Vitenskapskomiteen for mattrygghet
Norwegian Scientific Committee for Food Safety

# Food/feed and environmental risk assessment of insect-resistant and herbicide-tolerant genetically modified maize MIR604 x GA21 in the European Union under Regulation (EC) No 1829/2003 (EFSA/GMO/UK/2007/48)

**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.

## Acknowledgements

Monica Sanden, The National Institute of Nutrition and Seafood Research, is acknowledged for her valuable work on this opinion.

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# Summary

In preparation for a legal implementation of EU-regulation 1829/2003, the Norwegian Environment Agency (former Norwegian Directorate for Nature Management) has requested the Norwegian Food Safety Authority (NFSA) to give final opinions on all genetically modified organisms (GMOs) and products containing or consisting of GMOs that are authorised in the European Union under Directive 2001/18/EC or Regulation 1829/2003/EC within the Authority's sectoral 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 authorised 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 MIR604 x GA21 (Unique Identifier 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 2011/892/EC). Genetically modified maize 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/2007/48 in 2008 (VKM 2009a). In addition, 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, 2006, 2008, 2009b,c,d,e, 2010, 2012, 2013a,b,c,d).

The food/feed and environmental risk assessment of the maize MI604 x GA21 is based on information provided by the applicant in the application EFSA/GMO/UK/2007/48, 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 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), 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 scientific risk assessment of maize 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.

## Molecular characterisation

Southern blot and PCR analyses have indicated that the recombinant inserts in the parental maize lines MIR604 and GA21 are retained in the stacked maize MIR604 x GA21. Genetic stability of the inserts has previously been demonstrated in the parental events. Protein measurements show comparable levels of the mCry3A, PMI and mEPSPS proteins between the stacked and single maize lines. The VKM Panel on GMO considers the molecular characterisation of maize MIR604 x GA21 and its parental events MIR604 and GA21 as adequate.

## Comparative assessment

Comparative analyses of agronomic and phenotypic data from field trials located at representative sites and environments in USA in 2005 indicate that maize stack MIR604 x GA21 is equivalent to its conventional counterpart, with the exception of the insect resistance and the herbicide tolerance, conferred by the mCry3A, PMI and mEPSPS proteins. The field evaluations support a conclusion of no phenotypic changes indicative of increased plant weed/pest potential of maize MIR604 x GA21 compared to conventional maize varieties.

The applicant has performed a compositional analysis on the triple-stack Bt11 x MIR604 x GA21 instead of maize MIR604 x GA21. The analysis was performed on plant materials from maize Bt11 x MIR604 x GA21 and a near-isogenic control hybrid from field trials in USA in 2006. With the exception of small intermittent variations, no biologically significant compositional differences were found between the triple-stack and the near-isogenic control. The results of the study are considered valid by EFSA also for maize MIR604 x GA21, since maize Bt11 x MIR604 x GA21 encompasses the transgenic properties of maize MIR604 x GA21. This is in accordance with the EFSA guidance document for the risk assessment of genetically modified plants containing stacked transformation events (EFSA 2007b).

The VKM GMO Panel is of the opinion that the applicant should have performed a compositional analysis of maize MIR604 x GA21 and not only referred to analyses of the triple- stack Bt11 x MIR604 x GA21. However, based on all information available, including agronomic and phenotypic data from field trials with maize MIR604 x GA21, a feeding study on broilers showing nutritional equivalence to non-GM maize, and assessments of the single events MIR604 and GA21, the VKM GMO Panel concludes that forage and grain from maize MIR604 x GA21 are compositionally equivalent to its conventional counterpart.

## Food and feed risk assessment

A whole food feeding study on broilers has not indicated any adverse effects of maize MIR604 x GA21, and shows that maize MIR604 x GA21 is nutritionally equivalent to conventional maize. The mCry3A, PMI and mEPSPS, 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 MIR604 x GA21 is nutritionally equivalent to conventional maize varieties. It is unlikely that the mCry3A, PMI or mEPSPS proteins will introduce a toxic or allergenic potential in food or feed based on maize MIR604 x GA21 compared to conventional maize.

## Environmental risk assessment

The scope of the application EFSA/GMO/UK/2007/48 includes import and processing of maize stack MIR604 x GA21 for food and feed uses. Considering the intended uses of maize 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 MIR604 x GA21.

Maize 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 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 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 MIR604 x GA21 is nutritionally equivalent to conventional maize varieties. It is unlikely that the mCry3A, PMI or mEPSPS proteins will introduce a toxic or allergenic potential in food or feed based on maize MIR604 x GA21 compared to conventional maize.

The VKM GMO Panel likewise concludes that maize 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 MIR604 x GA21, EFSA/GMO/UK/2007/48, insect-resistance, herbicide-tolerance, Cry protein, *mcry3A, mepsps, pmi*, 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 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 MIR604 x GA21 (Unik kode SYN-IR6Ø4-5 x MON-ØØØ21-9) fra Syngenta Seeds Inc. ble godkjent til import, videreforedling og bruk som mat og fôr under EU-forordning 1829/2003 i 2011 (søknad EFSA/GMO/UK/2007/48). MIR604 x GA21 er resultat av konvensjonelle kryssinger mellom innavlede maislinjer med eventene MIR604 og GA21. Kryssingene er utført for å utvikle en maishybrid med resistens mot visse skadegjørere i billeordenen Coleoptera og toleranse mot herbicider med virkestoffet glyfosat. Maishybrid 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 2009a). Foreldrelinjene MIR604 og GA21 er også tidligere risikovurdert av VKM, både som enkelteventer og i en rekke andre hybrider (VKM 2005a,b, 2006, 2008, 2009b,c,d,e, 2010, 2012, 2013a,b,c,d).

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.

Mais MIR604 x Ga21 er fremskaffet ved konvensjonell kryssing av de to maislinjene MIR604 og GA21. Mais MIR604 ble utviklet via en *Agrobakterium*-mediert transformasjon av maisceller for resistens mot skadegjørere innen billeordenen Coleoptera ved introduksjon av genet *mcry3A,* en modifisert versjon av *cry3A-genet fra* jordbakterien *Bacillus thuringiensis* sp. *tenebrionis*. MIR604 uttrykker også genet *pmi* fra *E.coli* som er introdusert som seleksjonsmarkør, ved at den koder for et enzym som gjør det mulig for plantene å utnytte mannose som eneste karbonkilde, noe vanlige maisplanter ikke kan.

Maislinjen GA21 er framkommet 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 derved 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.

**Molekylær karakterisering**
Maishybriden MIR604 x GA21 er dannet ved konvensjonelle krysninger mellom maislinjene MIR604 og GA21. Spaltingsdata, Southern blot og PCR-analyser indikerer at de rekombinante innskuddene fra mais MIR604 og GA21 er stabilt nedarvet i mais MIR604 x GA21, og at antall innsatte gener, struktur og organiseringen av disse er ekvivalent med de som finnes i mais MIR604 og GA21. Nivåene av mCry3A, PMI og mEPSPS -proteiner i vegetativt vev og korn fra mais MIR604 x GA21er også sammenlignbare med nivåene i henholdsvis mais MIR604 og GA21. VKMs faggruppe for GMO anser den molekylære karakteriseringen av mais MIR604 x GA21 som adekvat.

**Komparative analyser**
Data fra feltforsøk i Nord-Amerika vekstsesongen 2005 indikerer, med unntak av insektsresistens og herbicidtoleranse, agronomisk og fenotypisk ekvivalens mellom maishybriden MIR604 x GA21 og korresponderende nær-isogen kontrollhybrid. Feltforsøkene understøtter konklusjonen om uendret sannsynlighet for spredning, etablering og invasjon av mais MIR604 x GA21 sammenliknet med konvensjonelle maissorter.
Søker har utført en ernæringsmessig komponentanalyse av trippel-maishybriden Bt11 x MIR604 x GA21 istedenfor mais MIR604 x GA21. Analysen ble utført på plantemateriale fra mais Bt11 x MIR604 x GA21 og korresponderende nær-isogen kontroll, fra feltforsøk i Nord-Amerika i 2006. Med unntak av små tilfeldige avvik ble det ikke avdekket forskjeller av biologisk betydning mellom mais Bt11 x MIR604 x GA21 og kontrollen. Ettersom de genetiske modifiseringene i GA21 x MIR604 er representert i Bt11 x MIR604 x GA21, anser EFSA resultatene som gyldige også for mais MIR604 x GA21. Dette er i tråd med EFSAs veiledende dokument for risikovurdering av genmodifiserte planter som inneholder stablete genmodifiserte egenskaper (EFSA 2007b). VKMs faggruppe for GMO mener søker heller burde ha utført en ernæringsmessig analyse av mais MIR604 x GA21 og ikke bare referert til analysene av trippel-maisen. Basert på tilgjengelig informasjon, inkludert feltforsøkene vedrørende agronomiske og fenotypiske egenskaper, fôringsforsøk med broilere, og tidligere vurderinger av maislinjene MIR604 og GA21, konkluderer VKMs faggruppe for GMO at mais MIR604 x GA21 er ernæringsmessig ekvivalent dens konvensjonelle motpart.

**Helserisiko**
I en fôringsstudie utført på broilere ble det vist at mais MIR604 x GA21 ikke førte til negative helseeffekter blant dyrene, og at maisen var ernæringsmessig ekvivalent konvensjonell mais. De introduserte proteinene mCry3A, PMI og mEPSPS 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 MIR604 x GA21 er ernæringsmessig ekvivalent konvensjonell mais. Det er lite sannsynlig at proteinene mCry3A, PMI eller mEPSPS vil introdusere et toksisk eller allergent potensiale i mat eller fôr basert på mais MIR604 x GA21sammenliknet med konvensjonelle maissorter.

**Miljørisiko**
Søknaden EFSA/GMO/UK/2007/49 gjelder godkjenning av maishybrid 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 maishybriden 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 MIR604 x GA21 er ernæringsmessig ekvivalent konvensjonell mais. Det er lite sannsynlig at proteinene mCry3A, PMI eller mEPSPS vil introdusere et toksisk eller allergent potensiale i mat eller fôr basert på mais MIR604 x GA21 sammenliknet med konvensjonelle maissorter.

Faggruppen finner at maishybrid 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

| | |
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| 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. |
| 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 |

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| EC | European Commission |
| 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 |
| 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. |

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| | 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 |
| 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. |

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| Maize growth stages | *Vegetative*<br>VE: emergence from soil surface<br>V1: collar of the first leaf is visible<br>V2: collar of the second leaf is visible<br>Vn: collar of the leaf number 'n' is visible<br>VT: last branch of the tassel is completely visible<br>*Reproductive*<br>R0: Anthesis or male flowering. Pollen shed begins<br>R1: Silks are visible<br>R2: Blister stage. The kernels are filled with a clear nourishing endosperm fluid and the embryo can be seen<br>R3: Milk stage. The kernels endosperm is milky white.<br>R4: Dough stage. The kernels endosperm has developed to a white paste<br>R5: Dent stage. If the genotype is a dent type, the grains are dented<br>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 14 November 2007, the European Food Safety Authority (EFSA) received from the Competent Authority of the United Kingdom an application (Reference EFSA/GMO/UK/2007/48) for authorisation of the insect-resistant and herbicide-tolerant genetically modified (GM) maize MIR604 x GA21 (Unique Identifier SYN-IR6Ø4-5 x MON-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 MIR604 x GA21
- GM plants for food and feed use
- Food and feed, containing or consisting of maize MIR604 x GA21
- Food and feed produced from maize MIR604 x GA21
- Food containing ingredients produced from maize MIR604 x GA21

After receiving the application EFSA/GMO/UK/2007/48 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 12 March 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 Panels scientific opinion was published in 29 April 2010 (EFSA 2010). The Commission Decision 2011/892/EC authorised the placing on the market of products containing, consisting of, or produced from maize MIR604 x GA21 pursuant to Regulation (EC) No 1829/2003 (EC 2008) on 22 December 2011.

Genetically modified maize 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/2007/48 in 2008 (VKM 2009a). In addition, 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, 2006, 2008, 2009b,c,d,e, 2010, 2012, 2013a,b,c,d).

*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 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 authorisation 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 authorised 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 authorised 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

Maize MIR604 x GA21 has been obtained from traditional breeding methods between progeny (inbred lines) of the genetically modified maize lines MIR604 and GA21.

The parental line MIR604 was developed to provide protection against certain coleopteran target pests belonging to the genus *Diabrotica* such as the larvae of western corn rootworm (WCRW; *D. virgifera virgifera* ), the northern corn rootworm (NCRW; *D. longicornis barberi*) by the introduction of a modified *cry3A* gene (*mcry3A*) derived from *Bacillus thuringiensis* subsp. *tenebrionis.* Maize MIR604 also contains the *pmi (manA*) gene from*Escherichia coli* which encodes the phosphomannose isomerise (PMI) protein as a selectable marker. PMI allows transformed maize cells to utilize mannose as a sole carbon source, while maize cells lacking the *pmi* gene fail to grow with mannose as single carbon source.

The parental line GA21 was developed to provide tolerance to the herbicidal active substance glyphosate by the introduction of a gene coding for the modified enzyme 5-enolpyruvylshikimate-3-phosphate synthase (mEPSPS). Glyphosate is normally phytotoxic to a broad range of plants. Its mode of action is to bind to and competitively inhibit the EPSPS protein, which is the key enzyme in the shikimate pathway that leads to the biosynthesis of the aromatic amino acids tyrosine, tryptophan and phenylalanine. The disruption of this pathway and the resulting inability to produce key amino acids prevents growth and ultimately leads to plant death. In the case of maize GA21, a gene has been introduced that codes for the expression of the mEPSPS protein, which is insensitive towards inhibition by glyphosate. This protein is similar to the native EPSPS in maize, but it is not inhibited by glyphosate thus allowing the crop to be protected from the recommended dosages of glyphosate.

The genetic modification in maize MIR604 x GA21 is intended to improve agronomic performance only, and is not intended to influence the nutritional properties, the processing characteristics and the overall use of maize as a crop.

Maize stack MIR604 x GA21 (Unique Identifier SYN-IR6Ø4-5 x MON-ØØØ21-9) 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 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), 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).

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 MIR604 x GA21

The stacked maize MIR604 x GA21 was developed through conventional breeding by crossing the single maize events MIR604 and GA21. Maize MIR604 x GA21 combines the insect resistance of maize MIR604 with the glyphosate tolerance of maize GA21, conferred through the expression of the *mcry3A* and *mepsps* genes, respectively. In addition, the stacked maize contains the selectable marker gene *pmi,* used in the development of maize MIR604.

### 2.1.2 Summary of evaluation of the single events

**Maize MIR604**

Maize MIR604 was developed by transforming immature maize embryos derived from a proprietary *Zea mays* line (A188) via *Agrobacterium*-mediated transformation, with the binary transformation vector pZM26. By this method, genetic elements within the left and right border regions (the T-DNA) of the transformation vector, are transferred and integrated into the genome of the plant cell, while genetic elements outside these border regions are (generally) not. The T-DNA genetic elements transferred to produce maize MIR604 are shown in Table 1 and Figure 1.

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*.

**Table 1. T-DNA genetic elements**

| Component | Size (bp) | Function and origin of the sequence |
|---|---|---|
| Right border | 25 | T-DNA right border region |
| MTL promoter | 2556 | Promoter derived from the metallothionein-like gene from Zea mays. Provides preferential expression in roots of Zea mays |
| *mcry3A* | 1797 | Modified version of the native *cry3A* gene (maize optimised) |
| NOS | 253 | Terminator sequence from nopaline synthase gene from *A. tumefaciens* |
| ZmUbiInt | 1993 | Promoter region and intron from the Zea mays polyubiquitin gene. Provides constitutive expression |
| *pmi* | 1176 | Phosphomannose isomerase gene from *E. coli*. Selectable marker gene |
| NOS | 253 | Terminator sequence from nopaline synthase gene from *A. tumefaciens* |
| Left border | 25 | T-DNA left border region |

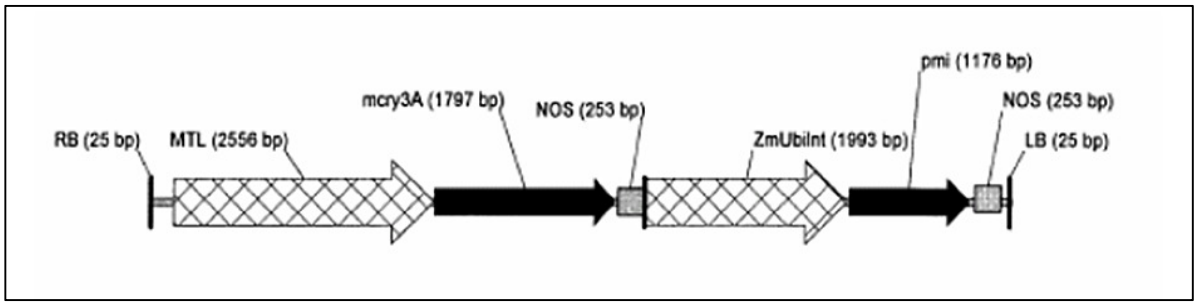


**Figure 1. Genes and regulatory elements inserted in MIR604**

Southern blot analyses have indicated that the maize event MIR604 occurred as an integration of a single intact T-DNA from plasmid pZM26 into the proprietary maize line genome, and that plasmid backbone DNA is not present in maize MIR604.

Sequence analyses of the entire T-DNA insert and flanking regions have shown that a total of 8416 bp of T-DNA was inserted in the maize genome, and that a 44bp segment was missing from the Right border region, as well as 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.

According to the applicant, 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 did not appear to disrupt any identified endogenous *Zea mays* genes. Analyses of six potential reading frames at both the 5' and 3' T-DNA to genome junctions did not show the presence of any novel ORF's.

Segregation analyses of 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, as analysed by Chi square analysis.

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 measured to be 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's were identified that spanned either the 5' or 3' junctions between the MIR604 T-DNA and *Zea mays* genomic sequence, no fusion proteins are expected.

In summary, the molecular characterisation of maize MIR604 indicates the presence of only single copies of the *mcry3A* and *pmi* genes, and that the T-DNA insert and phenotypic traits are stably inherited over several generations. The VKM GMO Panel considers the molecular characterisation of maize MIR604 as adequate.

## 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). The plasmid was derived from a pSK- vector, commonly used in molecular biology and is derived from pUC19. 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. 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 or truncated versions (fragments 1 to 6) of the 3.49 kb *Not*I restriction fragment. The insertions are located at a single locus. The absence of vector backbone sequences in GA21 plants has been demonstrated with 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. 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 *mepsps* 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 *mepsps* gene which

ends in a stop codon. Fragment 6 contains the rice actin promoter and the actin first exon truncated but no other elements. 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 an 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 and allergens. One potential new ORF was apparently created at the junction between fragment 5 and 6 but lacked the necessary components 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 GA21were 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 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.

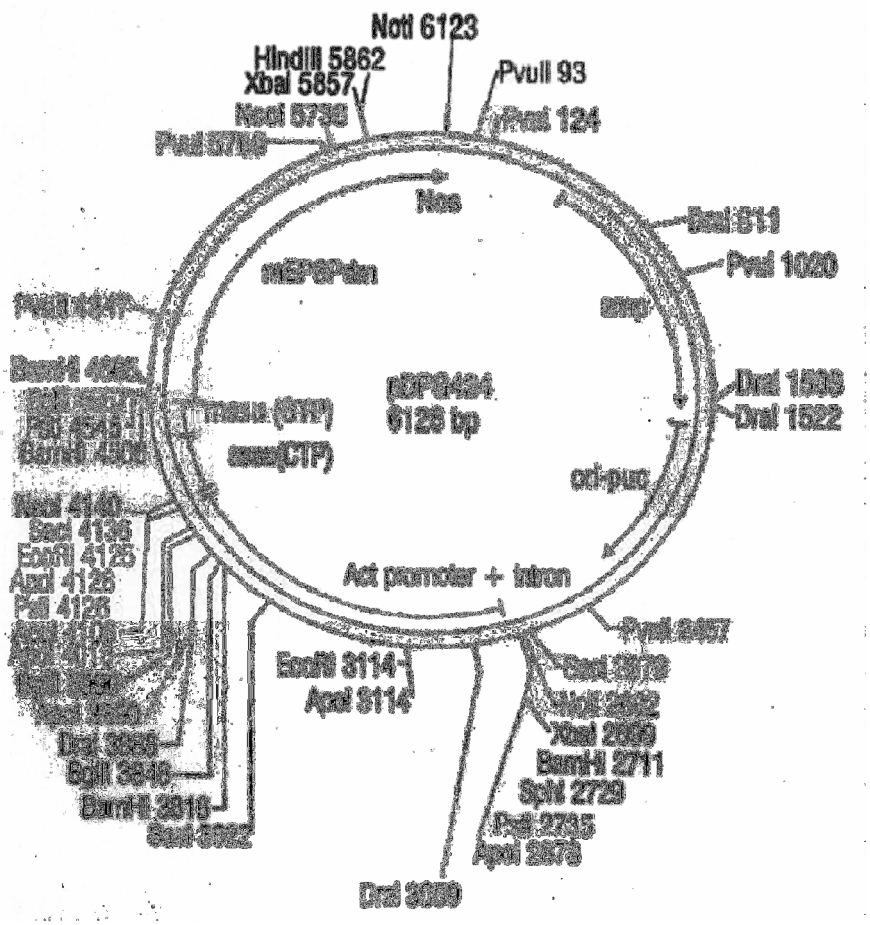


**Figure 2. Various gene elements of t transformation vector pDPG434 used for generation of the maize strain GA21.**

### 2.1.3 Transgene constructs in maize MIR604 x GA21

Maize MIR604 x GA21 was produced by combining MIR604 maize and GA21 maize through conventional breeding, and therefore expresses the three transgenic genes *mcry3A*, *pmi* and *mepsps*.

The applicant has performed a comparative Southern blot analysis of maize MIR604 x GA21 with the parental maize lines MIR604 and GA21, to investigate if the *mcry3A, pmi* and *mepsps* genes are intact and stably inherited by maize MIR604 x GA21.

***mcry3A specific probe***
Genomic DNA from MIR604 and MIR604 x GA21, were digested with the restriction enzymes *Kpn*I, *Eco*RV, and *Asc*I + *Xma*I, and then hybridised with the *mcry3A* specific probe. This produced single hybridisation bands of approximately 5.6 kb, 11.0 kb and 8.2 kb, respectively, corresponding to single copies of the *mcry3A* gene in both maize lines. These results together with negative (inbred hybrid and GA21) and positive control (plasmid pZM26) indicate that the *mcry3A* gene is intact in MIR604 x GA21, and equivalent to the *mcry3A* gene in MIR604.

***pmi* specific probe**
Genomic DNA from MIR604 and MIR604 x GA21 were digested with the restriction enzymes *Kpn*I, *Bam*HI, and *Asc*I + *Xma*I, and then hybridised with the *pmi* specific probe. This produced single hybridisation signals of approximately 5.2 kb, 2.7 kb and 8.2 kb, respectively, corresponding to single copies of the *pmi* gene in both maize lines. These results together with negative (inbred hybrid and GA21) and positive control (plasmid pZM26) indicate that the *pmi* gene is intact in MIR604 x GA21, and equivalent to the *pmi* gene in MIR604.

***mepsps* specific probe**
Genomic DNA from GA21 and MIR604 x GA21 were digested with the restriction enzymes *Hind*III, *Sac*I and *Sph*I, and then hybridised with the *mepsps* specific probe.

*Hind*III produced three unique hybridisation bands of approximately 3.5 kb, 4.7 kb, and 6.7 kb, corresponding to the multiple copies of the *mepsps* gene present in GA21 and MIR604 x GA2l. The digestion with *Hind*III also produced a hybridisation band at approximately 16.0 kb representing endogenous maize sequence in the parental lines GA21, MIR604, MIR604 x GA21, and the negative hybrid control.

Digestion with *Sac*I produced two unique hybridisation bands of approximately 2.1 kb and 3.5 kb corresponding to the multiple copies of the *mepsps* gene present in GA21 and MIR604 x GA21. The digestion with *Sac*I also produced hybridisation bands representing endogenous maize sequence at 4.3 kb in GA2l, 5.5 kb in MIR604, and both 4.3 kb and 5.5 kb in MIR604 x GA21 and the negative hybrid control.

Digestion with *Sph*I produced three unique hybridisation bands of approximately 2.1, 3.5 kb, and 16.0 kb corresponding to the multiple copies of the *mepsps* gene present in GA21 and MIR604 x GA21. The digestion with *Sph*I also produced hybridisation bands representing endogenous maize sequence at approximately 6.0 kb in GA21, 8.0 kb in MIR604, and both 6.0 kb and 8.0 kb in MIR604 x GA21 and the negative control.

These results indicate that the *mepsps* gene(s) in MIR604 x GA21 is intact and equivalent to the ones in GA21.

In summary, the results from the comparative Southern blot analysis show that *mcry3A, pmi* and *mepsps* genes are intact and stably inherited by maize MIR604 x GA21.

### 2.1.3.1 Information on the expression of the inserts

The maize plants used for this study were grown according to local agronomic practices at a Syngenta Seeds research station in Bloomington, IL, USA in 2005. All hybrids were grown in one plot containing two rows of 25 plants per hybrid. Samples were collected from plants grown in a single location at three sampling times across the growing season. To control for background effects, the corresponding tissues from a near-isogenic (nontransgenic) control maize were also analysed.

Five plants per maize hybrid, and two near-isogenic control plants, were collected at whorl, anthesis, and physiological maturity. From these plants, maize leaves and roots from all stages, pollen from anthesis, and kernels from physiological maturity, were analysed by ELISA to compare the concentrations of mCry3A, PMI and mEPSPS in maize MIR604, GA21 and MIR604 x GA21.

The transgenic protein concentration for each replicate sample was calculated with Microsoft Office Excel 2003. The expression data were subjected to statistical analysis with SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Each individual dataset, consisting of the data from MIR604 or GA2l and MIR604 x GA2l was subjected to analysis of variance, in which the effect of the genotype was assessed with an F-test. An F-test probability less than 5% indicates that the measured concentrations of transgenic proteins for the genotypes are significantly different at the customary 5% level. Only dry weight data were statistically analysed.

**mCry3A and PMI**

Except in leaf tissues at anthesis the levels of mCry3A were not significantly different between samples from MIR604 and MIR604 x GA2l. No statistically significant differences were observed in PMI levels between MIR604 and MIR604 x GA21 plant tissues.

**mEPSPS**

Statistically significant differences in levels of mEPSPS between maize GA2l and MIR604 x GA21 were observed for three out of seven maize plant tissues analysed.
According to the applicant, the antibodies used for the ELISA to quantify mEPSPS also detect endogenous maize EPSPS. The endogenous maize EPSPS is expressed at significantly lower levels than the mEPSPS in maize GA21. The endogenous maize EPSPS levels in the near-isogenic control samples were <LOD at the same assay dilutions at which mEPSPS was quantified in the corresponding transgenic samples. Therefore, the protein levels reported are essentially the levels of mEPSPS. The levels of mEPSPS in pollen are noticeably higher than those of the other plant tissues because the expression of the endogenous maize EPSPS protein is higher in pollen

Out of 21 statistical comparisons, only four significant differences were observed between the concentrations of the transgenic proteins expressed in the maize plant tissues of the parental maize lines MIR604 and GA21, and maize MIR604 x GA21. Maize MIR604 x GA21 had significantly higher mCry3A levels in leaves at anthesis than that of maize MIR604. However, no significant differences were seen in leaves at the developmental stages before or after anthesis, ruling out a consistent trend of significantly higher expression of mCry3A in leaves from the MIR604 x GA21 hybrid throughout the growing season.

Significant differences in mEPSPS levels were found in root samples from the whorl stage and at physiological maturity. The results showed that maize MIR604 x GA2l had significantly higher mEPSPS levels in roots at whorl, but significantly lower mEPSPS levels at physiological maturity than that of maize GA2l. No statistically significant differences were observed in root samples at anthesis. These results suggest no consistent significant difference of mEPSPS expression in roots

between the two hybrids. The mEPSPS concentrations in kernels at physiological maturity were also significantly different between maize MIR604 x GA2l and maize GA21; however, the difference between the two means is small.

For mCry3A and PMI, the overall concentrations were generally comparable between the MIR604 x GA21, and MIR604. Similarly, for the mEPSPS protein, the overall concentrations were also generally comparable between MIR604 x GA21 and GA21. Some statistically significant differences were noted, these differences were however small or not consistent across the growing season. The results indicate that the transgenic protein expression in maize MIR604 x GA21 is not substantially different from that of maize MIR604 and GA21.

#### 2.1.3.2 Parts of the plant where the insert is expressed

The range of expression of mCry3A, MIR604 PMI and mEPSPS proteins in MIR604 x GA21 maize plants, were determined by ELISA in samples from leaves, roots, kernels (grain) and pollen, as described above.

#### 2.1.3.3 Potential fusion proteins

Open Reading Frame (ORFs) analyses have been performed for the parental maize lines MIR604 and GA21. In these analyses no novel ORF's were identified in MIR604 that spanned either the 5' or 3' junctions between the maize MIR604 T-DNA and the *Zea mays* genomic sequence. As for maize GA21, bioinformatic analyses have revealed no biologically relevant homology to allergens or toxins for any of the putative polypeptides that might be produced from ORFs spanning the junction regions. No expression of potential fusion proteins are expected in maize MIR604 x GA21.

#### 2.1.3.4 Inheritance and genetic stability of inserted DNA

Genetic stability of the inserts has previously been demonstrated in the parental maize lines MIR604 and GA21. Comparative Southern blot analyses have indicated that the recombinant inserts in the parental maize lines are retained in the stacked maize MIR604 x GA21, and protein measurements with ELISA show comparable levels of the mCry3A, PMI and mEPSPS proteins between the stacked and single maize lines.

## 2.2 Conclusion

Conventional crossing methods were used to produce the stacked maize MIR604 x GA21. Southern blot and PCR analyses have indicated that the recombinant inserts in the parental maize lines MIR604 and GA21 are retained in the stacked maize MIR604 x GA21. Genetic stability of the inserts has previously been demonstrated in the parental events. Protein measurements show comparable levels of the mCry3A, PMI and mEPSPS proteins between the stacked and single maize lines. The VKM GMO Panel considers the molecular characterisation of maize MIR604 x GA21 and its parental events MIR604 and GA21 as adequate.

# 3 Comparative assessment

## 3.1. Summary of the previous evaluation of the single events

### 3.1.1. 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 2014a).

### 3.1.2. 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 2014b).

## 3.2 Choice of comparator and production of material for the compositional assessment

The applicant has measured key nutritional components in forage and grain from the triple stacked maize event Bt11 x MIR604 x GA21, instead of MIR604 x GA21. This is in accordence with the EFSA guidance document for the risk assessment of genetically modified plants containing stacked

transformation events (EFSA 2007b). The VKM GMO Panel commented the use of the triple stacked maize instead of maize MIR604 x GA21 during the EFSA official hearing in 2007 (Appendix). Maize Bt11 x MIR604 x GA21 expresses the genes *cry1Ab* and *pat* from maize Bt11, in addition to the *mcry3A, pmi* and *mepsps* from MIR604 and GA21. Forage and grain from maize Bt11 x MIR604 x GA21 were measured and compared with those from a near-isogenic control maize. No other control/reference maize was used in the study. The mazie were grown at six locations in USA in 2006.

All plant materials used in the srudy were from the two hybrids:

1. **Bt11 x MIR604 x GA21, [E1 (+)],** **Genotype: NP2673(GA2l)/NP2171(Bt11 + MIR604)**
2. **Nontransgenic hybrid, [E3 (-)],** **Genotype: NP2673/NP2171**

A pedigree chart of the two maize hybrids is shown in Appendix 3 of the Technical Dossier.
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.

### 3.2.1. Experimental design & statistical analysis

The Bt11 x MIR604 x GA2l maize and corresponding near-isogenic hybrid were grown at six locations in USA in 2006.

At each location, the hybrids were grown in a randomised complete block design, with three replicates per genotype. Forage and grain were harvested and analysed for key food and feed nutrients, antinutrients, and secondary plant metabolites chosen based on recommendations of OECD for comparative assessment of the composition of new varieties of maize (OECD 2002). Forage was analysed for proximates, calcium, and phosphorus (a total of 9 analytes), and grain was analysed for proximates, minerals, amino acids, fatty acids, vitamins, secondary metabolites, and antinutrients (a total of 56 analytes). Analysis of variance was used to test for genotype effects and location-by-genotype interactions. In addition, mean levels of nutritional components were compared with the ranges of variation for conventional maize hybrids published in the International Life Sciences Institute Crop Composition Database (ILSI 2006).

The Bt11 x MIR604 x GA21 maize was treated with glyphosate and glufosinate herbicides. Both the Bt11 x MIR604 x GA21 and the near-isogenic maize were treated with other conventional pesticides as needed to maintain optimal plant health. Plants were self-pollinated by hand, and the developing ears were bagged to prevent crosspollination.

According to the applicant, all compositional analyses were conducted by Covance Laboratories, Inc., according to methods published and approved by AOAC International, or other industry-standard analytical methods. Component levels were converted to equivalent units of dry weight (DW) based on the moisture content of each sample.

The data for each component were subjected to analysis of variance with the model

$Y_{ijk} = U + T_i + L_j + B(L)_{jk} + LT_{ij} + e_{ijk}$

where *Yijk* is the observed response for genotype *i* at location *j* block *k*, *U* is the overall mean, $T_i$ is the genotype effect, $L_j$ is the location effect, $B(L)_{jk}$ is the effect of block within location, $LT_{ij}$ is the location x genotype interaction effect and $e_{ijk}$ is the residual error.

For each quantifiable component, *F*-tests were used to assess the statistical significance of the genotype effect, with an alpha level of 0.05. An *F*-test was also used to assess the significance of the location by genotype interaction. An *F*-test probability of <0.05 suggests that the effect of genotype was not consistent across locations and that the comparison of genotypes averaged across locations may not be valid. Moisture content of grain was not statistically analysed, because the grain had been mechanically dried.

Details of the study can be found in Appendix 4 of Technical Dossier.

## 3.3. Compositional Analysis

### 3.3.1 Forage composition

**Proximates and fibres**
No statistically significant differences were found for moisture, protein, ash, carbohydrates, ADF and NDF. 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**
Phosphorus levels 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.

### 3.3.2 Grain composition

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

**Minerals**
Zinc levels were significantly different between the two genotypes and a significant location-by-genotype interaction was observed for calcium. Some sodium levels below the limit of quantitation (LOQ) precluded statistical analysis. No statistically significant differences were found for copper, iron, magnesium, manganese, phosphorus, potassium, or selenium between the two genotypes, and all Mean levels of all minerals across locations and for each location were within the ranges reported in the ILSI database.

**Vitamins**
A statistically significant difference was observed for vitamin $B_1$. 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 vitamin $B_2$ levels, which were slightly higher in the transgenic grain at location 8 and in control grain at location l, and the vitamin E levels that were <LOQ in both transgenic and control grain at some locations. Below LOQ values for

vitamin E are not represented in the ILSI database. Vitamins A, $B_2$, $B_3$, $B_6$, and $B_9$ did not differ significantly between the genotypes.

**Amino acids**
Most of the amino acid levels differed significantly between the genotypes. 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.

**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 furfuralbelow the LOQ precluded statistical analysis. All mean levels across locations and for each location were within the ranges reported in the ILSI database.

## 3.4 Agronomic and phenotypic characters

During field trials over at nine locations in the USA in the 2005 growth season, data on phenotypic characteristics, agronomic performance and disease susceptibility were collected for the maize stack MIR604 x GA21 and its conventional counterpart (near-isogenic conventional maize). Up to 20 separate agronomic parameters and three disease traits were assessed at each location, although not all parameters were recorded at all locations. Early growth, leaf color, gray leaf spot, northern corn leaf blight, southern corn leaf blight, and intactness were evaluated and recorded in qualitative scores on a scale of 1-9, where 1 is a good rating and 9 is a bad rating. Moisture, lodging, green snap, barrenness, dropped ears and stay green were recorded in percentages. Flowering data were recorded as heat units accumulated from date of planting.

The agronomic equivalence trials were conducted using two MIR604 x GA21 maize (field corn) hybrids. The MIR604 x GA21 maize hybrids are known as NP2672GA21/NP2171MIR604 (early maturity variety) and NP2673GA21/NP982MIR604 (mid maturity variety). The two corresponding near-isogenic non-transgenic hybrids are known as NP2672 x NP2171 (early maturity) and NP2673 x NP2391 (mid maturity).

Details on the trials conducted with Bt11 x MIR604 maize can be found in Appendix 3 of 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 four 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 (MIR604 x GA21 vs. the near-isogenic control) was determined with a standard F-test at the 5% probability.

Analyses of variance across trial locations showed no statistically significant differences between maize MIR604 x GA21 and the near-isogenic control hybrid for the agronomic and phenotypic characters recorded, except for "grain moisture at harvest" (data not shown). The MIR604 x GA21 hybrid had significant less grain moisture at harvest than the corresponding near-isogenic hybrid in the trials with the mid-maturity hybrids (MG877) (16.9 % vs. 17.8 %) ($p<0.05$). This difference was, however, relatively small and not observed in the early-maturling maize variety. A number of parameters showed statistically significant differences in the per-location statistical analysis of the comparisation between maize MIR604 x GA21 and its conventional counterpart, but non of these differences was consistently observed in each location. E.g. for grain yield, the effect of genotype within each of the two hybrids was not significant across locations indicating that the grain yield of the transgenic and nontransgenic hybrids was not different. The significant yield difference at two of the locations has been explained by a high occurrence of stalk lodging and a lower stand in the control, respectively. These results suggest that the two sets of hybrids essentially yielded equivalently in these trials.

## 3.5 Conclusion

Comparative analyses of agronomic and phenotypic data from field trials located at representative sites and environments in USA in 2005 indicate that maize stack MIR604 x GA21 is equivalent to its conventional counterpart, with the exception of the insect resistance and the herbicide tolerance, conferred by the mCry3A, PMI and mEPSPS proteins. The field evaluations support a conclusion of no phenotypic changes indicative of increased plant weed/pest potential of maize MIR604 x GA21 compared to conventional maize varieties.

The applicant has performed a compositional analysis on the triple-stack Bt11 x MIR604 x GA21 instead of maize MIR604 x GA21. The analysis was performed on plant materials from maize Bt11 x MIR604 x GA21 and a near-isogenic control hybrid from field trials in USA in 2006. With the exception of small intermittent variations, no biologically significant compositional differences were found between the triple-stack and the near-isogenic control. The results of the study are considered valid by EFSA also for maize MIR604 x GA21, since maize Bt11 x MIR604 x GA21 encompasses the transgenic properties of maize MIR604 x GA21. This is in accordance with the EFSA guidance document for the risk assessment of genetically modified plants containing stacked transformation events (EFSA 2007b).

The VKM GMO Panel is of the opinion that the applicant should have performed a compositional analysis of maize MIR604 x GA21 and not only referred to analyses of the triple-stack Bt11 x MIR604 x GA21. However, based on all information available, including agronomic and phenotypic data from field trials with maize MIR604 x GA21, a feeding study on broilers showing nutritional equivalence to non-GM maize, and assessments of the single events MIR604 and GA21, the VKM GMO Panel concludes that forage and grain from maize MIR604 x GA21 are compositionally equivalent to its conventional counterpart

# 4 Food /feed risk assessment

Both single maize events, MIR604 and GA21, have previously been evaluated by the VKM GMO Panel, and updated risk assessments were finalised in january 2014 (VKM 2014a,b)

## 4.1. Summary of the previous evaluations of the single events

### 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/2007/48 includes the import and processing of maize MIR604 x GA21 and its derived products for use as food and feed. The possible uses of maize MIR604 x GA21 include the production of animal feed and food products, such as starch, syrups and oils. The genetic modification of maize 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 MIR604 x GA21 as a food/feed plant.

## 4.3 Effects of processing

There are two basic methods employed in processing field maize kernels, 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. MIR604 x GA21 will be produced and processed in the same way as any field maize.

The food manufacturing of 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 mCry3A1, PMI and mEPSPS 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

In assessing the potential risks of GM foods, it is important to consider both adverse health effects that may arise from substances that are intentionally introduced or modified in food crops, and adverse effects that may be produced unexpectedly as a result of the genetic modification process (Chao &Krewski 2008).

### Toxicological assessment of the newly expressed protein

The VKM GMO Panel has previously evaluated the proteins mCry3A, PMI and mEPSPS in the risk assessments of the parental maize lines MIR604 and GA21 (VKM 2014a,b).

### 4.4.2 Toxicological assessment of the whole GM food/feed

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 broiler feeding study was conducted to compare the nutritional properties of maize MIR604 x GA21 (NP2673(GA21)/NP2391(MIR604) with its near-isogenic control (NP2673/NP2391), and a locally grown commercial maize NC 2006 (North Carolina, growing season 2006). Prior to the study, grain samples were analysed for proximates, amino acids and mycotoxins. The mycotoxin determinations showed low contamination by aflatoxins, fumonisin, T2 toxin, zearalenone, and deoxynivalenol (vomitoxin) in grain from all three maize lines.

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 7).

**Table 7. Composition of Starter, Grower and Finisher diets for the three maize lines tested.**

| | NC 2006 | | | Isogenic control | | | MIR604 x GA21 | | |
|---|---|---|---|---|---|---|---|---|---|
| **Ingredients** | **Starter** | **Grower** | **Finisher** | **Starter** | **Grower** | **Finisher** | **Starter** | **Grower** | **Finisher** |
| **Maize grain, %** | 51.36 | 58.07 | 63.94 | 49.61 | 56.12 | 61.84 | 49.99 | 56.55 | 62.31 |
| **Soybean oil cake (48%), %** | 38.77 | 31.55 | 26.49 | 38.12 | 30.78 | 25.63 | 38.03 | 30.68 | 25.52 |
| **Other, %** | 9.87 | 10.38 | 9.57 | 12.27 | 13.1 | 12.53 | 11.98 | 12.77 | 12.17 |
| **Total, %** | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

One day old male (commercial strain Ross344) and female (commercial strain Ross 508) 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 35-44.

The mCry3A concentrations in the starter, grower and finisher diets fell between LOD (limit of detection = 0.01 µg/g diet) and LOQ (limit of quantitation = 0.84 µg/g diet). The concentrations of mEPSPS ranged from 0.72 to 0.96 µg/g, and PMI from 0.45 µg/g to 0.57 µg/g in the corresponding diets.

At day 21, broilers fed the NC 2006 diet were significantly smaller in body weight than broilers fed either the MIR604 x GA21 or control diets, but not at any other measured time point. Overall mean body weights were comparable in all treatment groups on day 43 with the average male reaching 2,476 grams and the average female reaching 1,984 grams.

According to the applicant overall survival was good (>92 %), eventhough there was a heat-stress-related mortality due to an increase in ambient temperature over the summer months. The highest mortality was observed in broilers fed NC 2006 maize; neither mortality nor other differences were attributed to the different diets by the applicant.

Males fed the MIR604 x GA21 diet had slightly, but statistically significant, increased feed conversion ratios compared to the control and NC 2006 maize diets. The body weight gain was similar for all groups, with no statistically significant differences noted, and there were no significant differences in carcass yield. A slight difference in feed conversion ratios were not considered adverse by the applicant. Consumption of diets containing MIR604 x GA21 had no effect on carcass yield for males or females, and there was no overall effect of diets or gender on mortality.

At the end of the feeding period of the study, samples of Starting maize grain, Starter diets, Grower diets, and Finisher diets were again analysed for the concentrations of mCry3A, PMI, and mEPSPS.

In the EFSA Scientific Opinion adopted in 2010 this study was not considered by the EFSA GMO Panel.

## 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 with an array of bioinformatics tools. Sequence homology searches comparing the structure of novel proteins to known IgE-allergens in a database are conducted with 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 starts by analysing the primary amino acid sequences of the novel proteins and looking for similarites with sequences of known IgE allergens, followed by specific or targeted serum screens for IgE cross-reactions to known allergens, digestibility 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 mCry3A, PMI and mEPSPS present in maize MIR604 x GA21 have previously been evaluated and found unlikely to be allergenic. These assessments were described by the applicant for the single maize events MIR604 (EFSA-GMO--MIR604) and GA21 (EFSA-GMO-2005-19 and EFSA-GMO-RX-GA21), and were based on the following aspects:

PMI:

i) PMI enzymes are found in various plants and microorganisms.

ii) The *pm*i (*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 IgE 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 IgE 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 with serum IgE obtained from an allergic individual who displayed food-induce anaphylaxis against α-parvalbumin showed no cross-reactivity with PMI.

ix) 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 previously been assessed for genetically modified plants and found to have no potential for IgE allergenicity (EFSA 2009; Delany et al. 2008,).

mCry3A:

i) The Cry3A protein from *Bacillus thuringiensis* subsp.*tenebrionis* is not considered a common food allergen.

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

iii) 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.

iv) A comparison of amino acid sequence to known allergens indicated no homology between mCry3A and known allergens at the level of 8 contiguous amino acids.

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

vi) 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).

mEPSPS

i) EPSPS enzymes are ubiquitous in plants and microorganisms

ii) A gene encoding 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).

iii) The mEPSPS is functionally equivalent to other food derived EPSPS enzymes except for its tolerance to Roundup® herbicides.

iv) The EPSPS proteins have previously been assessed for genetically modified plants and found to have no potential for allergenicity by EPA, Canadian Food Inspection Agency and OECD.

v) The expressed mEPSPS protein is a single polypeptide with a 99.3 % sequence identity to the wild type.

vi) The mEPSPS protein lacks homology to known toxins or allergenic proteins (Meyer, 1999; Cressman, 2003).

vii) 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).

viii) The mEPSPS protein is rapidly degraded by simulated gastric fluids *in vitro* (OECD, 1999). No assay for degradation in gastrointestinal fluids has been performed by the applicant.

### 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 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.

An assessment of endogenous allergens in maize, i.e. 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 compositional analyses indicate nutritional equivalence between maize MIR604 x GA21, near isogenic non-GM control and a commercial maize line. This nutritional equivalence is further supported by the broiler feeding study.

### 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 mCry3A, PMI and mEPSPS proteins from maize MIR604 x GA21 is calculated to be 2.3 µg, 7.48 µg, and 13.2 µg, respectively, based on intake of maize staple (4.4 g/person/day) and the maximum protein levels in grain (kernel) at physiological maturity in Tables 5 and 6. The estimated maximum daily intake of mCry3A, PMI and mEPSPS proteins from sweet maize is calculated to be 9.1 µg, 29.8 µg and 52.5 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 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 MIR604 x GA21 and that the transgenic proteins are not denatured by processing.

### 4.6.2 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 of maize MIR604 x GA21 to non-GM maize shown in a broiler feeding study; and evaluation of the transgenic proteins produced by maize MIR604 x GA21, maize 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.

## 4.7 Conclusion

A whole food feeding study on broilers has not indicated any adverse effects of maize MIR604 x GA21, and shows that maize MIR604 x GA21 is nutritionally equivalent to conventional maize. The mCry3A, PMI and mEPSPS, 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 MIR604 x GA21 is nutritionally equivalent to conventional maize varieties. It is unlikely that the mCry3A, PMI or mEPSPS proteins will introduce a toxic or allergenic potential in food or feed based on maize 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 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 glyphosate based herbicides are applied. Similarly insect resistance against certain coleopteran pests provides a potential advantage in cultivation of MIR604 x GA21 under infestation conditions. It is considered very unlikely that maize 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 maize MIR604 x GA21 relative to its conventional counterpart. A series of field trials with maize MIR604 x GA21 were carried out across 6 locations in the USA in 2005 (application EFSA/GMO/UK/2007/48). 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 MIR604 x GA21 in comparison with its conventional counterpart (see section 3.1). Data from the field trials shows no statistical significant differences for the parameters assessed, except for grain moisure.

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 MIR604 x GA21, or changes to its survivability (including over-wintering), persistence or invasive capacity. Because the general characteristics of maize MIR604 x GA21 are unchanged, insect resistance 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 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 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 2005b).

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 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 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 *mcry3A, pmi* and *mepsps* genes from 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 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 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

Maize MIR604 was transformed to express a modified version of the Cry3A protein from *Bacillus thuringiensis* subsp. *tenebrionis*. The insecticidal toxin is active in the control of certain coleopteran insect pests belonging to the genus *Diabrotica,* such as larvae of western corn rootworm (WCR; *D. virgifera virgifera*) and the northern corn rootworm (NCR; *D. barberi*). WCR has been introduced to Europe from North America, where it is native and widespread (Miller et al. 2005, ref. EFSA 2013). *D. virgifera virgifera* was first detected in Serbia in 1992, but has since spread across the continent,

resulting in well-established populations in approximately 19 European countries (EC 2012). There have been no reports of *D. virgifera virgifera* in Norway (http://www.faunaeur.org/distribution.php

Considering the intended uses of maize 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 the mCry3A protein 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 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 mCry3A protein from GM plants in soil (Icoz & Stotzky 2009). Data supplied by the applicant indicate that a limited amount of the mCry3A protein enters the environment due to expression in the grains (mean value of 0.5 µg/g d.w). In addition, the data show that at least 99% of microbially produced Cry1Ab protein was rapidly degraded in simulated gastric fluid.

In conclusion, the VKM GMO Panel considers that the exposure of potentially non-target organisms to the mCry3A protein 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 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/2007/48 includes import and processing of maize MIR604 x GA21 for food and feed uses. Considering the intended uses of maize 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 MIR604 x GA21.

Maize 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 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 with 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 MIR604 and GA21 are retained in the stacked maize MIR604 x GA21. Genetic stability of the inserts has previously been demonstrated in the parental events. Protein measurements show comparable levels of the mCry3A, PMI and mEPSPS proteins between the stacked and single maize lines. The VKM Panel on GMO considers the molecular characterisation of maize MIR604 x GA21 and its parental events MIR604 and GA21 as adequate.

**Comparative assessment**
Comparative analyses of agronomic and phenotypic data from field trials located at representative sites and environments in USA in 2005 indicate that maize stack MIR604 x GA21 is equivalent to its conventional counterpart, with the exception of the insect resistance and the herbicide tolerance, conferred by the mCry3A, PMI and mEPSPS proteins. The field evaluations support a conclusion of no phenotypic changes indicative of increased plant weed/pest potential of maize MIR604 x GA21 compared to conventional maize varieties.

The applicant has performed a compositional analysis on the triple-stack Bt11 x MIR604 x GA21 instead of maize MIR604 x GA21. The analysis was performed on plant materials from maize Bt11 x MIR604 x GA21 and a near-isogenic control hybrid from field trials in USA in 2006. With the exception of small intermittent variations, no biologically significant compositional differences were found between the triple-stack and the near-isogenic control. The results of the study are considered valid by EFSA also for maize MIR604 x GA21, since maize Bt11 x MIR604 x GA21 encompasses the transgenic properties of maize MIR604 x GA21. This is in accordance with the EFSA guidance document for the risk assessment of genetically modified plants containing stacked transformation events (EFSA 2007b).

The VKM GMO Panel is of the opinion that the applicant should have performed a compositional analysis of maize MIR604 x GA21 and not only referred to analyses of the triple- stack Bt11 x MIR604 x GA21. However, based on all information available, including agronomic and phenotypic data from field trials with maize MIR604 x GA21, a feeding study on broilers showing nutritional equivalence to non-GM maize, and assessments of the single events MIR604 and GA21, the VKM GMO Panel concludes that forage and grain from maize MIR604 x GA21 are compositionally equivalent to its conventional counterpart

**Food and feed risk assessment**
A whole food feeding study on broilers has not indicated any adverse effects of maize MIR604 x GA21, and shows that maize MIR604 x GA21 is nutritionally equivalent to conventional maize. The mCry3A, PMI and mEPSPS, 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 MIR604 x GA21 is nutritionally equivalent to conventional maize varieties. It is unlikely that the mCry3A, PMI or mEPSPS proteins will introduce a toxic or allergenic potential in food or feed based on maize MIR604 x GA21 compared to conventional maize.

**Environmental risk assessment**
The scope of the application EFSA/GMO/UK/2007/48 includes import and processing of maize stack MIR604 x GA21 for food and feed uses. Considering the intended uses of maize 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 MIR604 x GA21.

Maize 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 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 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 MIR604 x GA21 is nutritionally equivalent to conventional maize varieties. It is unlikely that the mCry3A, PMI or mEPSPS proteins will introduce a toxic or allergenic potential in food or feed based on maize MIR604 x GA21 compared to conventional maize.

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

# References

Andreadis S (2011) Origin and taxonomic status of the Palearctic population of the stem borer *Sesamia nonagiodes* (Lefèbvre) Lepidoptera: Noctuidae. Biological Journal of the Linnean Society 103: 904–922

BEETLE report (2009). Long term effects of genetically modified (GM) crops on health and the environment (including biodiversity): prioritization of potential risks and delimitation of uncertainties. German Federal Office of Consumer Protection and Food Safety, BLaU-Umweltstudien and Genius GmbH. http://ec.europa.eu/environment/biotechnology/pdf/beetle_report.pdf

Bensasson D, Boore JL, Nielsen KM (2004) Genes without frontiers. Heredity 92: 483-489

Betz FS, Hammond BG, Fuchs RL (2000) Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests. Regul. Toxicol. Pharmacol. 32; 156–173.

Brandtzaeg P, Tolo K (1977) Mucosal penetrability enhanced by serum-derived antibodies. Nature 266: 262-263.

Brooks KJ, DeWildt PM (2000a) PS149B1 14KDa protein: Acute oral toxicity study in CD1 mice. Study number 001128. Unpublished technical report. Dow Chemical company.

Brooks KJ, DeWildt PM (2000b) PS149B1 14 kDa protein: acute oral toxicity study in CD-1 mice. Study number 001130. Unpublished technical report. Dow AgroSciences LLC

Brooks KJ, DeWildt PM (2000c) PS149B1 44 kDa protein: acute oral toxicity study in CD-1 mice. Study number 001129. Unpublished technical report. Dow AgroSciences LLC

Brooks KJ, DeWildt PM (2000d) PS149B1 14 kDa and 44 kDa proteins: acute oral toxicity study in CD-1 mice. Study number 001128. Unpublished technical report. Dow AgroSciences LLC

Chao E, Krewski D (2008) A risk-based classification scheme for genetically modified foods I: Conceptual development. Regul. Toxicol. Pharmacol. 52: 208–222.

Cressman RF (2003) Evaluation of the sequence similarities of the Cry34Ab1, Cry35Ab1, and PAT proteins the public protein sequence dataset. Pioneer Hi-Bred International, Inc.

Codex Alimentarius (2003) Guideline for the Conduct of Food Safety Assessment of Foods Derived From Recombinant-DNA Plants. CAC/GL 45-2003. Food and Agriculture Organization, Codex Alimentarius Commission, Rome.

de Vries J, Wackernagel W (2002) Integration of foreign DNA during natural transformation of Acinetobacter sp. by homology-facilitated illegitimate recombination. The Proceedings of the National Academy of Sciences USA 99: 2094-2099

Devos Y, Demont M, Dillen K, Reheul D, Kaiser M, Sanvido O (2009) The coexistence of genetically modified (GM) and non-GM crops in the European Union. Agronomy for Sustainable Development 29: 11-30

Dien BS, Bothast RJ,. Iten LB, Barrios L, Eckhoff SR (2002) Fate of Bt Protein and Influence of Corn Hybrid on Ethanol Production. Cereal Chem 79(4): 582-585.

Eastham K, Sweet J (2002) Genetically modified organisms (GMO): The significance of gene flow through pollen transfer. Environmental issue report. No 28. European Environment Agency (EEA), Copenhagen. http://reports.eea.eu.int/environmental_issue_report_2002_28/en

EFSA (2004) Opinion of the Scientific Panel on Genetically Modified Organisms on the use of antibiotic resistance genes as marker genes in genetically modified plants. The EFSA Journal, 48, 1-18. http://www.efsa.europa.eu/en/science/gmo/gmo_opinions/384.html.

EFSA (2006) Guidance document of the Scientific Panel on Genetically Modified Organisms for the risk assessment of genetically modified plants and derived food and feed. ISBN: 92-9199-019-1. European Food Safety Authority, Parma, Italy. 100 p. http://www.efsa.europa.eu/en/science/gmo/gmo_guidance/660.html

EFSA (2007) Opinion of the Scientific Panel on Genetically Modified Organisms on applications (references EFSA-GMO-UK-2005-19 and EFSA-GMO-RX-GA21) for the placing on the market of glyphosate-tolerant genetically modified maize GA21, for food and feed uses, import and processing and for renewal of the authorisation of maize GA21 as existing product, both under Regulation (EC) No 1829/2003 from Syngenta Seeds S.A.S. on behalf of Syngenta Crop Protection AG. The EFSA Journal 541, 1-25. http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/gmo_op_ej541_GA21Maize_en.pdf

EFSA (2009) Use of antibiotic resistance genes as marker genes in genetically modified plants. Scientific Opinion of the Panel on Genetically Modified Organisms (GMO) and the Panel on Biological Hazards (BIOHAZ). The EFSA Journal 1034: 1-82 http://www.efsa.europa.eu/cs/BlobServer/Statement/gmo_biohaz_st_ej1108_ConsolidatedARG_en.pdf?ssbinary=true

EFSA (2010) Guidance on the environmental risk assessment of genetically modified plants. Scientific option from the EFSA Panel on Genetically Modified Organisms (GMO). The EFSA Journal 8 (11):1-111 http://www.efsa.europa.eu/en/efsajournal/doc/1879.pdf

EFSA (2011a) Guidance for risk assessment of food and feed from genetically modified plants. The EFSA Journal 9(5): 2150. http://www.efsa.europa.eu/en/efsajournal/doc/2150.pdf

EFSA (2011b) EFSA Panel on Genetically Modified Organisms (GMO). Scientific Opinion on Guidance on selection of comparators for the risk assessment of genetically modified plants and derived food and feed. EFSA Journal 9(5):2149

EFSA (2011c) Guidance on the Post-Market Environmental Monitoring (PMEM) of genetically modified plants. The EFSA Journal 9(8):2316

EFSA (2013) Scientific Opinion on an application from Pioneer Hi-Bred International and Dow AgroSciences LLC (EFSA-GMO-NL-2005-23) for placing on the market of genetically modified maize 59122 for food and feed uses, import, processing and cultivation under Regulation (EC) No 1829/20031. The EFSA Journal 11: 3135 http://www.efsa.europa.eu/en/search/doc/3135.pdf

Erickson GE, Robbins ND, Simon JJ, Berger LL, Klopfenstein TJ, Stanisiewski EP, Hartnell GF (2003) Effect of feeding glyphosate-tolerant (Roundup-Ready events GA21 or nk603) corn compared with reference hybrids on feedlot steer performance and carcass characteristics. J. Anim. Sci.81:2600-2608.

Fard NA, Minuchehr Z, Mousawi A (2013) Allergenicity study of genetically modified herbicide resistant crops (Bioinformatics Assessment). Bull. Env. Pharmacol. Life Sci. 2 (3): 24-32.

FAO/WHO, (2001) Evaluation of allergenicity of genetically modified foods. In: Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology. Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO), January 22–25, Rome, Italy.

Fernandez A, Mills EN, Lovik M, Spoek A, Germini A, Mikalsen A, Wal JM (2013) Endogenous allergens and compositional analysis in the allergenicity assessment of genetically modified plants. Food Chem Toxicol. 16(62C):1-6. doi: 10.1016/j.fct.2013.08.023.

Folmer, J. D., R. J. Grant, C. T. Milton, and J. Beck. (2002). Utilization of Bt corn residues by grazing beef steers and Bt corn silage and grain by growing beef cattle and lactating dairy cows. J. Anim. Sci. 80:1352–1361.

Gruber S, Colbach N, Barbottin A, Pekrun C (2008) Post-harvest gene escape and approaches for minimizing it. Cab reviews: Perspective in Agriculture, Veterinary Science, Nutrition and Natural Resources, 3, No. 015, 17 pp.

Guertler P, Lutz B, Kuehn R, Meyer HHD, Einspanier R, Killermann B, Albrecht C (2008) Fate of recombinant DNA and Cry1Ab protein after ingestion and dispersal of genetically modified maize in comparison to rapeseed by fallow deer (*Dama dama*). European Journal of Wildlife Research 54: 36–43.

Hammond, B. G., Dudek, R., Lemen, J. K., Nemeth, M.A. (2006). Results of a 90-day safety assurance study with rats fed grain from corn borer-protected corn. Food and Chemical Toxicology 44(7): 1092-1099.

Hammond RG, Jez JM (2011) Impact of food processing on the safety assessment for proteins introduced into biotechnology-derived soybean and corn crops. Food Chem. Toxicol. 49;711-721.

Haryu Y, Taguchi Y, Itakura E, Mikami O, Miura K, Saeki T, Nakajima Y, (2009). Longterm biosafety assessment of a genetically modified (GM) plant: the genetically modified (GM) insect-resistant Bt11 corn does not affect the performance of multi-generations or life span of mice. Open Plant Science Journal 3, 49 – 53.

Icoz, Stotzky G (2008) Fate and effects of insect-resistant Bt crops in soil ecosystems. Soil Biology and Biochemistry 40: 559–586

Jung, H.G., Sheaffer, C.C. (2004) Influence of *Bt* transgenes on cell wall lignification and digestibility of maize stover for silage. Crop Science 44:1781-1789.

Kim J-H, Seo Y-J, Kim J-Y, Han Y-S, Lee K-S, et al. (2010) Allergenicity Assessment of Cry Proteins in Insect-resistant Genetically Modified Maize Bt11, MON810, and MON863. Food Sci Biotechnol 18: 1273–1278.

König A., Cockburn, A., Crevel, R.W.R., Debruyne, E., Grafstroem, R., Hammerling, U., Kimber, I., Knudsen, I., Kuiper, H.A., Peijnenburg, A.A.C.M., Penninks, A.H., Poulsen, M.,

Schauzu, M., Wal, J.M., (2004) Assessment of the safety of foods derived from genetically modified (GM) crops. Food Chem. Toxicol. 42: 1047–1088.

Lid J, Lid DT (2005) Norsk flora. Det Norske Samlaget, Oslo. 7. utgave. 1230s

Lim PL, Rowley D (1982) The effect of antibody on the intestinal absorption of macromolecules and on the intestinal permeability in adult mice. Int. Archs. Allergy Appl. Immun. 68: 41-46.

Lutz B, Wiedermann S, Einspanier R, Mayer J, Albrecht C (2005) Degradation of Cry1Ab protein from genetically modified maize in the bovine gastrointestinal tract. Journal of Agricultural and Food Chemistry 53: 1453–1456

Mesnage R., Clair E., Gress S., Then C., Székács A., Séralini G.-E. (2012) Cytotoxicity on human cells of mCry3A and Cry1Ac Bt insecticidal toxins alone or with a glyphosate-based herbicide. J. Appl. Toxicol. 33(7);695-699.

Meyer T (1999) Comparison of amino acid sequence similarity of Cry1F and PAT proteins to known allergen proteins. Pioneer Hi-Bred International, Inc

Netherwood T, Martín-Orúe SM, O'Donnell AG, Gockling S, Graham J, Mathers JC, Gilbert HJ (2004) Assessing the survival of transgenic plant DNA in the human gastrointestinal tract. Nature Biotechnology 22: 204-209

Nielsen KM, van Elsas JD, Smalla K. (2000) Transformation of *Acinetobacter* sp. 13(pFG4delta*nptII*) with transgenic plant DNA in soil microcosms and effects of kanamycin on selection of transformants. Applied Environmental Microbiology 66: 1237-42

Nielsen KM (2003) An assessment of factors affecting the likelihood of horizontal transfer of recombinant plant DNA to bacterial recipients in the soil and rhizosphere. Collection of Biosafety Reviews 1: 96-149

Nielsen KM, Townsend J P (2004) Monitoring and modeling horizontal gene transfer. Nature Biotechnology 22(9):1110-1114

OECD (2002) Consensus document on compositional considerations for new varieties of maize (*Zea mays*): Key food and feed nutrients, anti nutrients and secondary plant metabolites. Organisation for Economic Co-operation and Development, Paris. ENV/JM/MONO(2002)25

OECD (2003) Consensus Document on the biology of *Zea mays* subsp. *Mays* (Maize). Series on Harmonisation of Regulatory Oversight in Biotechnology (ENV/JM/MONO, No. 27, 1-9

Onose, J-i., Imai, T., Hasumura, M., Ueda, M., Ozeki, Y. and Hirose, M. (2008) Evaluation of subchronic toxicity of dietary administrated mCry3A protein from *Bacillus thuringiensis* var *kurustaki* HD-1 in F344 male rats with chemically induced gastrointestinal impairment. *Food and Chemical Toxicology* 46:2184-2189.

Panda R, Ariyarathna H, Amnuaycheewa P, Tetteh A, Pramod SN, Taylor SL, Ballmer-Weber BK, Goodman RE (2013) Challenges in testing genetically modified crops for potential increases in endogenous allergen expression for safety. Allergy 68: 142–151.

Palaudelmás M, Peñas G, Melé E, Serra J, Salvia J, Pla, M-, Nadal A, Messeguer J (2009) Effect of volunteers on maize gene flow. Transgenic Research 18: 583-594

Pfister T, Schmid H, Luetkemeier H, Biedermann K, Weber K (1996) PAT protein repeated dose oral toxicity (14-day feeding) study in rats.

Poulsen LK (2004) Allergy assessment of foods or ingredients derived from biotechnology, gene-modified organisms, or novel foods. Mol. Nutr. Food Res. 48: 413 – 423.

Randhawa GJ, Singh M, Grover M (2011) Bioinformatic analysis for allergenicity assessment of *Bacillus thuringiensis* Cry proteins expressed in insect-resistant food crops. Food Chem Toxicol 49:356-362.

Raybould A, Kilby P, Graser G. (2013) Characterising microbial protein test substances and establishing their equivalence with plant-produced proteins for use in risk assessments of transgenic crops. Transgenic Res 22:445–460. DOI 10.1007/s11248-012-9658-3

Rizzi AN, Raddadi C, Sorlini L, Nordgård L, Nielsen KM, Daffonchio D ( 2012) The stability and degradation of dietary DNA in the gastrointestinal tract of mammals - implications for horizontal gene transfer and the biosafety of GMOs. Crit. Rev. Food Science Nutr. 52:142-161

Schubbert GW, Lettmann C, Doerfler W (1994) Ingested foreign (phage M13) DNA survives transiently in the gastrointestinal tract and enters the bloodstream of mice. Molecular & general Genetics 242: 495-504

Shimada, N., Miyamoto, K., Kanda, K. and Murata, H. (2006a) *Bacillus thuringiensis* insecticidal mCry3A toxin does not affect the membrane integrity of the mammalian intestinal epithelial cells: an in vitro study. In vitro Cell. Dev. Biol. - Animal, 42 (1 & 2); 45-49.

Shimada, N., Miyamoto, K., Kanda, K.Murata, H. (2006b) Binding of mCry3A toxin, a *Bacillus thuringiensis* insecticidal toxin, to proteins of the bovine intestinal epithelial cell: An in vitro study. Appl. Entomol. Zool. 41(2);295-301.

Shimada, N., Murata, H., Mikami O, Yoshioka M, Guruge KS, Yamanaka N, Nakajima Y, Miyazaki S (2006c) Effects of feeding calves genetically modified corn Bt11: A clinic-biochemical study. J. Vet. Med. Sci. 68(10);1113-1115.

Shimada N, Murata H, Miyazaki S. (2008) Safety evaluation of Bt plants. JARQ 42(4); 251-259.¨

Sidhu, RS, Hammond BG, Fuchs RL, Mutz JN, Holden LR, George B, Olson T (2000) Glyphosate-tolerant corn: The composition and feeding value of grain from glyphosatetolerant corn is equivalent to that of conventional corn (*Zea mays* L.). J. Agric. Food Chem. 48:2305-2312.

US EPA (1996) *Bacillus thuringiensis* CryIA(b) Delta-Endotoxin and the genetic material necessary for its production in all plants; Exemption from requirement of a tolerance.

US EPA (1998) 6%?R Reregistration Eligibility Decision (RED) *Bacillus thuringiensis*.

US EPA (2010) Biopesticides registration action document. mCry3A and Cry1F *Bacillus thuringiensis* (*Bt*) Corn Plant-Incorporated Protectants. http://www.epa.gov/pesticides/biopesticides/pips/cry1f-cry1ab-brad.pdf

van de Wiel CCM, van den Brink L, Bus CB, Riemens MM, Lotz LAP (2011) Crop volunteers and climatic change. Effects of future climatic change on the occurrence of maize, sugar beet and potato in the Netherlands. COGEM report (CGM 2011-11).

Vazquez, R.I., Moreno Fierros, L., Neri Bazan, L., de la Riva, G.A., Lopez Revilla, R. (1999) *Bacillus thuringiensis* Cry1Ac protoxin is a potent systemic and mucosal adjuvant. Scandinavian Journal of Immunology, 49, 578-584.

Vermeire T, van de Bovenkamp M, de Bruin YB, Delmaar C, van Ehgelen J, Escher S, Marquart H, Meijsyer T (2010) Exposure based waiving under REACH. Regul. Toxicol. Pharmacol. 58 (3), 408–420.

Vijayakumar KR, Martin A, Gowda LR, Prakash V (2009) Detection of genetically modified soya and maize: Impact of heat processing. Food Chem 117: 514-521

Vikse R (2009) Betydning av mais i kostholdet i Norge. Unpublished report

VKM (2005a) Uttalelse om Syngenta Seeds genmodifiserte mais MIR604 (EFSA/GMO/UK/2005/11) http://www.vkm.no/dav/9ae41eee9e.pdf

VKM (2005b) Vurdering av Syngenta Seeds genmodifiserte åkermais GA21 (C/F/96/05-10) og EFSA GA21-søknad EFSA/GMO/UK/2005/19. http://www.vkm.no/dav/86543f0487.pdf

VKM (2005c) Report from an *Ad Hoc* Group appointed by the Norwegian Scientific Panel on Genetically Modified Organisms and Panel on Biological Hazards – An assessment on potentially long-term health effects caused by antibiotic resistance marker genes in genetically modified organisms based on antibiotic usage and resistance patterns in Norway. Opinion 05/302-1-final. Norwegian Scientific Committee for Food Safety, Oslo, Norway. 62 p. http://www.vkm.no/eway/default.aspx?pid=277&trg=Content_6500&Main_6177=6500:0:31,2296&Content_6500=6187:1685412::0:6295:14:::0:0

VKM (2009) Helse- og miljørisikovurdering genmodifisert maishybrid MIR604 x GA21 fra Syngenta Seeds Inc. (EFSA/GMO/UK/2007/48). http://www.vkm.no/dav/116ebd3dc5.pdf

VKM (2012) Helserisikovurdering av Cry-proteiners adjuvanseffekter. Uttalelse fra Faggruppe 3 i Vitenskapskomiteen for mattrygghet. http://www.vkm.no/dav/d6991c1718.pdf