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Assessment of genetically modified maize NK603 × T25 × DAS-40278-9 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2019-164)

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Abstract

Maize NK603 × T25 × DAS-40278-9 (three-event stack maize) was produced by conventional crossing to combine three single events: NK603, T25 and DAS-40278-9. The GMO Panel previously assessed the three single maize events and two of the subcombinations and did not identify safety concerns. No new data on the single maize events or the two subcombinations were identified that could lead to modification of the original conclusions on their safety. The molecular characterisation, comparative analysis (agronomic, phenotypic and compositional characteristics) and the outcome of the toxicological, allergenicity and nutritional assessment indicate that the combination of the single maize events and of the newly expressed proteins in the three-event stack maize does not give rise to food and feed safety and nutritional concerns. The GMO Panel concludes that the three-event stack maize, as described in this application, is as safe as the non-GM comparator and the selected non-GM reference varieties. In the case of accidental release of viable grains of the three-event stack maize into the environment, this would not raise environmental safety concerns. The GMO Panel assessed the likelihood of interactions among the single events in one of the maize subcombinations not previously assessed and concludes that these are expected to be as safe as the single events, the previously assessed subcombinations and the three-event stack maize. The post-market environmental monitoring plan and reporting intervals are in line with the intended uses of the three-event stack maize. Post-market monitoring of food/feed is not considered necessary. The GMO Panel concludes that the three-event stack maize and its subcombinations are as safe as the non-GM comparator and the selected non-GM reference varieties with respect to potential effects on human and animal health and the environment.

Keywords: GMO, herbicide tolerant, maize (*Zea mays*), NK603, T25, DAS-40278-9, import and processing

Requestor: Competent Authority of The Netherlands

Question number: EFSA-Q-2019-00808

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Summary

Following the submission of application EFSA-GMO-NL-2019-164 under Regulation (EC) No 1829/2003 from Pioneer Hi-Bred International, Inc. as represented by Pioneer Overseas Corporation (referred to hereafter as 'the applicant'), the Panel on Genetically Modified Organisms of the European Food Safety Authority (EFSA) (referred to hereafter as 'GMO Panel') was asked to deliver a Scientific Opinion on the safety of genetically modified (GM) glufosinate, glyphosate, 2,4-dichlorophenoxyacetic acid (2,4-D) and aryloxyphenoxypropionate (AOPP) herbicides tolerant maize (*Zea mays* L.) NK603 × T25 × DAS-40278-9 (referred to hereafter as 'three-event stack maize') and its subcombinations independently of their origin, according to Regulation (EU) No 503/2013 (referred to hereafter as 'subcombinations'). The scope of application EFSA-GMO-NL-2019-164 is for import, processing and food and feed uses within the European Union (EU) of maize NK603 × T25 × DAS-440278-9 and all its subcombinations independently of their origin, and does not include cultivation in the EU.

The term 'subcombination' refers to any combination of up to two of the events present in the three-event stack maize. The safety of subcombinations occurring as segregating progeny in the harvested grains of maize NK603 × T25 × DAS-40278-9 is evaluated in the context of the assessment of the three-event stack maize. The safety of subcombinations that have either been or could be produced by conventional crossing through targeted breeding approaches, and which can be bred, produced and marketed independently of the three-event stack, are risk assessed separately in the present scientific opinion.

The three-event stack maize was produced by conventional crossing to combine three single maize events: NK603 expressing CP4 EPSPS and CP4 EPSPS L214P to confer tolerance to glyphosate-containing herbicides; T25 expressing PAT to confer tolerance to glufosinate-ammonium containing herbicides; and DAS-40278-9 expressing AAD-1 to catalyse the degradation of the general class of herbicides known as aryloxyphenoxypropionates (AOPP) and to confer tolerance to 2,4-D containing herbicides.

The GMO Panel evaluated the three-event stack maize and its subcombinations with reference to the scope and appropriate principles described in its applicable guidelines for the risk assessment of GM plants and the post-market environmental monitoring. The GMO Panel considered the information submitted in application EFSA-GMO-NL-2019-164, additional information provided by the applicant during the risk assessment, the scientific comments submitted by the Member States and the relevant scientific literature.

For application EFSA-GMO-NL-2019-164, previous assessments of the three single events (NK603, T25 and DAS-40278-9), and three of the subcombinations provided a basis for the assessment of the three-event stack maize and all its subcombinations. No safety concerns were identified by the GMO Panel in the previous assessments. No safety issue concerning the three single maize events was identified by the updated bioinformatic analyses, nor reported by the applicant since the publication of the previous GMO Panel scientific opinions. Therefore, the GMO Panel considers that its previous conclusions on the safety of the single maize events remain valid.

For the three-event stack maize, the risk assessment included the molecular characterisation of the inserted DNA and analysis of protein expression. An evaluation of the comparative analysis of agronomic, phenotypic and compositional characteristics was carried out, and the safety of the newly expressed proteins and the whole food and feed were evaluated with respect to potential toxicity, allergenicity and nutritional characteristics. Environmental impacts and post-market environmental monitoring (PMEM) plan were also evaluated.

The molecular characterisation data establish that the events stacked in maize NK603 × T15 × DAS-40278-9 have retained their integrity. Protein expression analysis showed that the levels of the newly expressed proteins are similar in the three-event stack maize and in the single events.

The comparative analysis of agronomic and phenotypic characteristics and grain and forage composition identified no differences between maize NK603 × T25 × DAS-40278-9 and the non-GM comparator that required further assessment except for the change in plant lodging. This change was further assessed for environmental impact and raised no concern.

The molecular characterisation, the comparative analysis and the outcome of the toxicological, allergenicity and nutritional assessment indicate that the combination of the single maize events and of the newly expressed proteins in the three-event stack maize does not give rise to food and feed safety and nutritional concerns. The GMO Panel concludes that maize NK603 × T25 × DAS-40278-9, as

described in this application, is as safe as the non-GM comparator and the selected commercial non-GM maize reference varieties (referred to hereafter as non-GM reference varieties).

Considering the combined events and their potential interactions, the outcome of the comparative analysis, and the routes and levels of exposure, the GMO Panel concludes that maize NK603 × T25 × DAS-40278-9 would not raise safety concerns in the case of accidental release of viable GM maize grains into the environment.

Since no new safety concerns were identified for the two previously assessed subcombinations, and no new data leading to the modification of the original conclusions on safety were identified, the GMO Panel considers that its previous conclusions on these maize subcombinations remain valid. For the remaining subcombination included in the scope of application EFSA-GMO-NL-2019-164, no experimental data were provided. The GMO Panel assessed the possibility of interactions between the events in this subcombination and concludes that this subcombination would not raise safety concerns. This subcombination is therefore expected to be as safe as the single events, the previously assessed subcombinations and the three-event stack maize.

Given the absence of safety concerns for foods and feeds from maize NK603 × T25 × DAS-40278-9 and its subcombinations, the GMO Panel considers that post-market monitoring of these products is not necessary. The PMEM plan and reporting intervals are in line with the intended uses of the three-event stack maize and its subcombinations. The literature searches did not identify any relevant publications on maize NK603 × T25 × DAS-40278-9. In the context of annual PMEM reports, the applicant could further fine-tune future literature searches according to the GMO Panel recommendations provided in this scientific opinion.

The GMO Panel concludes that maize NK603 × T25 × DAS-40278-9 and its subcombinations, as described in this application, are as safe as the non-GM comparator and the selected non-GM reference varieties with respect to potential effects on human and animal health and the environment.

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1. Introduction

The scope of application EFSA-GMO-NL-2019-164 is for food and feed uses, import and processing in the European Union (EU) of the genetically modified (GM) herbicide-tolerant maize NK603 × T25 × DAS-40278-9 and all its subcombinations independently of their origin.

1.1. Background

On 13 December 2019, the European Food Safety Authority (EFSA) received from the Competent Authority of The Netherlands application EFSA-GMO-NL-2019-164 for authorisation of maize NK603 × T25 × DAS-40278-9 (hereafter referred to as 'the three-event stack maize') (Unique Identifier MON-ØØ6Ø3-6 × ACS-ZMØØ3-2 × DAS-4Ø278-9), submitted by Pioneer Hi-Bred International, Inc. as represented by Pioneer Overseas Corporation (hereafter referred to as 'the applicant') according to Regulation (EC) No 1829/2003[1].

Following receipt of application EFSA-GMO-NL-2019-164, EFSA informed EU Member States and the European Commission, and made the application available to them. Simultaneously, EFSA published the summary of the application.[2]

EFSA checked the application for compliance with the relevant requirements of Regulation (EC) No 1829/2003 and Regulation (EU) No 503/2013[3] and, when needed, asked the applicant to supplement the initial application. On 26 March 2020, EFSA declared the application valid.

From the validity date, EFSA and its scientific Panel on Genetically Modified Organisms (hereafter referred to as 'the GMO Panel') endeavoured to respect a time limit of 6 months to issue a scientific opinion on application EFSA-GMO-NL-2019-164. Such time limit was extended whenever EFSA and/or its GMO Panel requested supplementary information to the applicant. According to Regulation (EC) No 1829/2003, any supplementary information provided by the applicant during the risk assessment was made available to the EU Member States and European Commission (for further details, see the Section 'Documentation', below).

In accordance with Regulation (EC) No 1829/2003, EFSA consulted the nominated risk assessment bodies of EU Member States, including national Competent Authorities within the meaning of Directive 2001/18/EC[4]. The EU Member States had 3 months to make their opinion known on application EFSA-GMO-NL-2019-164 as of date of validity.

1.2. Terms of Reference as provided by the requestor

According to Articles 6 and 18 of Regulation (EC) No 1829/2003, EFSA and its GMO Panel were requested to carry out a scientific risk assessment of maize NK603 × T25 × DAS-40278-9 and all its subcombinations independently of their origin according to the scope of application EFSA-GMO-NL-2019-164.

According to Regulation (EC) No 1829/2003, this scientific opinion is to be seen as the report requested under Articles 6(6) and 18(6) of that Regulation including the opinions of the nominated risk assessment bodies of EU Member States.[5]

In addition to the present scientific opinion, EFSA and its GMO Panel were also asked to report on the particulars listed under Articles 6(5) and 18(5) of Regulation (EC) No 1829/2003. The relevant information is made available in Open. EFSA,[6] including the information required under Annex II to the Cartagena Protocol, a labelling proposal, a post-market environmental monitoring (PMEM) plan as provided by the applicant; the methods, validated by the European Union Reference Laboratory, for detection, including sampling, identification of the transformation events in the food-feed and/or foods-feeds produced from it and the appropriate reference materials.

[1] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. OJ L 268, 18.10.2003, p. 1–23.
[2] Available online: https://open.efsa.europa.eu/questions/EFSA-Q-2019-0080
[3] Commission Implementing Regulation (EU) No 503/2013 of 3 April 2013 on applications for authorization of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006. OJ L157, 8.6.2013, p. 1–48.
[4] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. OJ L 106, 12.3.2001, p. 1–38.
[5] Opinions of the nominated risk assessment bodies of EU Member States can be found at https://open.efsa.europa.eu/questions/EFSA-Q-2019-00808
[6] https://open.efsa.europa.eu/questions/EFSA-Q-2019-00808

2. Data and methodologies

2.1. Data

The GMO Panel based its scientific assessment of the three-event stack maize on the valid application EFSA-GMO-NL-2019-164, additional information provided by the applicant during the risk assessment, relevant scientific comments submitted by EU Member States and relevant peer-reviewed scientific publications. As part of this comprehensive information package, the GMO Panel received an additional unpublished study submitted by the applicant in order to comply with the specific provisions of Regulation (EU) No 503/2013. This additional unpublished study is provided in Appendix A.

2.2. Methodologies

The GMO Panel conducted its assessment in line with the principles described in Regulation (EU) No 1829/2003, the applicable guidelines (i.e. EFSA GMO Panel, 2010a,2011a,b,2015a; EFSA Scientific Committee, 2011) and explanatory notes and statements (i.e. EFSA GMO Panel, 2010b; EFSA, 2010, 2014, 2017a,b, 2019a,b) for the risk assessment of GM plants.

For this application, in the context of the contracts OC/EFSA/GMO/2018/04, OC/EFSA/GMO/2018/02 and EOI/EFSA/SCIENCE/2020/01 – CT02GMO the contractors performed preparatory work for the evaluation of the applicant`s literature search methods applied for the statistical analysis and statistical analysis of the 90-day toxicity study on T25 maize.

3. Assessment

3.1. Introduction

Application EFSA-GMO-NL-2019-164 covers the three-event stack maize NK603 × T25 × DAS-40278-9 and all its three subcombinations independently of their origin (Table 1).

Table 1: Stacked maize events covered by the scope of application EFSA-GMO-NL-2019-164

Degree of stacking	Event	Unique identifiers
3-event stack	NK603 × T25 × DAS-40278-9	MON-ØØ6Ø3-6 × ACS-ZMØØ3-2 × DAS-4Ø278-9
2-event stack	NK603 × T25	MON-ØØ6Ø3-6 × ACS-ZMØØ3-2
	NK603 × DAS-40278-9	MON-ØØ6Ø3-6 × DAS-4Ø278-9
	T25 × DAS-40278-9	DAS-4Ø278-9 × ACS-ZMØØ3-2

The term 'subcombination' refers to any combination of up to two of the maize events NK603, T25 and DAS-40278-9.

The safety of subcombinations occurring as segregating progeny in harvested grains of maize NK603 × T25 × DAS-40278-9 is evaluated in the context of the assessment of the three-event stack maize in Section 3.5 of the present scientific opinion.

'Subcombination' also covers combinations that have either been or could be produced by conventional crossing through targeted breeding approaches (EFSA GMO Panel, 2011a). These are maize stacks that can be bred, produced and marketed independently of the three-event stack maize. These subcombinations are assessed in Section 3.5 of this scientific opinion.

The three-event stack maize was produced by conventional crossing to combine three single maize events: NK603 expressing CP4 EPSPS and CP4 EPSPS L214P to confer tolerance to glyphosate-containing herbicides; T25 expressing PAT to confer tolerance to glufosinate-ammonium-containing herbicides; and DAS-40278-9 expressing AAD-1 to catalyse the degradation of the general class of herbicides known as aryloxyphenoxypropionates (AOPP) and to confer tolerance to 2,4-D containing herbicides.

All three single events were assessed previously (see Table 2) and no concerns for human and animal health or environmental safety were identified.

Table 2: Single maize events and subcombinations of maize NK603 × T25 × DAS-40278-9 previously assessed by the GMO Panel

Event	Application or mandate	EFSA Scientific Opinion
NK603	EFSA-GMO-NL-2005-22	EFSA GMO Panel (2009)
	Art4_NK603	EFSA (2004)
	CE/ES/00/01	EFSA GMO Panel (2007)
	EFSA-GMO-RX-NK603	EFSA GMO Panel (2009)
T25	EFSA-GMO-NL-2007-46	EFSA GMO Panel (2013)
	EFSA-GMO-RX-T25	EFSA GMO Panel (2013)
DAS-40278-9	EFSA-GMO-NL-2010-89	EFSA GMO Panel (2016)
NK603 × T25	EFSA-GMO-NL-2010-80	EFSA GMO Panel (2015b)
NK603 × DAS-40278-9	EFSA-GMO- NL-2013-112	EFSA GMO Panel (2019a)

3.2. Updated information on single events

Since publication of the scientific opinions on the single maize events by the GMO Panel (see Table 2), no safety issue concerning the three single events has been reported by the applicant. According to the Implementing Regulation EC 503/2013, sequencing information of the three events provided in the stack was assessed and confirms the originally submitted sequences of the single events (Section 3.4.1.2).

Updated bioinformatic analysis of the junction regions for maize events NK603, T25 and DAS-40278-9 confirmed that no known endogenous genes were disrupted by any of the inserts.

Updated bioinformatic analyses of the amino acid sequence of the newly expressed CP4 EPSPS, CP4 EPSPS L214P, PAT and AAD-1 proteins confirmed previous results indicating no significant similarities to known toxins and allergens. Updated bioinformatic analyses of the newly created open reading frames (ORFs) within the inserts or spanning the junctions between the insert and the flanking regions for events NK603, T25 and DAS-40278-9 confirmed previous analyses (Table 2). These analyses indicate that the production of a new peptide showing significant similarities to toxins or allergens for any of the events in maize NK603 × T25 × DAS-40278-9 is highly unlikely.

In order to assess the possibility for horizontal gene transfer (HGT) by homologous recombination (HR), the applicant performed a sequence identity analysis with microbial DNA for events NK603, T25 and DAS-40278-9. The likelihood and potential consequences of plant-to-bacteria gene transfer are described in Section 3.4.4.2.

Based on the above information, the GMO Panel considers that its previous conclusions on the safety of the single maize events remain valid.

3.3. Systematic literature review

The GMO Panel assessed the applicant's literature searches on maize NK603 × T25 × DAS-40278-9, which include a scoping review, according to the guidelines given in EFSA (2010, 2019b).

A systematic review as referred to in Regulation (EU) No 503/2013 has not been provided in support to the risk assessment of application EFSA-GMO-NL-2019-164. Based on the outcome of the scoping review, the GMO Panel agrees that there is limited value of undertaking a systematic review for maize NK603 × T25 × DAS-40278-9 at present.

The GMO Panel considered the overall quality of the performed literature searches is acceptable.

The literature searches identified no relevant publications on maize NK603 × T25 × DAS-40278-9.

3.4. Risk assessment of the three-event stack maize NK603 × T25 × DAS-40278-9

3.4.1. Molecular characterisation[7]

In line with the requirements laid down by Regulation (EU) 503/2013, the possible impact of the combination of the events on the integrity of the events, the expression levels of the newly expressed proteins or the biological functions conferred by the individual inserts are considered below.

[7] Dossier: Part II – Section 1.2 and additional information 7/8/2020, 30/11/2020, and 1/10/2021.

3.4.1.1. Genetic elements and their biological function[8]

Maize events NK603, T25 and DAS-40278-9 were combined by conventional crossing to produce the three-event stack maize NK603 × T25 × DAS-40278-9. The structure of the inserts introduced into the three-event stack maize is described in detail in the respective EFSA scientific opinions (Table 2) and no new genetic modifications were involved. Genetic elements in the expression cassettes of the single events are summarised in Table 3.

Intended effects of the inserts in maize NK603 × T25 × DAS-40278-9 are summarised in Table 4. Based on the known biological function of the newly expressed proteins (Table 4), no foreseen interactions at the biological level are identified.

Table 3: Genetic elements in the expression cassettes of the events stacked in maize NK603 × T25 × DAS-40278-9

Event	Promoter	5′ UTR	Transit peptide	Coding region	Terminator
NK603	*p-ract*1 (*O. sativa*)	*ract*1 (*Oryza sativa*)	CTP2 (*Arabidopsis thaliana*)	CP4 *epsps* (*Agrobacterium* sp.)	*nos* (*Agrobacterium tumefaciens*)
	*e*35S (CaMV)	*Zmhsp70* (*Zea mays*)	CTP2 (*A. thaliana*)	CP4 *epsps l214p* (*Agrobacterium* sp.)	*nos* (*A. tumefaciens*)
T25[(a)]	35S (CaMV)	–	–	*pat* (*Streptomyces viridochromogenes*)	35S (CaMV)
DAS-40278-9	ZmUbi1 (*Zea mays*)	–	–	*aad-1 (Sphingobium herbivorans)*	ZmPer5 (*Zea mays*)

CaMV: cauliflower mosaic virus; CTP: chloroplast transit peptide.
– : no element was specifically introduced to optimise expression.
(a): The event also contains fragments of the following elements: pUC18 cloning vector, the *bla* gene and the *ori*, as well as fragments of the 35S promoter, as described in EFSA-GMO-NL-2010-80.

Table 4: Characteristics and intended effects of the events stacked in maize NK603 × T25 × DAS-40278-9

Event	Protein	Donor organism and biological functions	Intended effects in GM plant
NK603	CP4 EPSPS	Based on a gene from *Agrobacterium* strain CP4 (Barry et al., 2001). 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) is an enzyme involved in the shikimic acid biosynthesis in plants and microorganisms (Hermann, 1995)	Event NK603 expresses the bacterial CP4 EPSPS protein which confers tolerance to glyphosate-containing herbicides as it has lower affinity towards glyphosate than the plant endogenous enzyme
	CP4 EPSPS L124P	Based on a gene from *Agrobacterium* strain CP4 (Barry et al., 2001). 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) is an enzyme involved in the shikimic acid biosynthesis in plants and microorganisms (Hermann, 1995)	Event NK603 expresses also the CP4 EPSPS L214P-this variant, compared to the CP4 EPSPS protein, contains a single amino acid substitution from the leucine to proline at position 214. The two CP4 EPSPS protein variants are structurally and functionally equivalent
T25	PAT	Based on a gene from *Streptomyces viridochromogenes* Tü494. Phosphinothricin-acetyl-transferase (PAT) enzyme acetylates L-glufosinate-ammonium (Thompson et al., 1987; Wohlleben et al., 1988; Eckes et al., 1989).	Event T25 expresses the PAT protein, which confers tolerance to glufosinate ammonium-based herbicides (Droge-Laser et al., 1994).

[8] Dossier: Part II –Section 1.2.1.2.

Event	Protein	Donor organism and biological functions	Intended effects in GM plant
DAS-40278-9	AAD-1	Based on a gene from *Sphingobium herbicidovorans*. Aryloxyalkanoate dioxygenase (AAD-1) facilitates the breakdown of phenoxy auxin and aryloxyphenoxypropionate (AOPP) herbicides into carbon sources for the bacterium (Wright et al., 2009)	Event DAS-40278-9 expresses AAD-1 protein which degrades 2,4-dichlorophenoxyacetic acid (2,4-D) and AOPP class of herbicides and thus confers tolerance to these herbicides.

3.4.1.2. Integrity of the events in the three-event stack maize NK603 × T25 × DAS-40278-9[9,10]

The genetic stability of the inserted DNA over multiple generations in the single maize events NK603, T25 and DAS-40278-9 was demonstrated previously (Table 2). Integrity of these events in maize NK603 × T25 × DAS-40278-9 was demonstrated by sequence analysis showing that the sequences of the events (inserts and their flanking regions) in the three-event maize stack are identical to the sequences originally reported for the three single events, thus confirming that the integrity of these events was maintained in the three-event stack maize. Overall, the quality of the methodology and data sets was assessed by the EFSA GMO Panel and is in compliance to the requirements listed in the EFSA Technical Note (EFSA GMO Panel, 2018).

3.4.1.3. Information on the expression of the inserts[11]

CP4 EPSPS (including variant CP4 EPSPS L214P), PAT and AAD-1 protein levels were analysed by enzyme-linked immunosorbent assay (ELISA) in material harvested from a field trial at six locations: five in the USA and one in Canada in 2018. Samples analysed included leaves (V2-V4, V9 and R1 growth stages), roots (R1 growth stage), pollen (R1 growth stage), forage (R4 growth stage) and grain (R6 growth stage), both those treated and not treated with intended herbicides.

In order to assess the changes in protein expression levels which may result from potential interactions between the events, protein levels were determined for the three-event stack and the corresponding single events in different parts of the plant.

The levels of all the newly expressed proteins in the three-event stack and the corresponding singles were comparable in all tissues (Appendix B). Therefore, there is no indication of an interaction that may affect the levels of the newly expressed proteins in this stack.

3.4.1.4. Conclusions of the molecular characterisation

The molecular data establish that the events stacked in maize NK603 × T25 × DAS-40278-9 have retained their integrity. Protein expression analyses showed that the levels of the newly expressed proteins are similar in the three-event stack maize and in the single events. Therefore, there is no indication of an interaction that may affect the integrity of the events or the levels of the newly expressed proteins in this stack.

Based on the known biological function (Table 4) of the newly expressed proteins, no potential functional interactions among proteins have been identified.

3.4.2. Comparative analysis

3.4.2.1. Overview of studies conducted for the comparative analysis

Application EFSA-GMO-NL-2019-164 presents data on agronomic and phenotypic characteristics, as well as on forage and grain composition of maize NK603 × T25 × DAS-40278-9 (Table 5).

[9] Dossier: Part II–Section 1.2.2.2.
[10] Dossier: Part II–Section 1.2.2.4.
[11] Dossier: Part II–Section 1.2.2.3.

Table 5: Main comparative analysis studies to characterise maize NK603 × T25 × DAS-40278-9 provided in the application EFSA-GMO-NL-2019-164

Study focus	Study details	Comparator	Non-GM reference varieties
Agronomic and phenotypic analysis	Field study, USA and Canada 2018, 12 sites[(a)]	SLB01/PHRDW	20[(b)]
Compositional analysis	Field study, USA 2018, eight sites[(a)]		

GM: Genetically Modified.

(a): The field trials in USA were located two in Iowa, Illinois, Indiana, Minnesota, Nebraska, Pennsylvania and Wisconsin. Four additional sites used only for agronomic and phenotypic analysis were included and were located in USA, one in Iowa, one in Illinois, one is South Dakota and in Canada the site was located in Ontario.

(b): Non-GM maize varieties used in the agronomic, phenotypic and compositional field trials, with their corresponding relative maturity indicated in brackets were 35A52 (107); 35P12 (103); BK5337 (103); BK5883 (108); MPS2R602 (106); MY09V40 (109); P0506 (105); P0589 (105); P0604 (106); P0760 (107); P0928 (109); P0993 (109); PB5385 (103); PB5466 (104); PB5624 (104); PB5646 (106); PRE1032 (103); XL5234 (102); XL5513 (105) and XL5939 (109).

3.4.2.2. Experimental field trial design and statistical analysis

At each field trial site, the following materials were grown in a randomised complete block design with four replicates: maize NK603 × T25 × DAS-40278-9 not exposed to the intended herbicides, maize NK603 × T25 × DAS-40278-9 exposed to the intended herbicides, the comparator SLB01 × PHRDW and four commercial non-GM maize reference varieties.

The agronomic, phenotypic and compositional data were analysed as specified by EFSA GMO Panel (2010b; 2011a,b). This includes, for each of the two treatments of maize NK603 × T25 × DAS-40278-9, the application of a difference test (between the GM maize and the non-GM comparator) and an equivalence test (between the GM maize and the set of non-GM commercial reference varieties). The results of the equivalence test are categorised into four possible outcomes (I–IV, ranging from equivalence to non-equivalence).[12]

3.4.2.3. Suitability of selected test materials

Selection of the test materials

To obtain the three-event stack maize, the single events NK603, T25 and DAS-40278-9 were transferred in the genetic background of two different non-GM maize inbred lines, SLB01 and PHRDW. In subsequent subsections, GM maize NK603 × T25 × DAS 40278-9 refers to hybrid (F_1 generation) obtained crossing GM inbred line SLB01 (carrying events NK603 and DAS-40278-9) with GM inbred line PHRDW (carrying event T25). The comparator used in the field trials is the non-GM maize hybrid SLB01 × PHRDW, which has a similar genetic background to that of maize NK603 × T25 × DAS-40278-9 (as documented by the pedigree), and is therefore considered to be an acceptable comparator.

Maize NK603 × T25 × DAS-40278-9 and the non-GM comparator, both with a comparative relative maturity (CRM) of 103, are considered appropriate for growing in environments across North America and Canada, where the comparative field trials were conducted.

Commercial non-GM reference varieties (see Table 5) with a CRM ranging from 102 to 109 were selected by the applicant and, at each selected site, four reference varieties were tested. On the basis of the information provided on relative maturity classes and year of commercialisation, the GMO Panel considers the selected non-GM reference varieties appropriate for the comparative assessment.

Seed production and quality

The seeds of the comparator used in the 2018 field trials (see Table 5) were produced, harvested and stored under similar conditions. The seed lots were verified for their identity via event specific PCR analysis. The germination of maize NK603 × T25 × DAS-40278-9 and the comparator was tested under warm and cold temperature conditions. The GMO Panel considers that the starting seed used as test material in the agronomic, phenotypic and compositional studies was of adequate quality.

[12] In detail, the four outcomes are category I (indicating full equivalence to the non-GM reference varieties); category II (equivalence is more likely than non-equivalence); category III (non-equivalence is more likely than equivalence); and category IV (indicating non-equivalence).

Conclusion on suitability

The GMO Panel is of the opinion that maize NK603 × T25 × DAS-40278-9, the comparator and the non-GM reference varieties were properly selected and are of adequate quality. Therefore, the test materials are considered appropriate for the comparative analysis.

3.4.2.4. Representativeness of the receiving environments

Selection of field trial sites

The selected field trial sites were located in commercial maize-growing regions of United States and Canada. Climate and soil characteristics of the selected fields were diverse,[13] corresponding to optimal, near-optimal and suboptimal conditions for maize cultivation (Sys et al., 1993). The GMO Panel considers that the selected sites, including the subset chosen for the compositional analysis, reflect commercial maize-growing regions in which the test materials are likely to be grown.

Meteorological conditions

Some exceptional weather conditions were reported at five of the selected sites.[14] However, due to the lack of major impacts on plant growth at these sites, the GMO Panel considers that the exceptional weather conditions did not invalidate the selection of the field trial sites for the comparative analysis.

Management practices

The field trials included plots containing maize NK603 × T25 × DAS-40278-9, plots with the comparator and plots with non-GM reference varieties, managed according to local agricultural practices. In addition, the field trials included plots containing the GM maize managed following the same agricultural practices, but conventional herbicides were replaced with two applications with a 2,4 D- and glyphosate-containing herbicide at pre-emergence and at growth stage BBCH 17 and two single applications with glufosinate- and quizalofop-containing herbicides at BBCH 12 and BBCH 14, respectively. The GMO Panel considers that the management practices, including sowing, harvesting and application of plant protection products, were appropriate for the field trials.

Conclusion on representativeness

The GMO Panel concludes that the geographical locations, soil and climate characteristics, meteorological conditions and management practices of the field trials are typical of the receiving environments where the test materials could be grown.

3.4.2.5. Agronomic and phenotypic analysis

Eleven agronomic and phenotypic endpoints[15] plus information on biotic and abiotic stressors were collected from the field trials (see Table 5).

The results of the test of difference and the test of equivalence were the following:

- For the three-event stack maize (not treated with the intended herbicides), the test of difference identified statistically significant differences with the comparator for days to maturity, lodging, harvest grain moisture, yield and 100-kernel weight. All the endpoints fell under equivalence category I or II.
- For the three-event stack maize (treated with the intended herbicides), the test of difference identified statistically significant differences with the comparator for plant height, days to maturity, lodging, harvest grain moisture, yield and 100-kernel weight. All the endpoints fell under equivalence category I or II except for lodging which fell under equivalence category III.[16]

[13] Soil types of the field trials were clay loam, sandy clay loam, silty clay loam, loam and silt loam; soil organic carbon ranged from 1.2% to 3.3%; pH ranged from 5.2 to 7.5; average temperatures and sum of precipitations during the usual crop growing season ranged, respectively, from 9.7°C to 14.6°C and from 554 mm to 899 mm.

[14] Wind was registered at one field trial in Iowa and in Illinois and frost at one field trial in South Dakota. Hail and wind were recorded at one field trial in Minnesota and Nebraska.

[15] Early stand count, days to flowering, plant height, days to maturity, lodging, final stand count, ear count, dropped ears, harvest grain moisture, yield and 100-kernel weight.

[16] The estimated means for lodging (plants/plot) were 1.5 (not treated GM), 0.9 (treated GM), 3.4 (comparator) and 5.4 (non-GM reference varieties); as a percentage of the final population, the values are 0.9% (not treated GM), 0.5% (treated GM), 2% (comparator) and 3.3% (non-GM reference varieties).

The GMO Panel considered that the result for lodging was too low to have an impact on the comparative assessment as the mean values for all the materials were at most only 3.3% of the final population.

3.4.2.6. Compositional analysis

Forage and grain of the three-event stack maize harvested from the field trials (Table 5) were analysed for 81 constituents (10 in forage and 71 in grain), including those recommended by OECD (OECD, 2002). The statistical analysis as described in Section 3.4.2.2 was not applied to nine grain constituents,[17] because more than half of the samples were below the limit of quantification.

The statistical analysis was applied to a total of 72 constituents (10 in forage[18] and 62 in grain[19]); a summary of the outcome of the test of difference and the test of equivalence is presented in Table 6:

- For the three-event stack maize not treated with the intended herbicides, statistically significant differences in the comparison with the non-GM comparator were found for 27 endpoints (four in forage and 23 in grains). All these endpoints fell under equivalence category I or II.
- For the three-event stack maize treated with the intended herbicides, statistically significant differences in the comparison with the non-GM comparator were found for 33 endpoints (five in forage and 28 in grains). All these endpoints fell under equivalence category I or II.

Table 6: Outcome of the comparative compositional analysis of forage and grain of maize NK603 × T25 × DAS 40278 9. The table shows the number of endpoints in each category

		Test of difference			
		Not treated[(c)]		Treated[(c)]	
		Not different	Significantly different	Not different	Significantly different
Test of equivalence[(b)]	Category I/II	42	27[(d)]	36	33[(d)]
	Category III/IV	1[(e)]	–	1[(e)]	–
	Not categorised	2[(f)]	–	2[(f)]	–
	Total endpoints	72		72	

(a): Comparison between the three-event stack maize and the non-GM comparator.
(b): Four different outcomes: category I (indicating full equivalence to the non-GM reference varieties); category II (equivalence is more likely than non-equivalence); category III (non-equivalence is more likely than equivalence); and category IV (indicating non-equivalence). Not categorised means that the test of equivalence was not applied because of the lack of variation among the non-GM reference varieties.
(c): Treated/not treated with the intended herbicide.
(d): Endpoints with significant differences between the three-event stack maize and the non-GM comparator and falling under equivalence category I–II. For forage, not treated only: crude fat. Treated only: crude protein and NDF. Both treated and not treated: carbohydrates, calcium and phosphorous. For grains, not treated only: crude fat, ash, arachidic acid (C20:0), eicosenoic acid (C20:1), arginine, glycine, tryptophan, copper, niacin and total tocopherols. Treated only: crude protein, carbohydrates, stearic acid (C18:0), alanine, glutamic acid, leucine, methionine, phenylalanine, proline, serine, calcium, magnesium, manganese, phosphorous and inositol. Both treated and not treated: ADF, palmitic acid (C16:0), palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2), α-linolenic acid (C18:3), lignoceric acid (C24:0), β-carotene, α-tocopherol, γ-tocopherol, p-coumaric acid, ferulic acid and raffinose.
(e): Levels of thiamin in grain (for both treated and not treated GM maize) fell under equivalence category IV, but no significant differences were identified between the GM maize and the non-GM comparator.
(f): Levels of TDF and lauric acid (C12:0) in grain (for both treated and not treated GM maize) were not categorised for equivalence, but no significant differences were identified between the GM maize and the non-GM comparator.

[17] Myristic acid (C14:0), heptadecanoic acid (C17:0), heptadecenoic acid (C17:1), eicosadienoic acid (C20:2), erucic acid (C22:1), riboflavin, β-tocopherol, δ-tocopherol and furfural. For these constituents (with the exception of erucic acid (C22:1), for which all the data were < LOQ), the Fisher exact test was applied in order to identify differences in the number of data points < LOQ; no statistically significant outcomes were identified.

[18] Moisture, crude protein, crude fat, crude fibre, acid detergent fibre (ADF), neutral detergent fibre (NDF), ash, carbohydrates (by calculation), calcium and phosphorus.

[19] Moisture, crude protein, crude fat, crude fibre, acid detergent fibre (ADF), neutral detergent fibre (NDF), total dietary fibre, ash, carbohydrates (by calculation), lauric acid (C12:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), α-linolenic acid (C18:3), arachidic acid (C20:0), eicosenoic acid (C20:1), behenic acid (C22:0), lignoceric acid (C24:0), alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, calcium, copper, iron, phosphorus, potassium, sodium, magnesium, manganese, zinc, β-carotene, thiamin, niacin, pantothenic acid, pyridoxine, folic acid, α-tocopherol, γ-tocopherol, total tocopherols (by calculation), p-coumaric acid, ferulic acid, inositol, phytic acid, raffinose and trypsin inhibitor.

The GMO Panel assessed all the significant differences between the three-event stack maize and the non-GM comparator, taking into account the potential impact on plant metabolism and the natural variability observed for the set of non-GM reference varieties. No endpoints were identified that showed significant differences between the three-event stack maize and the non-GM comparator and fell under category III/IV.

3.4.2.7. Conclusions on the comparative analysis

Considering the selection of test materials, the field trial sites and the associated management practices and the agronomic–phenotypic characterisation as an indicator of the overall field trial quality, the GMO Panel concludes that the field trials are appropriate to support the comparative analysis.

Taking into account the natural variability observed for the set of non-GM reference varieties, the GMO Panel concludes that:

- Regarding agronomic and phenotypic characteristics, none of the differences between the three-event stack maize and the non-GM comparator needs further assessment except for lodging, which is assessed for potential environmental impact in Section 3.4.4.
- None of the differences identified in forage and grain composition between the three-event stack maize and the non-GM comparator needs further assessment regarding food and feed safety.

3.4.3. Food/Feed safety assessment

3.4.3.1. Effects of processing

Maize NK603 × T25 × DAS-40278-9 will undergo existing production processes used for conventional maize. No novel production process is envisaged. Based on the outcome of the comparative assessment, processing of the three-event stack maize into food and feed products is not expected to result in products being different from those of conventional non-GM maize varieties.

3.4.3.2. Stability of newly expressed proteins

Protein stability is one of several relevant parameters to consider in the weight-of-evidence approach in protein safety assessment (EFSA GMO Panel, 2010c, 2011a, 2017, 2021a). The term protein stability encompasses several properties such as thermal stability, pH-dependent stability, proteolytic stability and physical stability (e.g. tendency to aggregate), among others (Li et al., 2019). It has been shown, for example, that when characteristics of known food allergens are examined, one prominent trait attributed to food allergens is protein stability (Helm, 2001; Breiteneder and Mills, 2005; Costa et al., 2021).

Effect of temperature and pH on newly expressed proteins

The effects of temperature and pH on the proteins CP4 EPSPS, PAT and AAD-1 have been previously evaluated by the GMO Panel (Table 2). No new information has been provided in the context of this application.

In vitro protein degradation by proteolytic enzymes

The resistance to degradation by pepsin of the newly expressed PAT, AAD-1 and CP4 EPSPS proteins has been previously evaluated by the GMO Panel (Table 2). No new information has been provided in the context of this application.

3.4.3.3. Toxicology

Testing of newly expressed proteins

Three proteins (CP4 EPSPS and its variant CP4 EPSPS L214P, PAT, and AAD-1) are newly expressed in the three-event stack maize NK603 × T25 × DAS-40278-9 (Section 3.4.1). The GMO Panel has previously assessed these proteins in the context of the single maize events (Table 2), and no safety concerns were identified for humans and animals. The GMO Panel is not aware of any other new information that would change its previous conclusions on the safety of these proteins. The potential for a functional interaction among the proteins newly expressed in maize NK603 × T25 × DAS-40278-9 has been assessed with regard to human and animal health. The three enzymatic proteins (CP4 EPSPS and its variant CP4 EPSPS L214P, PAT, and AAD-1) catalyse distinct biochemical reactions, acting on unrelated substrates and are not expected to interact. The CP4 EPSPS and its variant CP4 EPSPS L214P act on the

shikimic acid pathway for the biosynthesis of aromatic amino acids in plants, showing high substrate specificity. The PAT enzyme acts on the glufosinate-ammonium-based herbicides and the AAD-1 enzyme degrades 2,4-dichlorophenoxyacetic acid (2,4-D) and AOPP class of herbicides. On the basis of the known biological function of the individual newly expressed proteins (Table 4), there is currently no expectation for their possible interactions relevant to the food and feed safety of the three-event stack maize NK603 × T25 × DAS-40278-9.

The GMO Panel concludes that there are no safety concerns to human and animal health related to the newly expressed proteins CP4 EPSPS, including the variant CP4 EPSPS L214P, PAT, and AAD-1 in the three-event stack maize NK603 × T25 × DAS-40278-9.

Testing of new constituent other than proteins

No new constituents other than newly expressed proteins have been identified in seed and forage from maize NK603 × T25 × DAS-40278-9. Therefore, no further food/feed safety assessment of components other than the newly expressed proteins is required.

Information on altered levels of food and feed constituent

No altered levels of food/feed constituents have been identified in seed and forage from maize NK603 × T25 × DAS-40278-9. Therefore, no further food/feed safety assessment of components other than the newly expressed proteins is required.

Testing of the whole genetically modified food and feed

Based on the outcome of the molecular characterisation, comparative analysis and toxicological assessment, no indication of findings relevant to food/feed safety related to the stability and expression of the inserts or to interaction between the transformation events, and no modifications of toxicological concern in the composition of maize NK603 × T25 × DAS-40278-9 have been identified (see Sections 3.4.1, 3.4.2 and 3.4.3.3). Therefore, animal studies on food/feed derived from the three stacks are not necessary (EFSA GMO Panel, 2011a). In accordance to Regulation (EU) No 503/2013, the applicant provided a 90-day oral repeated-dose toxicity study in rats on whole food and feed from each of the maize single event composing the three-event stack maize.

90-Day studies on maize NK603

The GMO Panel had previously concluded that this study is in line with Regulation (EU) No 503/2013 and do not show adverse effects related to diets incorporating the single-event maize NK603 (EFSA GMO Panel, 2019b).

90-Day studies on maize T25

A 90-day study on maize T25 has been previously assessed by the GMO Panel in the context of the single-event renewal application dossier (EFSA GMO Panel, 2013), and was not consider adequate because of the low number of experimental units per treatment (two cages per sex with five animals per cage), reducing the power of the statistical analysis; moreover, the study was conducted with grains which were harvested from T25 maize plants not treated with the intended herbicide; upon EFSA's request to fulfil the requirements of Regulation (EU) No 503/2013, the applicant provided a new 90-day toxicity study on T25 maize.[20]

In this study, pair-housed Crl: WI (Han) rats (16/sex per group; 2 rats/cage) were allocated to three groups using a randomised complete block design with eight replications/sex.

Groups were fed diets containing 50% of incorporation rate of grains from maize T25 treated with the intended herbicide (glufosinate-ammonium-containing herbicide) (test material), the non-GM comparator (NIL, control material) or the non-GM commercial reference maize variety (5385, reference material).

The study was adapted from OECD test guideline 408 (OECD, 2018), aligned with EFSA Scientific Committee guidance (EFSA Scientific Committee, 2011) and complied with the principles of good laboratory practice (GLP) with some deviations not impacting the study results and interpretation (i.e. test item stability, homogeneity and concentration), which are detailed below.

Event-specific PCR analysis confirmed the presence of the event T25 in both the GM ground maize grains (meal) and diets and excluded the presence of the event in the respective controls. ELISA

[20] Additional information: 12/3/2021.

analyses also confirmed the presence of event T25 (i.e. PAT concentration) in the GM maize grains and GM diets.

Both GM and control maize grains and diets were analysed for nutrients, antinutrients and potential contaminants (e.g. selected heavy metals, mycotoxins and pesticides). Balanced diets were formulated based on the specifications for Kliba-modified maintenance diet.

The stability of the test and control materials was not verified; however, in accordance to product expiration declared by the diet manufacturer, the constituents of the diets are considered stable for the duration of the treatment. The GMO Panel considered this justification acceptable. Diet preparation procedures and regular evaluations of the mixing methods guaranteed the homogeneity and the proper concentration of the test or control substances in them. The applicant provided information on concentration of proximates and fibre in the formulated test diets, further supporting the homogeneity of the formulations.

Feed and water were provided ad libitum. In-life procedures and observations and terminal procedures were conducted in accordance to OECD test guideline 408 (OECD, 2018).

An appropriate range of statistical tests was performed on the results of the study. Detailed description of the methodology and of statistically significant findings identified in rats given a diet containing T25 maize is reported in Appendix C.

There were no test diet-related incidents of mortality or clinical signs. No test diet-related adverse findings were identified in any of the investigated parameters. A small number of statistically significant findings were noted, but these were not considered adverse effects of treatment for one or more of the following reasons:

- were within the normal variation[21] for the parameter in rats of this age;
- were of small magnitude;
- were identified at only a small number of time intervals with no impact on the overall value;
- exhibited no consistent pattern with related parameters or endpoints.

No gross pathology findings related to the administration of the test diet were observed at necropsy, and the microscopic examinations of a wide range of organs and tissues did not identify relevant differences in the incidence or severity of the histopathological findings related to the administration of the test diet compared to the control group. A notable, but not statistically significant, increase in basophilic renal tubules was noted in rats receiving T25 diet, but these animals had lower levels of lymphoid infiltration and tubular degeneration/regeneration and no indications of altered blood or urinary changes associated with impaired renal function. Overall, it is concluded that T25 did not have an adverse effect on the rat kidney.

The GMO Panel concludes that this study is in line with the requirements of Regulation (EU) No 503/2013 and that no treatment-related adverse effects were observed in rats after feeding diets including 50% grains from T25 maize for 90 days.

90-Day study on maize DAS-40278-9

In accordance with Regulation (EU) No 503/2013, the applicant provided a 90-day feeding study in rats receiving diets derived from maize DAS-40278-9.

In this study, pair-housed Crl: CD (SD) rats (12/sex per group; 2 rats/cage) were allocated to six groups with six replications/sex per group.[22]

Groups were fed diets incorporating DAS-40278-9 maize grains sprayed with 2,4-D- and quizalofop-containing herbicides at 33% and 11% of inclusion level (the latter supplemented with 22% of the non-GM comparator maize), the non-GM comparator (inclusion level 33%) or one of three non-transgenic commercial reference maize varieties (inclusion level 33%) (Master's choice commercial meal MC-535RF, Blue River commercial meal 71M36RF, Pioneer commercial meal 34M78RF).

[21] Although animals used in a toxicology study are of the same strain, from the same supplier and are closely matched for age and body weight at the start of the study, they exhibit a degree of variability in the parameters investigated during the study. This variability is evident even within control groups. To help reach a conclusion on whether a statistically significant finding in a test group is 'adverse' account is taken of whether the result in the test group is outside the normal range for untreated animals of the same strain and age. To do this, a number of sources of information are considered, including the standardized effect size, the standard deviations and range of values within test and control groups in the study and, if applicable, data from other studies performed in the same test facility within a small timeframe and under almost identical conditions (Historic Control Data).

[22] The treatment groups were arranged in blocks, with all diets equally represented in each block; there was no information available, however, on the randomisation of treatment groups within blocks.

The study was adapted from OECD test guideline 408 (OECD, 1998), aligned with EFSA Scientific Committee guidance (EFSA Scientific Committee, 2011) and complied with the principles of good laboratory practice (GLP) with some deviations not impacting the study results and interpretation (i.e. test item stability, homogeneity and concentration), which are detailed below.

Event-specific PCR analysis confirmed the presence of the event DAS-40278-9 in both the GM maize grains and diets and excluded the presence of the event in the non-GM comparator. ELISA analyses also confirmed the presence of event DAS-40278-9 (i.e. AAD-1 concentration) in the GM maize grains and GM diets.

Both GM and control maize grains and diets were analysed for nutrients, antinutrients and potential contaminants (e.g. selected heavy metals, mycotoxins and pesticides). Balanced diets were formulated based on the specifications for PMI Certified Rodent LabDiet® 5002. The stability of the test and control materials was not verified; however, in accordance to product expiration declared by the diet manufacturer, the constituents of the diets are considered stable for the duration of the treatment. The GMO Panel considered this justification acceptable. Diet preparation procedures and regular evaluations of the mixing methods guaranteed the homogeneity and the proper concentration of the test or control substances in them.

Feed and water were provided ad libitum. In-life procedures and observations and terminal procedures were conducted in accordance to OECD test guideline 408 (OECD, 1998).

An appropriate range of statistical tests was performed on the results of the study. Detailed description of the methodology and of statistically significant findings identified in rats given a diet containing DAS-40278-9 maize is reported in Appendix C.

There were no test diet-related incidents of mortality or clinical signs. No test diet-related adverse findings were identified in any of the investigated parameters. A small number of statistically significant findings were noted but these were not considered adverse effects of treatment for one or more of the following reasons:

- were within the normal variation[21] for the parameter in rats of this age;
- were of small magnitude;
- were identified at only a small number of time intervals with no impact on the overall value;
- exhibited no consistent pattern with related parameters or end-points.
- no consistency with increasing dietary incorporation level.

No gross pathology findings related to the administration of the test diet were observed at necropsy, and the microscopic examinations of a wide range of organs and tissues did not identify relevant differences in the incidence or severity of the histopathological findings related to the administration of the test diet compared to the control group.

The GMO Panel concludes that this study is in line with the requirements of Regulation (EU) No 503/2013, and that no treatment-related adverse effects were observed in rats after feeding diets including 11% or 33% grains from DAS 40278-9 maize for 90 days.

Although evidence is increasing that 50% maize incorporation rate is generally applicable as the high dose (EFSA 2014; Steinberg et al., 2019; EFSA GMO Panel, 2021b), the GMO Panel considers that further scientific evidence is needed that test diets incorporating 50% would not induce nutritional imbalance.

3.4.3.4. Allergenicity

For the allergenicity assessment, a weight-of-evidence approach was followed, taking into account all the information obtained on the newly expressed proteins, as no single piece of information or experimental method yields sufficient evidence to predict allergenicity and adjuvanticity (Codex Alimentarius, 2009; EFSA GMO Panel, 2011a; Commission Regulation (EU) No 503/2013). Furthermore, an assessment of the newly expressed proteins in relation to their potential to cause coeliac disease was also performed (EFSA GMO Panel, 2017).

Assessment of allergenicity of the newly expressed proteins[23]

For allergenicity, the GMO Panel has previously evaluated the safety of CP4 EPSPS, PAT and AAD-1 proteins individually, and no evidence of allergenicity was identified in the context of the applications assessed (Table 2). No new information on allergenicity of the proteins newly expressed in this three-event stack maize that might change the previous conclusions of the GMO Panel has become available.

[23] Technical dossier and additional information 18 December 2020.

Based on the current knowledge, and as there is no evidence of allergenicity of the newly expressed proteins, there are no expected concerns of allergenicity as a consequence of their interaction in this three-event stack maize.

The GMO Panel has previously evaluated the safety of the newly expressed proteins and has not found evidence of adjuvanticity (ability to enhance an allergic response) in the context of the applications assessed (Table 2). There was no indication that the proteins at the levels expressed in this three-event stack maize act as adjuvants.

Furthermore, the applicant provided information on the safety of CP4 EPSPS, PAT and AAD-1 proteins regarding their potential hazard to cause a coeliac disease response. For such assessment, the applicant followed the principles described in the EFSA GMO Panel guidance document (EFSA GMO Panel, 2017). The assessment of the CP4 EPSPS and AAD-1 proteins identified no perfect or relevant partial matches with known coeliac disease peptide sequences. The assessment of the PAT protein revealed partial matches containing the Q/E-X1-P-X2 motif and required further investigations. Based on additional considerations on position and nature of amino acids flanking the ELPA motif, such as the presence of two consecutive prolines and the charge and size of adjacent amino acids (EFSA GMO Panel, 2017), the two relevant peptides containing the motif do not raise concern as they fail to mimic gluten sequences. Therefore, no indications of safety concern were identified by the GMO Panel.

Assessment of allergenicity of the whole GM plant

The GMO Panel regularly reviews the available publications on food allergy to maize. However, maize is not considered a common allergenic food[24] (OECD, 2002). Therefore, the GMO Panel does not request experimental data to analyse the allergen repertoire of GM maize.

In the context of this application and considering the data from the molecular characterisation, the compositional analysis and the assessment of the newly expressed proteins (see Sections 3.4.1, 3.4.2 and 3.4.3), the GMO Panel identifies no indications of a potentially increased allergenicity of food and feed derived from this three-event stack maize with respect to that derived from the non-GM comparator.

3.4.3.5. Dietary exposure assessment to new constituents

In line with Regulation (EU) No 503/2013, the applicant provided dietary exposure estimates to CP4 EPSPS, PAT and AAD-1 proteins newly expressed in NK603 × T25 × DAS-40278-9 maize. Dietary exposure was estimated based on protein expression levels reported in this application for the three-event stack maize treated with the intended herbicides, the current available consumption data and feed practices, the foods and feeds currently available in the market and the described processing conditions.

For the purpose of estimating dietary exposure, the levels of newly expressed proteins in NK603 × T25 × DAS-40278-9 maize grains, forage and pollen were derived from replicated field trials (four replicates from six locations) in 2018 in the United States and Canada (see Section 3.4.1.3). Table 7 describes the protein expression levels used to estimate both human and animal dietary exposure.

Table 7: Mean values (n = 24, µg/g dry weight and µg/g fresh weight) for newly expressed proteins in grains, forage and pollen from NK603 × T25 × DAS-40278-9 maize treated with the intended herbicides[(a)]

Protein	Tissue/developmental stage		
	Grains/R6 (µg/g dry weight/µg/g fresh weight)	Pollen/R1 (µg/g fresh weight)[(b)]	Forage/R4 (µg/g dry weight)
CP4 EPSPS	14/11	385.4	110
PAT	0.27/0.21	0.12[(c)]	18
AAD-1	4.3/3.4	88.4	9

(a): Intended herbicides: glufosinate, glyphosate, 2,4-dichlorophenoxyacetic acid (2,4-D) herbicide and aryloxyphenoxypropionate (AOPP) herbicides.

[24] Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/200.

(b): Values refer to a moisture content of 6% derived from the reported concentrations in dry weight (study reports PHI-2018-017 and PHI-R066-Y19). These values were used to estimate dietary exposure to the different newly expressed protein via the consumption of pollen supplements.
(c): A total of 22 out of 24 samples were reported below the limit of quantification (LOQ, 0.22 μg/g). Half the LOQ value was assigned to the left-censored data to calculate the mean.

Human dietary exposure

Chronic and acute dietary exposure to CP4 EPSPS, PAT and AAD-1 proteins newly expressed in NK603 × T25 × DAS-40278-9 maize was provided. The applicant followed the methodology described in the EFSA Statement 'Human dietary exposure assessment to newly expressed protein in GM foods' (EFSA, 2019a) to estimate human dietary exposure in average and high consumers making use of summary statistics of consumption.

Human dietary exposure was estimated across different European countries on different population groups: young population (infants, toddlers, 'other children'), adolescents, adult population (adults, elderly and very elderly) and special populations (pregnant and lactating women). Since no specific consumption data were available on commodities containing, consisting of or obtained from NK603 × T25 × DAS-40278-9 maize grains, a conservative scenario with 100% replacement of conventional maize by the GM maize was considered. Consumption figures for all relevant commodities (e.g. corn flakes, sweet corn, popcorn, etc.) were retrieved from the EFSA Comprehensive European Food Consumption Database (EFSA consumption database).[25] Corn oil was excluded from the assessment since no proteins are expected to be present in the oil.

Mean protein expression values on fresh weight basis are considered as the most adequate to estimate human dietary exposure (both acute and chronic) when working with raw primary commodities that are commonly consumed as processed blended commodities (EFSA, 2019a). Different recipes and factors were considered to estimate the amount of maize in the consumed commodities before assigning newly expressed protein levels to the relevant commodities.[26] No losses in the newly expressed proteins during processing were considered, except for certain commodities excluded from the exposure estimations (maize oil, corn starch, corn syrup).

The highest acute dietary exposure (high consumers) was estimated in the age class 'Other children' with exposure estimates of 167 μg/kg body weight (bw) per day, 3.2 μg/kg bw per day and 51.7 μg/kg bw per day for CP4 EPSPS, PAT and AAD-1 proteins, respectively. The main contributor to the exposure in the dietary survey with the highest estimates was corn grains.

The highest chronic dietary exposure (high consumers) was estimated in the age class 'Infants' with exposure estimates of 62.1 μg/kg bw per day, 1.2 μg/kg bw per day and 19.2 μg/kg bw per day for CP4 EPSPS, PAT and AAD-1 proteins, respectively. The main contributor to the exposure in the dietary survey with the highest estimates was sweet corn.

An ad hoc dietary exposure scenario was carried out for consumers of pollen supplements under the assumption that these supplements might be made of pollen from NK603 × T25 × DAS-40278-9 maize. Consumption data on pollen supplements are available for few consumers across eight different European countries.[27] The low number of consumers available adds uncertainty to the exposure estimations which should be carefully interpreted, and it prevents from estimating exposure for high consumers of pollen supplements. In average consumers of pollen supplements, the highest acute dietary exposure would range from 0.09 μg/kg bw per day for PAT to 285.7 μg/kg bw per day for CP4 EPSPS, in the elderly population. Similarly, the highest chronic dietary exposure in average consumers would range from 0.06 μg/kg bw per day for PAT to 190.5 μg/kg bw per day for CP4 EPSPS, also in the elderly population.

Animal dietary exposure

Dietary exposure to CP4 EPSPS, PAT and AAD-1 proteins in maize NK603 × T25 × DAS-40278-9 was estimated across different animal species as below described, assuming the consumption of maize products commonly entering the feed supply chain (i.e. maize grains and forage). A conservative scenario with 100% replacement of conventional maize products by the three-event stack maize products was considered.

[25] https://www.efsa.europa.eu/en/applications/gmo/tools. Data accessed: September 2019.
[26] Example: 100 grams of maize bread are made with approximately 74 g of maize flour, and a reverse yield factor of 1.22 from the conversion of maize grains into flour is used. This results in 9.9 μg of CP4 EPSPS per gram of maize bread as compared to the 11 μg/g reported as mean concentration in the maize grains.
[27] https://www.efsa.europa.eu/en/food-consumption/comprehensive-database. Data accessed: March 2021.

Mean levels (dry weight) of the newly expressed proteins in grains and forage from the three-event stack maize treated with the intended herbicide used for animal dietary exposure are listed in Table 7.

The applicant estimated dietary exposure to CP4 EPSPS, PAT and AAD-1 proteins in livestock (i.e. poultry, swine, cattle and sheep), based on estimates for body weights, daily feed intakes and inclusion rates (percentage) of maize grains and forage in diets/rations (OECD, 2013).

Estimated dietary exposure in livestock animals was calculated based on the consumption of maize grain and forage alone or in combination, as reported in Appendix D.

3.4.3.6. Nutritional assessment of endogenous constituents

The intended traits of maize NK603 × T25 × DAS-40278-9 are herbicide tolerance, with no intention to alter nutritional parameters. Comparison of the seed and forage composition of maize NK603 × T25 × DAS-40278-9 with the conventional counterpart and the commercial non-GM maize reference varieties did not identify differences that would require a nutritional assessment as regards food/feed (see Section 3.4.2.7). From these data, the GMO Panel concludes that maize NK603 × T25 × DAS-40278-9 is nutritionally equivalent to the non-GM comparator and the non-GM reference varieties used.

3.4.3.7. Conclusion on the food/feed safety assessment

The CP4 EPSPS, PAT and AAD-1 proteins newly expressed in maize NK603 × T25 × DAS-40278-9 do not raise safety concerns for human and animal health. No interactions between the newly expressed proteins relevant for food and feed safety were identified, and no overall toxicological concerns on the three-event stack maize were identified. Similarly, the GMO Panel did not identify indications of safety concerns regarding allergenicity or adjuvanticity related to the presence of the newly expressed proteins in the three-stack maize NK603 × T25 × DAS-40278-9, or regarding the overall allergenicity of this three-event stack maize. Based on the outcome of the comparative assessment and the nutritional assessment, the GMO Panel concludes that the consumption of maize NK603 × T25 × DAS-40278-9 does not represent any nutritional concern, in the context of the scope of this application.

3.4.4. Environmental risk assessment

Considering the scope of application EFSA-GMO-NL-2019-164, which excludes cultivation, the environmental risk assessment (ERA) of maize NK603 × T25 × DAS-40278-9 mainly takes into account: (1) the exposure of microorganisms to recombinant DNA in the gastrointestinal tract of animals fed GM material and of microorganisms present in environments exposed to faecal material of these animals (manure and faeces); and (2) the accidental release into the environment of viable three-event stack maize grains during transportation and/or processing (EFSA GMO Panel, 2010a).

3.4.4.1. Persistence and invasiveness of the GM plant

Maize is highly domesticated, not winter hardy in colder regions of Europe, and generally unable to survive in the environment without appropriate management. Survival is limited mainly by a combination of low competitiveness, absence of a dormancy phase and susceptibility to plant pathogens, herbivores and cold climate conditions (OECD, 2002), even though occasional feral GM maize plants may occur outside cultivation areas in the EU (e.g. Pascher, 2016). Field observations indicate that maize grains may survive and overwinter in some EU regions, resulting in volunteers in subsequent crops (e.g. Gruber et al., 2008; Palaudelmàs et al., 2009; Pascher, 2016). However, maize volunteers have been shown to grow weakly and flower asynchronously with the maize crop (Palaudelmàs et al., 2009). Thus, the establishment and survival of feral and volunteer maize in the EU are currently limited and transient.

It is unlikely that the intended traits of the three-event stack maize and the observed decrease in lodged plants (see Section 3.4.2.5) will provide a selective advantage to maize plants, except when they are exposed to glyphosate- and/or glufosinate ammonium- and/or 2,4-D and/or AOPP-containing herbicides. However, if this was to occur, this fitness advantage will not allow the three-event stack maize to overcome other biological and abiotic factors (described above) limiting plant's persistence and invasiveness. Therefore, the presence of the intended traits and the observed differences in lodging will not affect the persistence and invasiveness of the GM plant.

In conclusion, the GMO Panel considers that the three-event stack maize will be equivalent to conventional maize hybrid varieties in their ability to survive until subsequent seasons, or to establish

occasional feral plants under European environmental conditions in case of accidental release into the environment of viable three-event stack maize grains.

3.4.4.2. 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 (HGT) of DNA or through vertical gene flow via cross-pollination from feral plants originating from spilled grains.

Plant to microorganism gene transfer

The probability and potential adverse effects of HGT of the recombinant DNA have been assessed in previous GMO Panel Scientific Opinions for the single events (see already assessed APs in Table 2). This assessment included consideration of homology-based recombination processes, as well as non-homologous end joining and microhomology-mediated end joining. Possible fitness advantages that the bacteria in the receiving environments would gain from acquiring recombinant DNA were considered. No concern as a result of an unlikely, but theoretically possible, HGT of the recombinant genes to bacteria in the gut of domesticated animals and humans fed GM material or other receiving environments was identified. The applicant submitted an updated bioinformatic analysis for each of the single events in order to assess the possibility for HGT by homologous recombination.

The updated bioinformatic analyses for NK603, T25 and DAS-40278-9 confirm the assessments provided in the context of previous Scientific Opinions (EFSA GMO Panel, 2015b, 2019a, 2021b).

Synergistic effects of the recombinant genes, for instance due to combinations of recombinogenic sequences, which would cause an increase in the likelihood for horizontal gene transfer or a selective advantage are not identified.

Therefore, the GMO Panel concludes that the unlikely, but theoretically possible, horizontal transfer of recombinant genes from this three-event stack maize to bacteria does not raise any environmental safety concern.

Plant-to-plant gene transfer

The GMO Panel assessed the potential for occasional feral three-event stack maize plants originating from grain import spills to transfer recombinant DNA to sexually compatible plants; the environmental consequences of this transfer were also considered.

For plant-to-plant gene transfer to occur, imported GM maize grains need to germinate and develop into plants in areas containing sympatric wild relatives and/or cultivated maize with synchronous flowering and environmental conditions favouring cross-pollination.

Maize is an annual predominantly cross-pollinating crop. Cross-fertilisation occurs mainly by wind (OECD, 2003). Vertical gene transfer from maize is limited to *Zea* species. Wild relatives of maize outside cultivation are not known/reported in Europe (Eastham and Sweet, 2002; OECD, 2003; EFSA, 2016; Trtikova et al., 2017). Therefore, potential vertical gene transfer is restricted to maize and weedy *Zea* species, such as teosintes and/or maize-teosinte hybrids, occurring in cultivated areas (EFSA, 2016; Trtikova et al., 2017; Le Corre et al., 2020).

The potential of spilled maize grains to establish, grow and produce pollen is extremely low and transient (see Section 3.4.4.1). Therefore, likelihood/frequency of cross-pollination between occasional feral GM maize plants resulting from grain spillage, and weedy or cultivated *Zea* plants is considered extremely low (EFSA, 2016). Even if cross-pollination would occur, the GMO Panel is of the opinion that environmental effects as a consequence of the spread of genes from occasional feral GM maize plants in Europe will not differ from that of conventional maize varieties for the reasons given in Section 3.4.4.1, even if exposed to the intended herbicides.

3.4.4.3. Interactions of the GM plant with target organisms

Taking the scope of application EFSA-GMO-NL-2019-164 (no cultivation) and thus the absence of target organisms into account, potential interactions of occasional feral three-event stack maize plants arising from grain import spills with target organisms are not considered a relevant issue.

3.4.4.4. Interactions of the GM plant with non-target organisms

Given that environmental exposure of non-target organisms to spilled GM grains or occasional feral GM maize plants arising from spilled GM grains is limited and because most proteins are degraded before entering the environment through faecal material of animals fed GM maize, potential interactions with non-target organisms are not considered a relevant issue by the GMO Panel.

3.4.4.5. Interactions with the abiotic environment and biogeochemical cycles

Given that environmental exposure to spilled grains or occasional feral maize NK603 × T25 × DAS-40278-9 plants arising from grain import spills is limited and because most proteins are degraded before entering the environment through faecal material of animals fed GM maize, potential interactions with the abiotic environment and biogeochemical cycles are not considered a relevant issue by the GMO Panel.

3.4.4.6. Conclusion of the environmental risk assessment

The GMO Panel concludes that it is unlikely that the maize NK603 × T25 × DAS-40278-9 would differ from conventional maize varieties in its ability to persist under EU environmental conditions. Considering the scope of the application EFSA-GMO-NL-2019-164, interactions of occasional feral maize NK603 × T25 × DAS-40278-9 plants with the biotic and abiotic environment are not considered to be relevant issues. The analysis of horizontal gene transfer from maize NK603 × T25 × DAS-40278-9 to bacteria does not indicate a safety concern. Therefore, considering the combined traits and their interactions, the outcome of the agronomic and phenotypic analysis, the routes and levels of exposure, the GMO Panel concludes that maize NK603 × T25 × DAS-40278-9 would not raise safety concerns in the event of accidental release of viable GM maize grains into the environment.

3.5. Risk assessment of the subcombinations

Subcombinations previously assessed in the frame of other applications are discussed in Section 3.5.1. The subcombination that has not been previously assessed is discussed in Section 3.5.2.

3.5.1. Subcombinations previously assessed

The GMO Panel has previously assessed two subcombinations (see Table 1) and no safety concerns were identified. Literature searches covering the 10 years before submission of the application and the period since the time of validity of the application (January 2009–April 2021) revealed no new scientific information relevant to the risk assessment of these maize stacks.[28] Consequently, the GMO Panel considers that its previous conclusions on these subcombinations remain valid.

3.5.2. Subcombinations not previously assessed

One of the three subcombinations included in the scope of this application has not been previously assessed by the GMO Panel, and no experimental data were provided for this maize stack (see Table 8). In this case, following the strategy defined by the GMO Panel,[29] the risk assessment takes as its starting point the assessment of the single maize events, and uses the data generated for the three-event stack as well as all the additional data available on subcombinations previously assessed by the GMO Panel (Table 2) and the additional studies provided by the applicant (Appendix A).

Table 8: Maize stacks not previously assessed and covered by the scope of application EFSA-GMO-NL-2019-164

Degree of stacking	Event
2-event stack	DAS-40278-9 × T25

3.5.2.1. Stability of the events

The genetic stability of the inserted DNA over multiple generations in the three single maize events was demonstrated previously (see Table 2). Integrity of the events was demonstrated in the three-event stack maize NK603 × T25 × DAS-40278-9 (Section 3.4.1.2) and the previously assessed maize subcombinations (see Table 2). The GMO Panel finds no reasons to expect the loss of integrity of the events in the maize subcombination not previously assessed (see Table 8).

3.5.2.2. Expression of the events

The GMO Panel assessed whether any combination of the three-events by conventional crossing could result in significant changes in expression levels of the newly expressed proteins, as this could

[28] Dossier: Part II – Section 7; additional information: 7/8/2020.
[29] 115th GMO Panel meeting (Annex 1 of the minutes: https://www.efsa.europa.eu/sites/default/files/event/170517-m.pdf).

indicate an unexpected interaction between the events. Based on current knowledge of the molecular elements introduced, there is no reason to expect interactions that would affect the levels of the newly expressed proteins in this subcombination compared with those in the single maize events. This assumption was confirmed by comparing the levels of the newly expressed proteins of each single maize event with those of the three-event stack maize. The levels were comparable in the three-event stack maize and in the single events (Section 3.4.1.3 and Appendix B). This supports the conclusion that interactions affecting the expression levels of the newly expressed proteins are not expected in the subcombination not previously assessed and included in the scope of application EFSA-GMO-NL-2019-164.

3.5.2.3. Potential functional interactions between the events

The GMO Panel assessed the potential for interactions between maize events in the subcombination not previously assessed (Table 8), taking into consideration intended traits and unintended effects.

Based on the known biological functions of the individual newly expressed proteins (Table 4), there is currently no expectation for possible interactions relevant for the food and feed or environmental safety between these proteins in those subcombinations. The GMO Panel took into account all the intended and potential unintended effects considered in the assessment of the three single events, the previously assessed subcombinations (Table 2) and the three-event stack maize. It is concluded that none of these events would raise safety concerns when combined in any of these maize subcombinations. The GMO Panel considers that no further data are needed to complete the assessment of subcombinations from the three-event stack maize.

3.5.3. Conclusions

Since no new safety concerns were identified for the previously assessed subcombinations, the GMO Panel considers that its previous conclusions on these maize subcombinations remain valid. For the remaining subcombination included in the scope of application EFSA-GMO-NL-2019-164, for which no experimental data have been provided, the GMO Panel assessed the possibility of interactions between the events and concluded that these combinations would not raise safety concerns. These subcombinations are therefore expected to be as safe as the single maize events, the previously assessed subcombinations and the three-event stack maize.

3.6. Post-market monitoring

3.6.1. Post-market monitoring of GM food/feed

The GMO Panel concluded that the three-event stack maize, as described in this application, does not raise any nutritional concern and is as safe as the non-GM comparator and the non-GM reference varieties tested (Section 3.4.3). Two of the subcombinations have been previously assessed and no safety concerns were identified. The subcombination not previously assessed and included in the scope of this application is expected to be as safe as the single maize events, the previously assessed maize subcombinations and the three-event stack maize (Section 3.5.2). Therefore, the GMO Panel considers that post-market monitoring of food and feed from the three-event stack maize and its subcombinations, as described in this application, is not necessary.

3.6.2. Post-market environmental monitoring

The objectives of a post-market environmental monitoring (PMEM) plan, according to Annex VII of Directive 2001/18/EC, are: (1) to confirm that any assumption regarding the occurrence and impact of potential adverse effects of the GMO, or its use, in the ERA are correct; and (2) to identify the occurrence of adverse effects of the GMO, or its use, on human health or the environment that were not anticipated in the ERA.

Monitoring is related to risk management, and thus, a final adoption of the PMEM plan falls outside the mandate of EFSA. However, the GMO Panel gives its opinion on the scientific rationale of the PMEM plan provided by the applicant (EFSA GMO Panel, 2011b).

As the ERA does not identify potential adverse environmental effects from the three-event stack maize, no case-specific monitoring is required.

The PMEM plan proposed by the applicant for the three-event stack maize and its subcombinations includes: (1) the description of a monitoring approach involving operators (federations involved in

import and processing), reporting to the applicant, via a centralised system, any observed adverse effect(s) of GMOs on human health and the environment; (2) a coordinating system established by Croplife Europe for the collection of information recorded by the various operators; and (3) the review of relevant scientific publications retrieved from literature searches (Lecoq et al., 2007; Windels et al., 2008). The applicant proposes to submit a PMEM report on an annual basis and a final report at the end of the authorisation period.

The GMO Panel considers that the scope of the PMEM plan provided by the applicant is consistent with the intended uses of the three-event stack maize. The GMO Panel agrees with the reporting intervals proposed by the applicant in its PMEM plan. The PMEM plan and reporting intervals are in line with the intended uses of the three-event stack maize and its subcombinations.

3.6.3. Conclusion on post-market monitoring

No PMM of food and feed is necessary. The scope of the PMEM plan provided by the applicant and the reporting intervals are in line with the intended uses of maize NK603 × T25 × DAS-40278-9.

4. Overall conclusions

The GMO Panel was asked to carry out a scientific assessment of maize NK603 × T25 × DAS-40278-9 and subcombinations for import, processing and food and feed uses in accordance with Regulation (EC) No 1829/2003.

No new information was identified on the three single maize events (NK603, T25 and DAS-40278-9) that would lead to a modification of the original conclusions on their safety.

The molecular characterisation, the comparative analysis (agronomic, phenotypic and compositional characteristics) and the outcome of the toxicological, allergenicity and nutritional assessment indicate that the combination of the single maize events and of the newly expressed proteins in the three-event stack maize does not give rise to food/feed safety and nutritional concerns. The GMO Panel concludes that the three-event stack maize, as described in this application, does not raise any nutritional concern and is as safe as its non-GM comparator and the selected non-GM reference varieties.

The GMO Panel concludes that there is a very low likelihood of environmental effects resulting from the accidental release of viable grains from the three-event stack maize into the environment.

Since no new data were identified on the two previously assessed subcombinations that would lead to a modification of the original conclusions on their safety, the GMO Panel considers that its previous conclusions on these maize stacks remain valid. For the remaining subcombination included in the scope of application EFSA-GMO-NL-2019-164, no information has been provided. The GMO Panel assessed the possible interactions between the events in this subcombination and concludes that these combinations of events NK603, T25 and DAS-40278-9 would not raise safety concerns. This subcombination is therefore expected to be as safe as the maize single events, the previously assessed subcombinations and the three-event stack maize.

The literature searches did not identify any relevant publications on maize NK603, T25 and DAS-40278-9. In the context of annual PMEM reports, the applicant could further fine-tune future literature searches according to the GMO Panel recommendations.

In addition, the GMO Panel considered the additional unpublished study listed in Appendix A. This new information does not raise any concern for human and animal health and the environment regarding the three-event stack maize and its subcombinations.

Given the absence of safety and nutritional concerns for foods and feeds from the three-event stack maize and all its subcombinations, the GMO Panel considers that PMM of these products is not necessary. The PMEM plan and reporting intervals are in line with the intended uses of the three-event stack maize and its subcombinations.

In conclusion, the GMO Panel considers that maize NK603 × T25 × DAS-40278-9 and its subcombinations, as described in this application, are as safe as the non-GM comparator and the selected non-GM reference varieties with respect to potential effects on human and animal health and the environment.

5. Documentation as provided to EFSA

- Letter from the Competent Authority of the Netherlands received on 13 December 2019 concerning a request for authorization of the placing on the market of maize

NK603 × T25 × DAS-40278-9 submitted in accordance with Regulation (EC) No 1829/2003 by submitted by Pioneer Hi-Bred International, Inc. as represented by Pioneer Overseas Corporation (application EFSA-GMO-NL-2018-153 - EFSA-Q-2019-00808).

- Application validated by EFSA, 26 March 2020.
- Request for supplementary information to the applicant, 1 April 2020.
- Receipt of supplementary information from the applicant, 8 May 2020.
- Request for supplementary information to the applicant, 12 June 2020.
- Receipt of supplementary information from the applicant, 7 August 2020.
- Request for supplementary information to the applicant, 5 October 2020.
- Receipt of supplementary information from the applicant, 27 November 2020.
- Receipt of supplementary information from the applicant, 30 November 2020.
- Receipt of supplementary information from the applicant, 29 January 2021.
- Receipt of supplementary information from the applicant, 12 March 2021.
- Receipt of spontaneous information from the applicant, 12 March 2021.
- Request for supplementary information to the applicant, 21 April 2021.
- Request for supplementary information to the applicant, 26 April 2021.
- Receipt of supplementary information from the applicant, 15 June 2021.
- Receipt of supplementary information from the applicant, 28 June 2021.
- Request for supplementary information to the applicant, 3 August 2021.
- Request for supplementary information to the applicant, 28 September 2021.
- Receipt of supplementary information from the applicant, 1 October 2021.
- Receipt of spontaneous information from the applicant, 1 October 2021.
- Receipt of supplementary information from the applicant, 6 October 2021.
- Request for supplementary information to the applicant, 14 October 2021.
- Receipt of supplementary information from the applicant, 15 October 2021.

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Abbreviations

AAD-1	Aryloxyalkanoate dioxygenase
ADF	acid detergent fibre
AOPP	aryloxyphenoxypropionate
Bw	body weight
CaMV	cauliflower mosaic virus
CRM	comparative relative maturity
CTP	chloroplast transit peptide
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EPSPS	5-enolpyruvylshikimate-3-phosphat synthase
ERA	environmental risk assessment
FOB	functional observational battery
GLP	Good Laboratory Practice
GM	genetically modified
GMO Panel	EFSA Panel on Genetically Modified Organisms
GMO	genetically modified organism
HGT	horizontal gene transfer
HR	homologous recombination
Hsp	heat shock proteins
kg	kilogram
LOQ	limit of quantification
mg	milligram
MS	Member States
NDF	neutral detergent fibre
NL	The Netherlands
Nos	nopaline synthase
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame
Ori	origin of replication
PAT	Phosphinothricin-acetyl-transferase
PCR	polymerase chain reaction

PMEM	post-market environmental monitoring
SES	standardised effect sizes
TDF	total dietary fibre
UTR	untranslated region

Appendix A – List of additional studies

List of additional studies performed by or on behalf of the applicant with regard to the evaluation of the safety of maize NK603 × T25 × DAS-40278-9 for humans, animal or the environment

Study identification	Title
PHI-2018-137	Evaluation of Zea m 14 Concentrations in Grain from a Maize Line Containing the Combined Trait Product MON-ØØ6Ø3-6xACS-ZMØØ3-2xDAS-4Ø278-9 Using Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS): U.S. and Canada Test Sites

Appendix B – Protein expression data

Table B.1: Mean, standard deviation and range of protein levels (μg/g dry weight) from maize NK603 × T25 × DAS-40278-9 (not treated) and NK603, T25 and DAS-40278-9 (not treated), from field trials performed across six locations in USA and Canada in 2018 (n = 24)[a]

Protein	Event(s)	Leaf (V2–V4)	Leaf (V9)	Leaf (R1)	Pollen (R1)	Root (R1)	Forage (R4)	Grain (R6)
CP4 EPSPS[f]	**NK603 × T25 × DAS-40278-9**	310[b] ± 87[c] (160–490)[d]	200 ± 34 (120–260)	220 ± 29 (150–280)	280 ± 43 (150–340)	81 ± 19 (39–120)	98 ± 32 (54–180)	8.6 ± 2.5 (3.4–13)
	NK603	330 ± 85 (190–470)	200 ± 38 (130–290)	220 ± 41 (150–290)	280 ± 48 (120–350)	60 ± 12 (33–75)	96 ± 22 (61–150)	8.0 ± 2.1 (4.2–13)
PAT	**NK603 × T25 × DAS-40278-9**	50 ± 15 (23–91)	50 ± 11 (30–70)	55 ± 17 (15–91)	< LOQ[e]	13 ± 4.4 (4.1–23)	18 ± 7.6 (8.5–33)	0.24 ± 0.13 (< 0.054–0.51)
	T25	53 ± 11 (33–77)	50 ± 9.8 (34–77)	59 ± 13 (38–98)	0.13 ± 0.03[e] (< LOQ–0.26)	16 ± 3.9 (9–25)	19 ± 6.8 (10–33)	0.22 ± 0.09 (0.073–0.48)
AAD-1	**NK603 × T25 × DAS-40278-9**	13 ± 5.2 (4.6–22)	6.6 ± 2.5 (2.8–11)	10 ± 2.5 (4.9–13)	99 ± 19 (57–120)	5.4 ± 2.2 (0.87–10)	8.6 ± 1.6 (5.3–13)	3.9 ± 1.1 (2.0–6.4)
	DAS-40278-9	12 ± 5.6 (4.5–22)	6.1 ± 1.8 (3.0–9.0)	8.9 ± 3.1 (4.2–16)	100 ± 18 (71–130)	5.4 ± 1.9 (1.6–9.8)	9.0 ± 1.3 (7.1–12)	3.7 ± 1.3 (1.7–6.4)

(a): Number of samples is n = 24.
(b): Mean.
(c): Standard deviation.
(d): Range.
(e): Some, but not all, sample results were below the LLOQ. A value equal to half the LLOQ value was assigned to those samples to calculate the mean and standard deviation. LOQ = 0.22; LOQ: limit of quantification.
(f): EPSPS levels in the maize NK603 × T25 × DAS-40278-9 are a sum of two protein variants CP4 EPSPS and CP4 EPSPS L214P, both expressed in maize NK603.

Appendix C – Statistical analysis and statistically significant findings in the 90-day toxicity studies in rats on the whole food/feed

C.1. Statistical analysis of the 90-day study on T25 in rats

The following endpoints were statistically analysed: body weights, body weight changes, water and food consumption, clinical pathology values (as applicable), absolute and relative organ weights, functional observational battery (FOB) data, locomotor activity, ophthalmological examinations and histopathological data. For all continuous endpoints, mean, standard deviation in terms of the standardised effect sizes (SES) of each dose group for each sex, variable, and period or time interval were reported. The main statistical analysis compared rats consuming the test diet (T25) with those consuming the control diet (NIL). The statistical analysis was performed using linear mixed models with treatment, sex and their interaction as fixed effects whereas the random effects included only the block. If the interaction of the treatment per sex was significant, the comparison of the test vs. the control group was conducted separately for the two genders. In case of pathology and clinical pathology data where a statistical difference between test (T25) and test the control group (NIL) was identified, an additional comparison between the test (T25) and the reference (5385) group was performed. If this latest analysis also shows a significant difference, a direct comparison of test data (T25) to the historical control data was provided. Historical control data were provided for endpoints belonging to clinical pathology and pathology. Missing data were considered by the Panel and found not impacting the results.

Table C.1: Statistically significant findings in 90-day study on T25 in rats

Statistically significant parameter/endpoint	Finding	GMO Panel interpretation
RBC count and haematocrit & haemoglobin levels	Statistically significant decreases in males (< 5%)	Low magnitude, within normal variation. Not an adverse effect of treatment with maize T25.
Basophil count	Statistically significant decrease in females	Within normal variation. Not an adverse effect of treatment with maize T25.
Monocyte (% of WBC)	Statistically significant increase in both sexes combined (< 20%)	Low magnitude, absolute count not changed significantly. Within normal variation. Not an adverse effect of treatment.
Blood urea	Statistically significant decrease in females (25%)	Decrease is not adverse in isolation. Within normal variation. Not an adverse effect of treatment.
Creatinine	Statistically significant decrease in both sexes combined (20%)	Decrease is not adverse in isolation. Within normal variation Not an adverse effect of treatment.
Total bilirubin	Statistically significant increase in both sexes combined (15%)	Low magnitude, within normal variation. No consistent pattern with other markers of liver toxicity. Not an adverse effect of treatment.
Albumin	Statistically significant increase in females (1%)	Low magnitude, within normal variation. Not an adverse effect of treatment.
Sodium	Statistically significant increase in females (1%)	Low magnitude, within normal variation. Not an adverse effect of treatment.
Urinary pH	Statistically significant increase in both sexes combined (20%)	Value is within the normal physiological range. Not an adverse effect of treatment.
Kidney weight (absolute and relative to body weight)	Statistically significant increase in males and females (10–20%)	Low magnitude, within normal variation. No pattern of adverse effects on other markers of kidney function. Not an adverse effect of treatment.
Pituitary weight (relative to body weight)	Statistically significant decrease in both sexes combined (20%)	Low magnitude, within normal variation. Not an adverse effect of treatment. No associated pathological changes.
Thyroid weight (relative to body weight)	Statistically significant decrease in both sexes combined (15%)	Low magnitude, within normal variation. Not an adverse effect of treatment. No associated pathological changes.

Statistically significant parameter/endpoint	Finding	GMO Panel interpretation
Uterus weight	Statistically significant increase (40%)	Within normal variation. No associated pathological changes. Not an adverse effect of treatment.

C.2. Statistical analysis of the 90-day study on DAS 40278-9 in rats

The following endpoints were statistically analysed: body weight, body weight gain, feed consumption and feed efficiency, forelimb and hindlimb strength, motor activity data, sensory evaluation data, rectal temperature, haematology, coagulation, urinalysis and clinical chemistry values, absolute and relative organ weights. For all continuous endpoints, mean and standard deviation were provided for each dose group for each sex, variable and period or time interval. In the statistical analysis, rats consuming the low- and high-dose test diets were compared with those consuming the control diet. For continuous parameters, a linear mixed model was applied to data for individual animals for the two sexes combined (fixed effects: diet, sex and sex-by-diet interaction; random effects: block-within-sex and cage). Test-control comparisons were done both across sexes and separately for males and females; in case a significant sex-by-diet interaction was identified, only the sex-specific results were considered for the assessment. The model was modified as needed for the analysis of sex-specific endpoints and cage-level data (food consumption and food efficiency). The data for the three reference groups (not included in the linear mixed model analysis) were used to calculate ranges of variability for the parameters. For each comparison, point estimates and 95% confidence intervals of the SES were reported to aid the assessment. Missing data were considered by the Panel and found not to affect the results.

Table C.2: Statistically significant findings in 90-day study on DAS-40278-9 in rats

Statistically significant parameter/endpoint	Finding	GMO Panel interpretation
Potassium	Statistically significant increase in males and females at the top dose (< 10%)	Within normal variation compared with reference diet results. Not an adverse effect of treatment with maize DAS 40278-9.
Brain weight (absolute)	Statistically significant decrease (5%) in low dose males	Low magnitude. No significant change in top dose males. Within normal variation. Not an adverse effect of treatment.
Spleen weight (relative to body weight)	Statistically significant increase in low dose groups in both sexes (15%)	Low magnitude, within normal variation. No significant change at top dose. No associated haematology or pathological findings. Not an adverse effect of treatment.
Thymus weight (relative to body weight)	Statistically significant increase in low dose groups in both sexes (< 20%)	Low magnitude. No significant change at top dose. No associated haematology or pathological findings Not an adverse effect of treatment.

Appendix D – Animal dietary exposure

Table D.1: Dietary exposure to CP4 EPSPS, PAT and AAD-1 proteins (mg/kg bw per day) in livestock, based on the consumption of maize grain and forage

	Dietary exposure (mg/kg bw per day)								
	CP4 EPSPS			PAT			AAD-1		
	Grain (G)	Forage (F)	G + F	Grain (G)	Forage (F)	G + F	Grain (G)	Forage (F)	G + F
Broiler	0.69	NA	NA	0.013	NA	NA	0.21	NA	NA
Layer	0.67	75.3	75.9	0.013	12.3	12.3	0.21	6.2	6.4
Turkey	0.50	NA	NA	0.0096	NA	NA	0.15	NA	NA
Breeding pigs	0.23	50.8	51	0.0044	8.3	8.3	0.069	4.2	4.2
Finishing pigs	0.29	NA	NA	0.0057	NA	NA	0.090	NA	NA
Beef cattle[(a)]	0.27	2.11	2.38	0.0052	0.35	0.35	0.083	0.17	0.26
Dairy cattle	0.16	2.54	2.70	0.0031	0.42	0.42	0.050	0.21	0.26
Ram/ewe	0.14	NA	NA	0.0027	NA	NA	0.043	NA	NA
Lamb	0.18	1.40	1.58	0.0034	0.23	0.23	0.055	0.11	0.17

(a): The inclusion rate for beef cattle would be 160% of the diet, resulting the DDE to each protein an overestimation.
NA indicates that a forage inclusion rate was not provided in the reference, and therefore, no exposure calculations were done.