already provided by the applicant. Analyses of variance across trial locations showed statistically significant differences between maize Bt11 x MIR604 x GA21 and the near-isogenic control hybrid for grain yield, grain moisture and plant height. The transgenic maize Bt11 x MIR604 x GA21 had significant higher yield compared the corresponding control at four of the ten sites (data not shown). According to the applicant the significant differences between the hybrids was likely due to the effects from specific locations where the hybrids were grown. Although insecticide was applied to both the control and the stacked hybrids at planting, insect pressure from the target pests European corn borer or corn rootworm in these locations may have caused damage to the control hybrids which may have contributed to the lower yields. The differences in the average values for plant height and grain moisture across locations were of minor magnitude. The statistical analysis showed additional statistically significant differences at individual field trial sites. However, when data from all locations were considered there were no consistent statistically significant differences that occurred in each separate location.

Details on the agronomic and phenotypic characters can be found in the technical dossier from the applicant (Appendix 3), considered confidential by Syngenta.

3.4 Conclusion

Comparative analyses of data from field trials located at representative sites and environments in North America during the 2006 growing season indicate that maize stack Bt11 x MIR604 x GA21 is compositionally, agronomically and phenotypically equivalent to its conventional counterpart, with the exception of the insect resistance and the herbicide tolerance, conferred by the expression of Cry1Ab, mCry3A, PAT, PMI and mEPSPS proteins.

Based on the assessment of available data, the VKM GMO Panel is of the opinion that conventional crossing of maize Bt11, MIR604 and GA21 to produce the hybrid Bt11 x MIR604 x GA21 does not result in interactions between the newly expressed proteins affecting composition and agronomic characteristics.

4 Food /feed risk assessment

4.1. Summary of the previous evaluation of the single events

The single maize events Bt11, MIR604 and GA21 have previously been evaluated by the VKM GMO Panel, and updated risk assessments were finalised in January 2014 (VKM 2014a,b,c).

Maize Bt11
Maize Bt11 has a long history of use, and has been evaluated extensively by The VKM GMO Panel. In the latest risk assessment (VKM 2014) it was concluded that Bt11 is nutritionally equivalent to conventional maize varieties and that it is unlikely that the Cry1Ab or PAT proteins will introduce a toxic or allergenic potential in food or feed based on maize Bt11 compared to conventional maize.

With regard to animal studies with the whole product, feeding studies with maize Bt11 grain with different target animals, such as rats (Hammond et al 2006), broilers (Brake et al. 2003a) and laying hens, mice (Brake et al. 2004), dairy cows (Folmer et al. 2002) and beef cattle fed silage (Folmer et al. 2002), have all indicated nutritional equivalence between maize Bt11 and its non-GM maize counterpart and to conventional maize (Chowdhury et al. 2003 a,b; 2004; Shimada et al 2006 a,b,c; 2008).

Furthermore, in a multi-generation study (5 generations) with ICR mice, performance and life span was investigated on mice fed diets containing 68% of either Bt11 maize or isogenic non-Bt maize. Multiple parameters were measured e.g. feed intake and growth, mating, gestation, milking periods, reproduction, longivety and pathology. No significant differences were found between the Bt11- and non-Bt -fed mice in any of the generations (Haryu et al. 2009).

Maize MIR604
In the latest risk assessment of maize MIR604 the VKM GMO Panel concluded, based in part on data from whole food feeding studies on rats, rainbow trout and broilers, that maize MIR604 is nutritionally equivalent to conventional maize varieties. It is unlikely that the mCry3A or PMI proteins will introduce a toxic or allergenic potential in food or feed based on maize MIR604 compared to conventional maize.

Maize GA21
In the latest risk assessment of maize GA21 the VKM GMO Panel concluded, based in part on data from whole food feeding studies on rats, feedlot cattle and broilers, that maize GA21 is nutritionally equivalent to conventional maize varieties. It is unlikely that the mEPSPS protein will introduce a toxic or allergenic potential in food or feed based on maize GA21 compared to conventional maize.

4.2 Product description and intended uses

The scope of application EFSA-GMO-UK-2008-56 includes the import and processing of maize Bt11 x MIR604 x GA21 and its derived products for use as food and feed. Thus, the possible uses of maize

Bt11 x MIR604 x GA21 include the production of animal feed, but it also includes valuable food products, such as starch, syrups and oils. The genetic modification of maize Bt11 x MIR604 x GA21 is intended to improve agronomic performance only and is not intended to influence the nutritional properties, processing characteristics and overall use of maize as a crop.

4.3 Effects of processing

There are two basic methods employed in processing field maize grain, dry milling and wet milling. In dry milling, maize is separated into flour, maize-meal, grits and other products. Wet milling is the process by which maize is separated into starch, germ to produce oil and fiber, and gluten for animal feed. Bt11xGA21 will be produced and processed in the same way as any field maize.

The food manufacturing of Bt11 x MIR604 x GA21 field maize includes processing steps that are harsh, e.g. cooking, heating, high pressures, pH treatments, physical shearing, extrusion at high temperatures etc. under which the majority of proteins are denatured, which also applies to Cry1Ab1, PAT, mEPSPS, mCry3A and MIR604 PMI proteins (Hammond & Jez 2011). Concentrations of these proteins will be below the limit of detection in wet-milled fractions, in maize chips and maize oil. In the unprocessed kernel, and all of dry-milled fractions these protein will probably been found in quantifiable amounts.

4.4 Toxicological assessment

4.4.1 Toxicological assessment of the newly expressed protein

No new constituents other than the Cry1Ab, PAT, mEPSPS mCry3A and PMI proteins are expressed in maize Bt11 x MIR604 xGA21 and no relevant changes in the composition of maize Bt11 x MIR604 x GA21 were detected by the compositional analysis.

Following a request from the EFSA GMO Panel the applicant submitted an updated bioinformatics analysis comparing the amino acid sequences of the newly expressed proteins Cry1Ab, PAT, mCry3A, PMI and mEPSPS in maize Bt11 x MIR604 x GA21 with the sequences of known toxic and general proteins using an updated database. These analyses confirmed the results of the previous studies, which showed no similarities between the newly expressed proteins Cry1Ab, PAT, mCry3A, PMI and mEPSPS and known proteins toxic to mammals.

Determination of the levels of the newly expressed proteins in grains of maize Bt11 x MIR604 x GA21, Bt11, MIR604 and GA21 showed comparable expression levels in the stacked maize events and the respective single maize events.

4.4.2 Toxicological assessment of the whole GM food/feed

49-day feeding study on broiler chickens
Poultry studies are considered useful because chickens are fast growing organism that can consume large quantities of maize in the diet and thus are sensitive to potentially toxic effects of maize dietary components (OECD 2003).

A 49-day poultry feeding study was conducted to evaluate whether standard poultry diets prepared with Bt11 x MIR604 x GA21 maize grain had any adverse effects on male or female broiler chickens

as compared to diets prepared with near isogenic control grain and other conventional maize lines commercially available.

A broiler feeding study was conducted to compare the nutritional properties of maize MIR604 x GA21 (NP2673(GA21)/ NP2171(BT11+MIR604) with its near-isogenic control NP2673/NP2171, and a locally grown commercial maize NC 2007 (North Carolina, growing season 2006). One day old male (commercial strain Ross344) and female (commercial strain Ross 708) birds were distributed into 36 pens assigned in a randomised complete block design. Male and female birds were housed separately. Each test group (GM, control, and reference) consisted of six replicated pens of 15 birds/gender, - a total of 540 birds. The birds were fed *ad libitum* the Starter diets from day 0 - 16, Grower diets from day 17 – 35, and Finisher diets from day 35-49.

Three different diets: 1) Starter, 2) Grower, and 3) Finisher, were prepared for each of the three maize lines. Maize grain was mixed with soybean oil cake (48%) and other nutrients with an increasing inclusion of maize from starter to finisher diets (Table 1).

Table 1. Composition of Starter, Grower and Finisher diets for the three maize lines tested.

	NC 2007			Isogenic control			Bt11 x MIR604 x GA21		
Ingredients	**Starter**	**Grower**	**Finisher**	**Starter**	**Grower**	**Finisher**	**Starter**	**Grower**	**Finisher**
Maize grain, %	51.10	57.97	63.32	49.55	55.72	60.87	49.23	56.36	60.48
Soybean oil cake (48%), %	38.72	31.46	26.49	37.78	30.49	25.43	37.95	30.67	25.63
Other, %	10.18	10.57	10.19	12.67	13.79	13.7	12.82	12.97	13.89
Total, %	100	100	100	100	100	100	100	100	100

Animal performance on the various diets was evaluated by measuring mortality, body weight gain (overall final weight of males: 3359 g/animal; of females: 2764 g/animal), feed consumption, feed conversion ratio (FCR) (cumulative FCR of males 1.69 g/g; of females 1.77 g/g) and carcass yields (fat pad, drums, thighs, wings and breasts).

At the end of the study feeding period, samples of the Bt11 x MIR604 x GA21 starting grain and the nontransgenic starting grain were analysed for the concentrations of Cry1Ab, PAT, mCry3A, MIR604 PMI, and mEPSPS by ELISA. The Cry1Ab, PAT, mCry3A, MIR604 PMI, and mEPSPS proteins were not detected in the non-transgenic starting grain. The mean Cry1Ab concentration measured in the Bt11 x MIR604 x GA21 starting grain was 1.79 µg/g sample; the PAT concentration fell between the limit of detection (LOD; 0.02 µg/g grain) and the limit of quantitation (LOQ; 0.07 µg/g grain); the mCry3A concentration fell between the LOD (0.02 µg/g grain) and the LOQ (0.36 µg/g grain); the mean MIR604 PMI concentration was 2.59 µg/g sample; and the mean concentration of mEPSPS was 3.91 µg/g sample.

According to the applicant, there were no body weight differences among broilers consuming Bt11 x MIR604 x GA21 diets, non-transgenic diets, or NC 2007 diets. Further, there was no maize grain source-by-sex interaction for body weight.

Broilers fed the Bt11 x MIR604 x GA21 diets had significantly improved feed conversion compared with broilers fed the NC 2007 diets during the 16-35 day and 0-35 day time periods while the broilers fed the non-transgenic diets performed intermediately between the two.

The overall survival was good (>97%) and there were no differences between male and female broilers. There also were no significant effects between maize grain sources or maize grain source-by-sex interactions.

The source of the maize grain had no effect on carcass yield (presented in either grams or as a percentage) among males or females. The yield of carcass portions was proportional to body weight for males and females in all cases.

The broiler feeding study supported the results of the comparative compositional analysis and indicates that maize Bt11 x MIR604 x GA21 is nutritionally equivalent to grain from its conventional counterpart and a commercial non-GM maize variety when used in adjusted diets.

4.5 Allergenicity assessment

Most food allergies are mediated by immunoglobulin E (IgE, type-I reactions). The strategies used when assessing the potential allergenic risk focuses on the characterisation of the source of the recombinant protein, the potential of the newly expressed protein to induce sensitisation, or to elicit allergic reactions in already sensitised individuals and whether the transformation may have altered the allergenic properties of the modified food. A weight-of-evidence approach is recommended, taking into account all of the information obtained with various test methods, since no single experimental method yields decisive evidence for allergenicity (EFSA 2010).

Most of the major food and respiratory IgE-allergens have been identified and cloned, and their protein sequences incorporated into various databases. As a result, novel proteins can be routinely screened for amino acid sequence homology and structural similarity to known human IgE-allergens using an array of bioinformatics tools. Sequence homology searches comparing the structure of novel proteins to known IgE-allergens in a database are conducted using various algorithms such as FASTA to predict overall structural similarities. According to FAO/WHO (2001) in cases where a novel protein and a known IgE-allergen have more than 35% identity over a segment of 80 or greater amino acids, IgE cross-reactivity between the novel protein and the allergen should be considered a possibility.

4.5.1 Assessment of IgE-mediated allergenicity of the newly expressed protein

A weighted risk analysis based on the decision tree approach has been performed by the applicant. The individual steps of this analysis comprise sequence homology to known allergens, specific or targeted serum screens for IgE cross-reactions to known allergens, digestability studies of the proteins in simulated gastric and/or intestinal fluids, and animal studies (FAO/WHO 2001; Codex Alimentarius 2003; König et al. 2004; Poulsen 2004).

The proteins Cry1Ab, PAT, mEPSPS, mCry3A and MIR604 PMI present in maize Bt11 x MIR604 x GA21 have been evaluated previously and it was found unlikely that they are allergenic.

These assessments have previously been described by the applicant for the single maize events Bt11 (Notification C/F/96/05.10 and EFSA-GMO-RX-Bt11), MIR604 (EFSA-GMO-2005-11) and GA21 (EFSA-GMO-2005-19 and EFSA-GMO-RX-GA21), and were based on the following aspects:

The proteins expressed by the transgenes in maize (*Zea mays*) are not considered common food allergens.

<u>Cry1Ab and PAT</u>

i) The sources of the transgene genes: *B. thuringiensis* (*cry-genes*) and *S. viridochromogenes* (*pat*) have no history of causing allergy

ii) History of safe use of Cry proteins as microbial pesticides (US EPA, 1998), no indications of Cry proteins originating from *Bacillus thuringiensis* having harmful effects on the health of humans and animals (US EPA, 1996).
iii) The PAT protein has been subjected to previous safety assessments for genetically modified plants and found to have no allergenic potential
iv) The PAT protein has no homology to known toxins or IgE-allergenic proteins
v) The microbially produced Cry1Ab and PAT proteins were rapid degraded in simulated gastric fluids *in vitro*. No degradation assay in gastrointestinal fluids has been performed by the applicant.
vi) PAT and Cry1Ab do not resemble any characteristics of known IgE-allergens, and no significant homologies between the amino acid sequences of the PAT and Cry1Ab proteins and IgE-allergenic proteins have been found (Fard et al, 2013, Kim et al, 2010, Randhawa et al 2011, Meyer, 1999, US EPA, 2010).
vii) The PAT and Cry1Ab protein are not glycosylated (Raybold et al, 2013, US EPA, 2010)
viii) Cry1Ab and PAT are considered heat labile (US EPA 2010)

mEPSPS

ix) The sources of the transgene gene is maize (*Zea mays*), which is not considered a common food allergen.
x) EPSPS enzymes are ubiquitous in plants and microorganisms
xi) A gene coding for the mEPSPS was expressed in bacteria and the resulting enzyme compared to the plant derived mEPSPS by Western blot. The enzymes expressed from the two sources were shown to be identical (Raybould et al. 2013).
xii) The mEPSPS is functionally equivalent to other food derived EPSPS enzymes except for its tolerance to Roundup® herbicides.
xiii) The EPSPS proteins have been previously assessed for genetically modified plants and found to have no potential for allergenicity by EPA, Canadian Food Inspection Agency and OECD.
xiv) The expressed mEPSPS protein is a single polypeptide with a 99.3 % sequence identity to the wild type.
xv) The mEPSPS protein lacks homology to known toxins or allergenic proteins (Meyer, 1999; Cressman, 2003).
xvi) Immunoblot glycosylation analyses of mEPSPS derived from recombinant E.coli and from extracts of leaf material from transgenic GA21 maize, indicate that both mEPSPS proteins are not glycosylated (Raybould et al. 2013).
xvii) Rapid degradation of the mEPSPS protein in simulated gastric fluids *in vitro* (OECD, 1999). No degradation assay in gastrointestinal fluids has been performed by the applicant.
xviii) The sources of the transgene genes: *B. thuringiensis* (*cry-genes*), *E. coli* (*pmi*), and *Zea mays (mepsps)* have no history of causing allergy

PMI

i) PMI enzymes are found in various plants and microorganisms.
ii) the *pmi* (*manA*) gene came from *Escherichia coli*,
iii) the manA protein is a member of the superfamily of "cupins," which are proteins with a specific 3-D structure. Some members of this super family are known allergens.
iv) the gene coding for the PMI in the MIR604 was expressed in bacteria and the resulting enzyme compared to the MIR604 derived PMI by Western blot. The enzymes expressed from the two sources were shown not to be identical, two amino acids were changed, valine-61 was substituted by alanine, and glutamine-210 by histidine.
v) Bioinformatic analysis did not reveal any relevant sequence homology between the PMI expressed in maize MIR604 and known allergens of the cupin superfamily.

vi) No significant similarity was found between any of the PMI 80-amino acid peptides and any entries in the SBI Allergen Database.

vii) In the eight or more contiguous amino acids homology search, there was an alignment between the PMI protein and a recently identified allergen, α-parvalbumin from *Rana* species CH2001 (a frog of Indonesian origin).

viii) Serum screening concluded that there is no cross-reactivity between PMI and serum IgE (obtained from an allergic individual who displayed food-induce anaphylaxis from α-parvalbumin)

ix) The PMI protein is rapidly degraded by simulated gastric fluids *in vitro*. No degradation assay in gastrointestinal fluids has been performed by the applicant

x) The *E. coli* expressed PMI protein is also found in human intestinal microbiota, e.g. *E. coli*

x) There has always been a background of human exposure and a low quantity of PMI found in the human diet.

xi) The PMI-protein has been previously assessed in genetically modified plants and found to have no potential for allergenicity (EFSA 2009; Delany et al. 2008,).

mCry3A

The sources of the transgene *mcry3A* is *Bacillus thuringiensis* subsp.*tenebrionis*. A *cry3A* gene from *Bt* subsp. *tenebrionis* was recreated synthetically to optimize for expression in corn. The Cry3A protein from *Bt* subsp. *tenebrionis* is not considered a common food allergen.

i) The expressed mCry3A protein is a single polypeptide with a 92.9 % sequence identity to the wild type.

ii) Immunoblot and glycosylation analysis of mCry3A derived from recombinant *E.coli* and from extracts of leaf material from transgenic MIR604 maize, indicate that post-translational glycosylation of mCry3A protein has not occurred.

iii) A comparison of amino acid sequences of known allergens uncovered no evidence of any homology with mCry3A, even at the level of 8 contiguous amino acids residues.

iv) The mCry3A protein is rapidly degraded by simulated gastric fluids *in vitro*. No degradation assay in gastrointestinal fluids has been performed by the applicant.

v) At 4°C, 25°C, and 37° C there was little or no effect on mCry3A bioactivity, while at 65°C there was some reduction in the bioactivity. At 95°C mCry3A protein was completely inactivated (US EPA 2010).

4.5.2 Assessment of the IgE-mediated allergenicity of the whole GM plant

Food allergies to maize are of low frequency and mainly occur in populations of specific geographic areas. Rare cases of occupational allergy to maize dust have been reported. There is no reason to expect that the use of maize Bt11 x MIR604 x GA21 will significantly increase the intake and exposure to maize. According to the applicant, a possible overexpression of any endogenous protein, which is not known to be allergenic, would be unlikely to alter the overall allergenicity of the whole plant or the allergy risk for consumers.

However, an assessment of endogenous allergens in maize, ie mLTP (maize lipid transfer protein), has been carried out with immunoassays based on rabbit anti-mLPT-peptide serum (Panda et al, 2013). According to Panda et al. (2013) the intent of this study was to demonstrate that natural variation exists between varieties of commodity crops, demonstrating a 15-fold variation in mLTP concentration between nine maize varieties. The allergenicity assessment of GM plants is not meant to address the adventitious presence of an allergen in a given food but rather to understand whether a GM plant might be more allergenic than its non-GM comparator(s) to such an extent to be of concern for human and animal health (Fernandez et al. 2013). A major concern for the allergenicity assessment of

GM plants, however, is to evaluate whether the genetic modification introduces new allergens into the GM plant, and to verify that an increased expression of endogenous allergens in the GM plant has not taken place (Fernandez et al. 2013).

4.5.3 Adjuvanticity

According to the EFSA Opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed (EFSA 2010) adjuvants are substances that, when co-administered with an antigen increase the immune response to the antigen and therefore might increase the allergic response. In cases when known functional aspects of the newly expressed protein or structural similarity to known strong adjuvants may indicate possible adjuvant activity, the possible role of these proteins as adjuvants should be considered. As for allergens, interactions with other constituents of the food matrix and/or processing may alter the structure and bioavailability of an adjuvant and thus modify its biological activity.

Only two of the ~ 10 Cry proteins that are currently used in genetically modified plants, Cry1Ab and Cry1Ac, have been studied experimentally regarding adjuvant effects. To the knowledge of the VKM GMO Panel, adjuvant effects have not been investigated for the other Cry proteins normally used in GM plants, or other groups of Cry proteins.

Studies with immunological mapping of the systemic and mucosal immune responses to Cry1Ac have shown that mice produce both systemic IgM and IgG and secretory IgA following intraperitonal (i.p.), intragastric (i.g.) or intranasal (i.n.) immunisation, and that the adjuvant effects of Cry1Ac is comparable to that of cholera toxin (CT) (Guerrero et al., 2004; Vazquez-Padron et al., 1999a, b; 2000; Moreno-Fierros et al., 2003). The adjuvant effect of CT is thus a relevant basis for comparison in a risk assessment of Cry1Ac. It is uncertain whether this applies to the same extent to other Cry proteins. A possible immunogenicity and adjuvanticity of Cry proteins has been considered by EFSA and VKM (EFSA 2009, VKM 2012).

“Bystander sensitisation”
"Bystander sensitisation” can occur when an adjuvant in food, or an immune response against a food antigen, results in an increased permeability of the intestinal epithelium for other components in food. Traditionally it was assumed that the epithelial cells of the intestine were permanently "glued together" by the so-called "tight junctions". Studies have however shown that these complex protein structures are dynamic and that they can be opened up by different stimuli.
Both *in vitro* and *in vivo* experiments have demonstrated that when an IgG response which can result in a complement activation (among other) is not balanced by an IgA response, the epithelial barrier can be opened and unwanted proteins are able to enter the body (bystander-penetration) and lead to allergic sensitisation (Brandtzaeg P, Tolo K 1977; Lim PL, Rowley D1982).

Additional information can be found in the report by VKM on Cry-proteins and adjuvanticity: “Health risk assessment of the adjuvant effects of Cry proteins from genetically modified plants used in food and fodder” (VKM 2012)

4.6 Nutritional assessment of GM food/feed

The broiler feeding study supported the results of the comparative compositional analysis, which showed that grain from maize Bt11 x MIR604 x GA21 is compositionally and, therefore, nutritionally equivalent to grain from the non-GM maize counterpart and conventional maize. The whole feed test indicates that no unexpected alterations in nutrients and other feed components have occurred and that no nutritional imbalances were introduced in Bt11 MIR604 xGA21.

4.6.1 Intake information/exposure assessment

Net import of maize staple, e.g. flour, starch and mixed products, in Norway in 2007 was 7600 tons, corresponding to 4.4 g dry weight/person/day or an estimated daily energy intake for adults to be 0.6 % (Vikse 2009). The estimated median daily intake of sweet maize is 3.25 g/day, with a 97,5 % percentile of 17.5 g/day. The production of maize porridge for children in 2007 was about 37.5 tons, corresponding to a daily intake of 1.7 g/day or an estimated daily energy intake to be 0.6 % for a 6 month child (Vikse 2009).

Since most foods and foodstuffs from maize are derived from field maize grains, an estimated maximum daily intake for a Norwegian adult of Cry1Ab, mCry3A, PAT, PMI and mEPSPS proteins from maize staple is calculated to be 8,36 µg, 2,64 µg, 7,04 µg, 21,56 µg and 31,24 µg, based on intake of maize staple (4.4 g/person/day) and the maximum protein levels in grain at physiological maturity in Table 1. The estimated maximum daily intake of Cry1Ab, mCry3A, PAT, PMI and mEPSPS proteins from sweet maize is calculated to be 33,25 µg, 28 µg, 10,5 µg, 85,75 µg and 124,25 µg, respectively, based on a daily intake of 17.5 g fresh sweet maize/day (97.5 % percentile). These levels are several orders of magnitude below the levels shown to have no effect in laboratory toxicology testing. Also, these levels are considerably below the proposed threshold of toxicological concern (TTC) level of 1800 µg/person/day (Class 1, oral exposure) for chemicals considered to have a low potential for toxicity based on metabolism and mechanistic data (Vermeire et al., 2010). Transgenic proteins produced by genetically modified plants are generally considered non-toxic to humans.

The VKM GMO Panel notes that farm (production) animals e.g. pigs and poultry often are fed diets with a substantial inclusion of unprocessed maize grain, and that the exposure to transgenic proteins from maize Bt11 x MIR604x GA21 may be higher for these animals.

This dietary exposure assessment is very conservative as it assumes that all maize consumed comes from maize Bt11 x MIR604 x GA21 and that the transgenic proteins are not denatured by processing.

4.5.2 Nutritional assessment of feed derived from the GM plant

Based on the compositional analyses of forage and grain samples from maize Bt11 x MIR604 x GA21; nutritional equivalence to non-GM maize shown in a broiler feeding study; and evaluation of the transgenic proteins produced by the maize, maize Bt11 x MIR604 x GA21 and derived food and feed products seem to be substantially and nutritionally comparable to conventional maize and maize products, except for the expression of the transgenic proteins.

4.6 Conclusion

A whole food feeding study on broilers has not indicated any adverse health effects of maize Bt11 x MIR604 x GA21, and shows that maize Bt11 x MIR604 x GA21 is nutritionally equivalent to conventional maize. The Cry1Ab, PAT, mEPSPS, mCry3A or PMI proteins do not show sequence resemblance to other known toxins or IgE allergens, nor have they been reported to cause IgE mediated allergic reactions. Some studies have however indicated a potential role of Cry-proteins as adjuvants in allergic reactions.

Based on current knowledge, the VKM GMO Panel concludes that maize Bt11 x MIR604 x GA21 is nutritionally equivalent to conventional maize varieties. It is unlikely that the Cry1Ab, PAT, mEPSPS,

mCry3A or PMI proteins will introduce a toxic or allergenic potential in food or feed based on maize Bt11 x MIR604 x GA21 compared to conventional maize.

5 Environmental risk assessment

5.1 Unintended effects on plant fitness due to the genetic modification

Maize (*Zea mays* L.) is an annual plant and member of the grass family Poacea. The species, originating from Central America, is highly domesticated and generally unable to survive in the environment without management intervention (Eastham & Sweet 2002). Maize propagates entirely by seed produced predominantly by cross-pollination (OECD 2003). In contrast to weedy plants, maize has a pistillate inflorescence (ear) with a cob enclosed with husks. Due to the structure of the cob, the seeds remain on the cob after ripening and natural dissemination of the kernels rarely occurs.

The survival of maize in Europe is limited by a combination of absence of a dormancy phase resulting in a short persistence, high temperature requirements for germination, low frost tolerance, low competitiveness and susceptibility to plant pathogens, herbivores and climatic conditions (van de Wiel et al. 2011). Maize plants cannot survive temperatures below 0ºC for more than 6 to 8 hours after the growing point is above ground (OECD 2003), and in Norway and most of Europe, maize kernels and seedlings do not survive the winter cold (Gruber et al. 2008). Observations made on cobs, cob fragments or isolated grains shed in the field during harvesting indicate that grains may survive and overwinter in some regions in Europe, resulting in volunteers in subsequent crops. The occurrence of maize volunteers has been reported in Spain and other European regions (e.g. Gruber et al. 2008). However, maize volunteers have been shown to grow weakly and flower synchronously with the maize crop (Palaudelmás et al. 2009). Cross-pollination values recorded were extremely variable among volunteers, most probably due to the loss of hybrid vigour and uniformity. Overall cross-pollination to adjacent plants was estimated as being low.

Despite cultivation in many countries for centuries, seed-mediated establishment and survival of maize outside cultivation or on disturbed land in Europe is rare (BEETLE Report 2009). Maize plants occasionally grow in uncultivated fields and by roadsides. However the species is incapable of sustained reproduction outside agricultural areas in Europe and is non-invasive of natural habitats (Eastham & Sweet 2002; Devos et al. 2009). There are no native or introduced sexually cross-compatible species in the European flora with which maize can hybridise and form backcross progeny (Eastham & Sweet 2002; OECD 2003). The only recipient plants that can be cross-fertilised by maize are other cultivated maize cultivars.

It is considered very unlikely that the establishment, spread and survival of maize Bt11 x MIR604 x GA21 would be increased due to the insect resistance and herbicide tolerance traits. The herbicide tolerant trait can only be regarded as providing a selective advantage for the GM maize plant where and when glufosinate ammonium-based herbicides are applied. Glufosinate ammonium-containing herbicides have been withdrawn from the Norwegian market since 2008, and the substance will be phased out in the EU in 2017 for reasons of reproductive toxicity. Similarly insect resistance against certain lepidopteran and coleopteran pests provides a potential advantage in cultivation of Bt11 x MIR604 x GA21 under infestation conditions. It is considered very unlikely that maize Bt11 x MIR604 x GA21 plants or their progeny will differ from conventional maize cultivars in their ability to survive as volunteers until subsequent seasons, or to establish feral populations under European environmental conditions.

Field trials carried out by the applicant do not indicate altered fitness of the single maize events Bt11, MIR604 and GA21 and the stacked event Bt11 x MIR604 x GA21 relative to its conventional

counterpart. A series of field trials with maize Bt11 x MIR604 x GA21 were carried out across ten locations in the USA in 2006. Information on phenotypic (e.g. crop physiology, morphology, development) and agronomic (e.g. grain yield) characteristics was provided to assess the agronomic performance of maize Bt11 x MIR604 x GA21 in comparison with its conventional counterpart (see section 3.1). Data from these field trials showed some statistical significant differences for grain yield, grain moisture and plant height, and enhanced biomass production when glufosinate-ammonium/or glyphosate-based herbicides were applied and/or under infestation of target pests. These differences were however small in magnitude and were not consistently observed over locations. The VKM GMO Panel is of the opinion that they do not raise any environmental safety concern. No changes in plant characteristics indicating altered fitness and invasiveness of maize stack Bt11 x MIR604 x GA21 were observed.

In addition to the data presented by the applicant, the VKM GMO Panel is not aware of any scientific reports indicative of increased establishment or spread of maize Bt11 x MIR604 x GA21, or changes to its survivability (including over-wintering), persistence or invasive capacity. Because the general characteristics of maize Bt11 x MIR604 x GA21 are unchanged, insect resistance, glufosinate and glyphosate tolerance are not likely to provide a selective advantage outside of cultivation in Europe. The VKM GMO Panel is of the opinion that the likelihood of unintended environmental effects based on establishment and survival of maize Bt11 x MIR604 x GA21 will not differ from that of conventional maize varieties.

5.2 Potential for gene transfer

A prerequisite for any gene transfer is the availability of pathways for the transfer of genetic material, either through horizontal gene transfer of DNA, or vertical gene flow via pollen or seed dispersal. Exposure of microorganisms to transgenic DNA occurs during decomposition of plant material remaining in the field after harvest or comes from pollen deposited on cultivated areas or the field margins. Transgenic DNA is also a component of a variety of food and feed products derived from maize Bt11 x MIR604 x GA21. This means that micro-organisms in the digestive tract in humans and animals (both domesticated animals and other animals feeding on fresh or decaying plant material from the transgenic maize line) may be exposed to transgenic DNA.

Maize is the only representative of the genus *Zea* in Europe, and there are no cross-compatible wild or weedy relatives outside cultivation with which maize can hybridise and form backcross progeny (Eastham & Sweet 2002; OECD 2003). Vertical gene transfer in maize therefore depends on cross-pollination with other conventional or organic maize varieties. All maize varieties which are cultivated in Europe can interbreed. In addition, unintended admixture/adventitious presences of genetically modified material/transgenes in seeds represent a possible way for gene flow between different production systems.

5.2.1 Plant to micro-organisms gene transfer

Experimental studies have shown that gene transfer from transgenic plants to bacteria rarely occurs under natural conditions and that such transfer depends on the presence of DNA sequence similarity between the DNA of the transgenic plant and the DNA of the bacterial recipient (Nielsen et al. 2000; De Vries & Wackernagel 2002, reviewed in EFSA 2004, 2009a; Bensasson et al. 2004; VKM 2005c).

Based on established scientific knowledge of the barriers for gene transfer between unrelated species and the experimental research on horizontal transfer of genetic material from plants to microorganisms, there is today little evidence pointing to a likelihood of random transfer of the transgenes present in maize Bt11 x MIR604 x GA21 to unrelated species such as bacteria.

It is however pointed out that there are limitations in the methodology used in these experimental studies (Nielsen & Townsend 2004). Experimental studies of limited scale should be interpreted with caution given the scale differences between what can be experimental investigation and commercial plant cultivation.

Experiments have been performed to study the stability and uptake of DNA from the intestinal tract in mice after M13 DNA was administered orally. The DNA introduced was detected in stool samples up to seven hours after feeding. Small amounts (<0.1%) could be traced in the blood vessels for a period of maximum 24 hours, and M13 DNA was found in the liver and spleen for up to 24 hours (Schubbert et al. 1994). By oral intake of genetically modified soybean it has been shown that DNA is more stable in the intestine of persons with colostomy compared to a control group (Netherwood et al. 2004). No GM DNA was detected in the faeces from the control group. Rizzi et al. (2012) provides an extensive review of the fate of feed-derived DNA in the gastrointestinal system of mammals.

In conclusion, the VKM GMO Panel consider it is unlikely that the introduced gene from maize Bt11 x MIR604 x GA21 will transfer and establish in the genome of microorganisms in the environment or in the intestinal tract of humans or animals. In the rare, but theoretically possible case of transfer of the *cry*, *pat, pmi* and *mepsps* genes from Bt11 x MIR604 x GA21 to soil bacteria, no novel property would be introduced into or expressed in the soil microbial communities; as these genes are already present in other bacteria in soil. Therefore, no positive selective advantage that would not have been conferred by natural gene transfer between bacteria is expected.

5.2.2 Plant to plant gene flow

Considering the intended uses of maize Bt11 x MIR604 x GA21 (excluding cultivation) and the physical characteristics of maize seeds, possible pathways of gene dispersal are grain spillage and dispersal of pollen from potential transgenic maize plants originating from accidental grain spillage during transport and/or processing.

The extent of cross-pollination to other maize cultivars will mainly depend on the scale of accidental release during transportation and processing, and on successful establishment and subsequent flowering of the maize plant. For maize, any vertical gene transfer is limited to other varieties of *Zea mays* plants as populations of sexually compatible wild relatives of maize are not known in Europe (OECD 2003).

Survival of maize plants outside cultivation in Europe is mainly limited by a combination of low competitiveness, absence of a dormancy phase and susceptibility to plant pathogens, herbivores and frost. As for any other maize cultivars, GM maize plants would only survive in subsequent seasons in warmer regions of Europe and are not likely to establish feral populations under European environmental conditions. In Norway, maize plants from seed spillage occasionally grow on tips, waste ground and along roadsides (Lid & Lid 2005).

The flowering of occasional feral GM maize plants origination from accidental release during transportation and processing is however unlikely to disperse significant amounts of GM maize pollen to other maize plants. Field observations performed on maize volunteers after GM maize cultivation in Spain revealed that maize volunteers had a low vigour, rarely had cobs and produced pollen that cross-pollinated neighbour plants only at low levels (Palaudelmás et al. 2009).

As maize Bt11 x MIR604 x GA21 has no altered survival, multiplication or dissemination characteristics, the VKM GMO Panel is of the opinion that the likelihood of unintended environmental

effects as a consequence of spread of genes from this GM maize in Norway will not differ from that of conventional maize varieties. The likelihood of cross-pollination between cultivated maize and the occasional feral maize plants resulting from grain spillage is considered extremely low.

5.3 Interactions between the GM plant and target organisms

Considering the intended uses of maize Bt11 x MIR604 x GA21, excluding cultivation, the environmental exposure is limited to exposure through manure and faeces from the gastrointestinal tract mainly of animals fed on the GM maize as well as to the accidental release into the environment of GM seeds during transportation and processing and subsequently to potential occurrence of sporadic feral plants. Thus the level of exposure of target organisms to Cry1Ab and mCry3Aproteins is likely to be extremely low and of no ecological relevance.

5.4 Interactions between the GM plant and non-target organisms (NTOs)

Considering the intended uses of maize stack Bt11 x MIR604 x GA21, excluding cultivation, the environmental risk assessment is concerned with accidental release of GM maize viable grains into the environment during transportation and processing, and exposure through manure and faeces from the gastrointestinal tracts of animals fed the GM maize.

Cry proteins are degraded by enzymatic activity in the gastrointestinal tract, meaning that only very low amounts would remain intact to pass out in faeces (e.g. Lutz et al. 2005, Guertler et al. 2008; Paul et al. 2010). There would subsequently, be further degradation of the Cry proteins in the manure and faeces due to microbial processes. In addition, there will be further degradation of Cry proteins in soil, reducing the possibility for the exposure of potentially sensitive non-target organisms. Although Cry proteins bind rapidly on clays and humic substances in the soil and thereby reducing their availability to microorganisms for degradation, there is little evidence for the accumulation of Cry proteins from GM plants in soil (Icoz & Stotzky 2009).

Data supplied by the applicant indicate that a limited amount of the Cry1Ab and mCry3A proteins enters the environment due to expression in the grains (mean value of 1.6 and 3.1 µg/g d.w., respectively). In addition, the data show that at least 99% of microbially produced Cry1Ab and mCry3A proteins were rapidly degraded in simulated gastric fluid.

In conclusion, the VKM GMO Panel considers that the exposure of potentially non-target organisms to the Cry1Ab and mCry3A proteins is likely to be very low and of no ecological relevance.

5.5 Potential interactions with the abiotic environment and biochemical cycles

Considering the intended uses of maize Bt11 x MIR604 x GA21, which exclude cultivation, and the low level of exposure to the environment, potential interactions of the GM plant with the abiotic environment and biogeochemical cycles were not considered an issue by the VKM GMO Panel.

5.6 Conclusion

The scope of the application EFSA/GMO/UK/2008/56 includes import and processing of maize Bt11 x MIR604 x GA21 for food and feed uses. Considering the intended uses of maize Bt11 x MIR604 x

GA21, excluding cultivation, the environmental risk assessment is concerned with accidental release into the environment of viable grains during transportation and processing, and indirect exposure, mainly through manure and faeces from animals fed grains from maize Bt11 x MIR604 x GA21.

Maize Bt11 x MIR604 x GA21 has no altered survival, multiplication or dissemination characteristics, and there are no indications of an increased likelihood of spread and establishment of feral maize plants in the case of accidental release into the environment of seeds from maize Bt11 x MIR604 x GA21. Maize is the only representative of the genus *Zea* in Europe, and there are no cross-compatible wild or weedy relatives outside cultivation. The risk of gene flow from occasional feral GM maize plants to conventional maize varieties is negligible. Considering the intended use as food and feed, interactions with the biotic and abiotic environment are not considered to be an issue.

6 Data gaps

Adjuvanticity

There are many knowledge gaps related to assessment of adjuvants. Most of the immunologic adjuvant experiments have been performed using Cry1Ac. Whether the other Cry proteins have similar adjuvant properties is unknown.

The quantities of Cry proteins in genetically modified maize and soya are marginal compared with the amounts of other adjuvants that are natural components of food. However, the extent to which these naturally occurring adjuvants and Cry proteins contribute to the development of allergies is largely unknown. Determination of their importance is hampered by the lack of validated methods for measuring adjuvant effects.

The possibility that Cry proteins might increase the permeability of the intestinal epithelium and thereby lead to "bystander" sensitization to strong allergens in the diet of genetically susceptible individuals cannot be completely excluded. This possibility could be explored in a relevant animal model.

One element of uncertainty in exposure assessment is the lack of knowledge concerning exposure via the respiratory tract and the skin, and also the lack of quantitative understanding of the relationship between the extent of exposure to an adjuvant and its effects in terms of development of allergies.

Herbicide residue levels

Herbicide residue levels on plants with engineered **r**esistance to one or two broad spectrum herbicides could entail higher levels of herbicide residue cocktails compared to plants produced by conventional farming practice.

Since it is difficult to predict the toxicity of cocktails from the toxicity of the single components, there is uncertainty related to risk of confounding effects such as additive or synergistic effects between the residues in herbicide resistant plants.

The transgene technology used can possibly lead to different metabolic products of the applied herbicides from what is expected from conventional usage. The risk assessment of herbicides should take into account plants with altered metabolism.

At present the changes related to herbicide residues of stacked plants as a result of the application of plant-protection products fall outside the remit of the Norwegian VKM Panels.

7 Conclusions

Molecular characterisation
Southern blot and PCR analyses have indicated that the recombinant inserts in the parental maize lines Bt11, MIR604 and GA21 are retained in the stacked maize Bt11 x MIR604 x GA21. Genetic stability of the inserts has previously been demonstrated in the parental maize lines. Protein levels measured by ELISA show comparable levels of the Cry1Ab, PAT, mCry3A, PMI and mEPSPS proteins between the stacked and single maize lines. Phenotypic analyses also indicate stability of the insect resistance and herbicide tolerance traits in the stacked maize. The VKM Panel on GMO considers the molecular characterisation of maize Bt11 x MIR604 x GA21 and its parental events Bt11, MIR604 and GA21 as adequate.

Comparative assessment
Comparative analyses of data from field trials located at representative sites and environments in North America during the 2006 growing season indicate that maize stack Bt11 x MIR604 x GA21 is compositionally, agronomically and phenotypically equivalent to its conventional counterpart, with the exception of the insect resistance and the herbicide tolerance, conferred by the expression of Cry1Ab, mCry3A, PAT, PMI and mEPSPS proteins.

Based on the assessment of available data, the VKM GMO Panel is of the opinion that conventional crossing of maize Bt11, MIR604 and GA21 to produce the hybrid Bt11 x MIR604 x GA21 does not result in interactions between the newly expressed proteins affecting composition and agronomic characteristics.

Food and feed risk assessment
A whole food feeding study on broilers has not indicated any adverse health effects of maize Bt11 x MIR604 x GA21, and shows that maize Bt11 x MIR604 x GA21 is nutritionally equivalent to conventional maize. The Cry1Ab, PAT, mEPSPS, mCry3A or PMI proteins do not show sequence resemblance to other known toxins or IgE allergens, nor have they been reported to cause IgE mediated allergic reactions. Some studies have however indicated a potential role of Cry-proteins as adjuvants in allergic reactions.

Based on current knowledge, the VKM GMO Panel concludes that maize Bt11 x MIR604 x GA21 is nutritionally equivalent to conventional maize varieties. It is unlikely that the Cry1Ab, PAT, mEPSPS, mCry3A or PMI proteins will introduce a toxic or allergenic potential in food or feed based on maize Bt11 x MIR604 x GA21 compared to conventional maize.

Environmental risk assessment
The scope of the application EFSA/GMO/UK/2008/56 includes import and processing of maize stack Bt11 x MIR604 x GA21 for food and feed uses. Considering the intended uses of maize Bt11 x MIR604 x GA21, excluding cultivation, the environmental risk assessment is concerned with accidental release into the environment of viable grains during transportation and processing, and indirect exposure, mainly through manure and faeces from animals fed grains from maize Bt11 x MIR604 x GA21.

Maize Bt11 x MIR604 x GA21 has no altered survival, multiplication or dissemination characteristics, and there are no indications of an increased likelihood of spread and establishment of feral maize plants in the case of accidental release into the environment of seeds from maize GA21. Maize is the only representative of the genus Zea in Europe, and there are no cross-compatible wild or weedy relatives outside cultivation. The VKM GMO Panel considers the risk of gene flow from occasional

feral GM maize plants to conventional maize varieties to be negligible in Norway. Considering the intended use as food and feed, interactions with the biotic and abiotic environment are not considered by the GMO Panel to be an issue.

Overall conclusion
Based on current knowledge, the VKM GMO Panel concludes that maize Bt11 x MIR604 x GA21 is nutritionally equivalent to conventional maize varieties. It is unlikely that the Cry1Ab, PAT, mEPSPS, mCry3A or PMI proteins will introduce a toxic or allergenic potential in food or feed based on maize Bt11 x MIR604 x GA21 compared to conventional maize.

The VKM GMO Panel likewise concludes that maize Bt11 x MIR604 x GA21, based on current knowledge, is comparable to conventional maize varieties concerning environmental risk in Norway with the intended usage.

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