Evaluation of existing guidelines for their adequacy for the food and feed risk assessment of genetically modified plants obtained through synthetic biology

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Evaluation of existing guidelines for their adequacy for the food and feed risk assessment of genetically modified plants obtained through synthetic biology

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Abstract

Synthetic biology (SynBio) is an interdisciplinary field at the interface of molecular engineering and biology aiming to develop new biological systems and impart new functions to living cells, tissues and organisms. EFSA has been asked by the European Commission to evaluate SynBio developments in agri-food with the aim of identifying the adequacy and sufficiency of existing guidelines for risk assessment and determine if updated guidance is needed. In this context, the GMO Panel has previously adopted an Opinion evaluating the SynBio developments in agri-food/feed and the adequacy and sufficiency of existing guidelines for the molecular characterisation and environmental risk assessment of genetically modified plants (GMPs) obtained through SynBio and reaching the market in the next decade. Complementing the above, in this Opinion, the GMO Panel evaluated the adequacy and sufficiency of existing guidelines for the food and feed risk assessment of GMPs obtained through SynBio. Using selected hypothetical case studies, the GMO Panel did not identify novel potential hazards and risks that could be posed by food and feed from GMPs obtained through current and near future SynBio approaches; considers that the existing guidelines are adequate and sufficient in some Synbio applications; in other cases, existing guidelines may be just adequate and hence need updating; areas needing updating include those related to the safety assessment of new proteins and the comparative analysis. The GMO Panel recommends that future guidance documents provide indications on how to integrate the knowledge available from the SynBio design and modelling in the food and feed risk assessment and encourages due consideration to be given to food and feed safety aspects throughout the SynBio design process as a way to facilitate the risk assessment of SynBio GMPs and reduce the amount of data required.

Keywords: agri-food use, food, feed, genetically modified plant (GMP), risk assessment, synthetic biology (SynBio)

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Summary

Synthetic biology (SynBio) is an interdisciplinary field at the interface of molecular engineering and biology aiming to develop new biological systems and impart new functions to living cells, tissues and organisms. SynBio has potential applications in agri-food systems, requiring a pre-market authorisation according to the European Union (EU) GMO legislation. This Opinion addresses four Terms of Reference (ToRs) requested by the European Commission on the food and feed risk assessment of genetically modified plants obtained through SynBio (SynBio GMPs), complementing the previous GMO Panel Opinion on molecular characterisation (MC) and environmental risk assessment (ERA) (EFSA GMO Panel, 2021a): (1) identify sectors/advances in the agri-food system considered among SynBio GMP developments; (2) identify potential risks and potential novel hazards that SynBio GMPs could pose for humans and animals (farmed and companion animals); (3) evaluate the adequacy and sufficiency of existing guidelines for risk assessment of current and near-future SynBio GMPs arriving in the EU market in the next decade; and (4) identify specific areas for which updated guidance is needed. To this aim, the existing guidelines are challenged by a selection of four case studies representing plant products likely to be developed using state-of-the-art SynBio approaches and potentially reaching the market within the next decade. These hypothetical case studies were developed using existing techniques (transgenes insertion and genome editing), but the technological complexity of the engineering process or the products themselves would require the application of SynBio approaches. Three of the four cases had already been used for the assessment of the adequacy of guidelines for the MC and ERA of SynBio GMPs (EFSA GMO Panel, 2021a); the fourth case (*de novo* domestication of a wild species) has been selected to further assess the adequacy and sufficiency of guidelines in the case where a single appropriate comparator is not available. EFSA consulted EU Member States and interested parties during a public consultation and addressed the comments received. The GMO Panel addressed the ToRs concluding that (1) previous conclusions regarding new sectors/advances in the agri-food system among SynBio GMP developments remain applicable; (2) no novel potential hazards and risks for humans and animals (farmed and companion animals) that could be posed by food and feed from GM plants obtained through current and near future SynBio approaches are identified; (3) the existing guidelines are adequate and sufficient in some SynBio applications, in other cases are adequate but need updating; and (4) areas that may need updating are those related to the safety assessment of new proteins and the comparative analysis. The guidelines should be reviewed as new approaches are becoming available for use in risk assessment to streamline and strengthen the protein safety assessment; comparative analysis as requested by the existing guidelines may not always be applicable to some SynBio GMPs. The comparative strategy would remain the preferred approach for the risk assessment of SynBio GMPs, though updated guidance documents are needed (choice of the comparator, identification of multiple comparators, comparative analysis protocol and related statistical analysis); in case a stand-alone assessment is conducted, an appropriate procedure should also be developed, e.g. integrating risk assessment approaches as for other novel foods, as already indicated by the existing guidelines. Considering the range of GMPs obtained by SynBio, the GMO Panel highlights that a case-by-case approach is pivotal to complete the risk assessment. The GMO Panel recommends that future guidance documents provide indications on how to integrate the knowledge available from the SynBio design and modelling in the food and feed risk assessment and encourages due consideration to be given to food and feed safety aspects throughout the SynBio design process as a way to facilitate the risk assessment of SynBio GMPs and reduce the amount of data required.

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1. Introduction

Synthetic biology (SynBio) is an interdisciplinary field at the interface of molecular engineering and biology aiming to develop new biological systems and impart new functions to living cells, tissues and organisms. SynBio is not a technique, or a plain combination of techniques, rather it is a process or strategy comprising theoretical and experimental approaches. It employs engineering principles such as standardisation, modularity, modelling and computer-aided design to improve the predictability of the process and to achieve the desired characteristics of the product (see also EFSA GMO Panel, 2021a). The principles of standardisation and modularity facilitate the iterative engineering cycles of 'design–build–test–learn' (Appendix A). So, by bridging molecular engineering, life sciences and computational modelling, SynBio is expanding the range of applications and products that can be developed and improves the predictability of biotechnology.

SynBio has potential applications in the food and feed chain. These applications entail the alteration of genetic material of a plant in a way that does not occur naturally by mating or natural recombination and are therefore subject to pre-market authorisation according to the EU GMO legislation.

The European Commission's Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Consumer Safety (SCCS) previously published[1] three Opinions (SCENIHR, SCCS and SCHER, 2014, 2015a,b) on SynBio, addressing six SynBio developments: (1) genetic part libraries and methods; (2) minimal cells and designer chassis; (3) protocells and artificial cells; (4) xenobiology; (5) DNA synthesis and genome editing; and (6) citizen science (Do-It-Yourself Biology). The Opinions addressed the definition of SynBio, risk assessment methodologies and safety aspects, risks to the environment and biodiversity and research priorities in the field of SynBio. These non-food Scientific Committees concluded in their Opinions that new SynBio applications may be assessed using current risk assessment methodology for GMOs and that the rapidly evolving technologies may require existing methodologies to be revisited at regular intervals and improved when necessary to continue ensuring safety.

Therefore, as a proactive measure, the European Commission requested the European Food Safety Authority (EFSA) for an opinion on GMOs developed using SynBio approaches and the implications, if any, for risk assessment methodologies. EFSA identified a total of six work packages (WP) to be reflected in the development of six Opinions, according to organism group and risk assessment aspects (see Section 1.2). In this context, the GMO Panel adopted in 2020 a Scientific Opinion evaluating the SynBio developments in agri-food/feed with the aim of identifying the adequacy of existing molecular characterisation (MC) and environmental risk assessment (ERA) guidelines for genetically modified plants (GMPs) obtained through SynBio and to determine if updated guidance is needed (EFSA GMO Panel, 2021a). Of relevance in this context, considerations on developments regarding targeted mutagenesis were addressed in the EFSA GMO Panel (2020) opinion on 'Applicability of the EFSA Opinion on site-directed nucleases type 3 for the safety assessment of plants developed using site-directed nucleases type 1 and 2 and oligonucleotide-directed mutagenesis'. For microorganisms, the Scientific Committee adopted in 2020 a Scientific Opinion evaluating the SynBio developments in different types of microorganisms for deliberate release into the environment and the adequacy of existing guidelines for MC and ERA (EFSA Scientific Committee, 2020).

1.1. Definitions for SynBio for the Terms of Reference

Synthetic biology (SynBio) has been previously defined as follows by the joint SCENIHR, SCCS and SCHER committees upon request of the EC1: 'Synthetic biology is the application of science, technology and engineering to facilitate and accelerate the design, manufacture and/or modification of genetic materials in viable organisms.' Similar to the EFSA Scientific Opinions addressing WP1 (MC and ERA SynBio microorganisms) and WP2 (MC and ERA SynBio plants), this definition is used as a starting

[1] SCENIHR, SCCS, SCHER (2014) Synthetic Biology I Definition, Opinion, September 2014. Available online: http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_044.pdf, SCENIHR, SCCS, SCHER (2015) Synthetic Biology II – Risk assessment methodologies and safety aspects, Opinion, May 2015. Available from: http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_048.pdf and SCENIHR, SCCS, SCHER (2015a,b) Synthetic Biology III – Research priorities, Opinion, December 2015. Available from: http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_050.pdf

point for the present opinion due to the request of the European Commission to build on the three Opinions of SCENIHR, SCCS and SCHER (2014, 2015a,b).

The Convention on Biological Diversity[2] further clarified that 'While there is no internationally agreed definition of 'synthetic biology', key features of synthetic biology include the *de novo* synthesis of genetic material and an engineering-based approach to develop components, organisms and products.' This further clarification establishes the link for the request to support the EU in the work under the Convention on Biological Diversity and the Cartagena Protocol on Biosafety (2000/2003).

Further background and technical specificities on Synthetic Biology are provided in Section 3 of the Opinion on SynBioM (MC and ERA) (EFSA Scientific Committee, 2020).

In the context of this opinion, and in line with EFSA GMO Panel (2021a), 'SynBio GMP' is used as synonymous of 'GMP obtained through SynBio' and means a GM plant developed through the application of SynBio approaches.

1.2. Background and Terms of Reference as provided by the requestor

Building on SCENIHR, SCCS and SCHER Opinions (2014, 2015a,b) and taking into account available literature and previous analyses carried out by EU Member States and at international level, the Commission asked EFSA,[3] in accordance with Article 29 (1) of Regulation (EC) No 178/2002, for an opinion on GMOs developed through SynBio and their implications for risk assessment methodologies. The scope of the present mandate is limited to agri-food uses.[4] In this context:

1) EFSA was asked to consider whether and which newer sectors/advances should be considered among SynBio developments, in addition to the six identified by the Scientific Committees (ToR1).
2) EFSA was requested to identify, where possible, potential risks in terms of impact on humans, animals and the environment that current and near future SynBio developments could pose; in this respect EFSA is also asked to identify potential novel hazards compared to established techniques of genetic modification[5] (ToR2).
3) EFSA was requested to determine whether the existing guidelines for risk assessment are adequate and sufficient for current and near future SynBio developments or whether there is a need for updated guidance (ToR3).
4) In the latter case EFSA was requested to identify the specific areas where such updated guidance is needed (ToR4).

EFSA was also requested to provide technical and scientific expertise on the risk assessment of GMOs obtained through SynBio to support the EU in the work under the Convention on Biological Diversity and the Cartagena Protocol on Biosafety.

The mandate received from the EC was split in six WPs by EFSA to be reflected in six Opinions:

1) Molecular characterisation (MC) and environmental risk assessment (ERA) of GMMs obtained through SynBio (EFSA Scientific Committee, 2020).
2) MC and ERA of GMPs obtained through SynBio (EFSA GMO Panel, 2021a).
3) Food and feed risk assessment of GMMs obtained through SynBio (ongoing).
4) Food and feed risk assessment of GMPs obtained through SynBio (current opinion).
5) MC and ERA of GM animals obtained through SynBio.
6) Food and feed risk assessment of GM animals obtained through SynBio.

The current Opinion is addressing WP4 (evaluates the adequacy of existing guidelines for food and feed risk assessment of food and feed from GMPs obtained through SynBio) and it is intended to complement the GMO Panel Opinion on MC and ERA of SynBio GMPs developed under WP2 (EFSA GMO Panel, 2021a).

[2] https://www.cbd.int/doc/meetings/cop/cop-12/information/cop-12-inf-11-en.pdf
[3] See correspondence under mandate M-2018-0205 in the EFSA register of questions: http://registerofquestions.efsa.europa.eu/roqFrontend/wicket/page?3
[4] For the purpose of this mandate, agri-food uses means agri/food/feed products falling within the remit of EFSA.
[5] For the purpose of this mandate, the term 'established techniques of genetic modification' refers to various genetic engineering techniques that have been significantly used over the last 30 years to produce genetically modified organisms, such as those that have been authorised under Directive 2001/18/EC and Regulation (EU) No 1829/2003.

1.3. Interpretation of the Terms of Reference and scope

The interpretation of the Terms of Reference (ToRs) of the mandate was previously made in agreement with the EC[6] (EFSA GMO Panel, 2021a). This interpretation is considered applicable to this Opinion, with a clarification on ToR2 in line with the food/feed scope of this Opinion:

- Not all of the six developments previously identified by the non-food Scientific Committees were considered relevant: citizen science was excluded as a concept not being linked to a technique or a product.
- 'Near future': for this mandate this is interpreted as products with the potential to reach the EU market in the next decade. In this Opinion this is reflected in Section 2.3 when selecting hypothetical case studies.
- 'Agri-food uses': on footnote 5 of the mandate 'For the purpose of this mandate agri-food uses means agri/food/feed products falling within the remit of EFSA', further clarifications were needed to determine which applications fall within the remit of EFSA, within this mandate and within the available time frame. The limited time frame led to the explicit exclusion of bioremediation applications from this mandate. By extrapolation, the following applications are also excluded from this mandate: de-extinction, bioweapons/biopreparedness, medical use, and biofuels (see Section 2.2 of EFSA GMO Panel, 2021a and the EFSA External Scientific Report 'Mapping of plant SynBio developments in the agri-food sector'[7]).
- For this Opinion, ToR2 is focused on the evaluation of potential novel hazards and risks for humans and animals (farmed and companion animals) that could be posed by food and feed from GM plants obtained through current and near future SynBio approaches. The MC and ERA for SynBio GMPs was previously addressed (EFSA GMO Panel, 2021a), see Section 1.4 below.
- 'Existing guidelines for risk assessment': for this Opinion see Section 2.1.

Addressing ToR3, when a guideline is considered **adequate and sufficient** it contains the necessary information for conducting the risk assessment. When a guideline is considered **adequate**, it can be used but modifications or additions are necessary.

This Opinion is produced not only to support the European Commission, but is also meant for the public, scientific community and stakeholders, companies and institutions interested in the safety of SynBio (GMP) developments, all of which were given the opportunity to provide comments on the final draft opinion during the public consultation.

1.4. Summary of the conclusions of the previous SynBio opinion (EFSA GMO Panel, 2021a)

Based on selected hypothetical case studies, the GMO Panel had not identified novel potential risks in terms of the impact of SynBio GMPs on humans, animals and the environment, and no novel hazards, compared with established techniques of genetic modification. Furthermore, requirements for the MC and ERA of SynBio GMPs were found to be adequate. However, it was concluded that as SynBio developments are constantly evolving, the analysed cases may not be representative for all future applications. Some aspects were identified as possible challenges for future SynBio developments such as the potential scale of the changes introduced and the potentially increases in the complexity and diversity of the new traits in future SynBio applications compared with GMP applications assessed to date. It was also noted that the comparative analysis of SynBio GMPs with complex traits might face challenges with regards to the choice of the comparator (conventional counterpart). Conclusions and recommendations included: (1) the concept of comparator might need to evolve, as finding a suitable comparator with a genetic background as close as possible to the SynBio GMP may present challenges in more complex future SynBio cases; (2) other risk assessment approaches that do not rely on the current comparative approach may need to be considered. Modelling of the characteristics of engineered organisms, including their expected behaviour in different environments might aid, facilitate and improve the comparative analysis and ERA of SynBio GMPs in a given receiving environment; (3) the 'design' factor in the SynBio GMP offers the possibility to address safety issues already identified during the design–build–test–learn cycle of the SynBio

[6] See correspondence under mandate M-2018-0205 in the EFSA register of questions: https://open.efsa.europa.eu/questions/EFSA-Q-2020-00768

[7] https://doi.org/10.2903/sp.efsa.2020.EN-1687

approach ('safe by design'); and (4) more emphasis may be given on the possible interactions between the SynBio GMP genotypes, their management and the receiving environments in which they are to be cultivated, by exploiting the advancement of approaches in ecosystem modelling.

2. Data and methodologies

2.1. Ad hoc expert Working Group and its methodology

EFSA established an ad hoc expert Working Group of the GMO Panel (from this point forwards referred to as WG) for the development of this opinion on food and feed risk assessment aspects of GMPs obtained by SynBio that met regularly to address the mandate of the European Commission.[8]

The WG has adopted a methodology based on a four-phase approach, that is described in Table 1.

Table 1: Methodology of the SynBio GMP Food Feed Working Group to address the mandate

Phase 1	**Aims**	**Confirm the use of case studies** to identify potential novel hazards and risks for humans and animals that could be posed by food and feed from GMPs obtained through current and near future SynBio approaches (ToR2); and to determine whether the existing guidelines for food and feed risk assessment are adequate and sufficient or whether there is a need for update (ToR3). **Confirm the relevance of the case studies previously identified** (EFSA GMO Panel, 2021a) for this working package (WP4), i.e., their relevance for food and feed risk assessment.
	Approach	The WG analysed the three test cases previously identified (EFSA GMO Panel, 2021a) as regards their relevance for food and feed risk assessment.
	Outcome	See Section 2.2 of the Opinion.
Phase 2	**Aim**	**Expand the pool of case studies** relevant for food and feed risk assessment and possibly coming to the market in the near future, based on the outcome of Phase 1.
	Approach	Building on the opinion of EFSA GMO Panel (2021a), and the horizon scanning exercises performed in WP2,[(a)] the WG identified an additional, hypothetical case study to challenge further previously identified aspects (such as the 'concept of the comparator') of relevance for food and feed risk assessment.
	Outcome	See Section 2.2 of the Scientific Opinion.
Phase 3	**Aim**	**Verify if existing guidelines are adequate and sufficient** for the evaluation of potential novel hazards and risks for humans and animals that could be posed by food and feed from GMPs obtained through current and near future SynBio approaches, **or whether there is a need for updated guidance** (addressing ToR2 and ToR3).
	Approach	The WG conducted a detailed scrutiny of the relevant guidelines using the four case studies identified in Phase 2.
	Outcome	See Section 3 of the Opinion.
Phase 4	**Aim**	If updated guidance is needed, **identify the specific areas where such updated guidance is needed** (ToR4).
	Approach	Based on the analysis of the outcome of Phase 3, the WG identified areas where guideline updates are needed.
	Outcome	See Section 4 of the Opinion.

(a): https://efsa.onlinelibrary.wiley.com/doi/abs/10.2903/sp.efsa.2020.en-1687

2.2. Selection of case studies to address WP4

Based on a 2019 horizon scanning exercise,[9] the GMO Panel had previously selected three hypothetical case studies (Table 2) representing plant products that were likely to be developed using state-of-the-art SynBio approaches with the potential to reach the market within the next decade. The GMPs described in these hypothetical case studies were developed using existing techniques (transgenes insertion and genome editing); however, the technological complexity of the engineering process or the product itself would require the application of SynBio approaches. The three cases had

[8] http://www.efsa.europa.eu/sites/default/files/wgs/gmo/wg-plant-synbio-ERA.pdf to be updated.
[9] https://efsa.onlinelibrary.wiley.com/doi/abs/10.2903/sp.efsa.2020.en-1687

been considered suitable and used for the assessment of the adequacy of guidelines for the MC and ERA of SynBio GMPs (EFSA GMO Panel, 2021a).

The GMO Panel considered the previously selected Synbio case studies also appropriate to address WP4, since they could show a range of characteristics of relevance for the food and feed risk assessment (e.g. case study 1 – maize expressing several new proteins and producing a new compound compared with conventional maize; case study 2 – wheat with altered levels of multiple endogenous constituents; case study 3 – oilseed rape with enhanced agronomic traits). In addition, the GMO Panel included a case study reflecting the rapid pace of developments in SynBio applications and which could be used to further assess the adequacy and sufficiency of the comparator-based approach in the case of extensive compositional, agronomic or phenotypic changes as compared to the original material, as for example the *de novo* domestication of wild species. The new example identified is a *de novo* domestication product in which multiplex genome editing (CRISPR/Cas9 for loss/gain of function) is used to rapidly 'domesticate' *Solanum pimpinellifolium* (a wild species closely related to domesticated tomato) so that the developed material has the nutritional and high yield characteristics of cultivated tomato varieties (*S. lycopersicum*) combined with the increased pathogen resistance and stress tolerance intrinsic to *S. pimpinellifolium* (Table 2, case study 4).

Table 2: Case studies selected to address WP4

Case study	Crop and intended trait/phenotype	Technology
1	Sweet maize producing vitamin B12[(a)]	Transgenic insertion of a molecular stack containing multiple engineered genes from one or more bacterial vitamin B12 biosynthesis pathways
2	Low-gluten wheat[(a)]	Targeted disruptive mutations of multiple alpha-gliadin genes using genome editing
3	Fungus-resistant oilseed rape[(a)]	Transgenic insertion of a plant resistance gene and genome engineering of susceptibility genes
4	*De novo* domesticated tomato	Targeted gene editing of wild tomato to complement useful endogenous traits with acquired traits of current domesticated, commercial crop variants

(a): EFSA GMO Panel (2021a).

While showing a technological complexity analogous to case study 2, in this case instead of introducing new traits in a domesticated crop a wild plant was modified to introduce typical domestication traits.

This hypothetical example is based on two reported studies, Li et al. (2018) and Zsögön et al. (2018). In both studies, multiplex CRISPR-Cas9 editing of coding sequences, *cis*-regulatory regions and upstream open reading frames were used to introduce into wild species domestication traits present in commercial plants currently used as food/feed. This approach permits the synergistic combination of useful traits from wild lines typically lost during selection, with agronomically desirable traits already present in commercial varieties. In this example, the stress-tolerant *S. pimpinellifolium* was modified (by loss/gain of function) to deliver a plant with characteristics most similar to those present in current domesticated cultivars, including for example compact architecture, synchronised fruit ripening, day-length insensitivity, large fruit size and high nutrient content (vitamin C and lycopene levels).

2.3. Existing guidance documents and guidelines considered in this Opinion

In delivering this Opinion, the GMO Panel together with the WG considered the current GMO legislation and related EFSA guidance documents and statements relevant for the food and feed risk assessment[10] (Table 3).

For the development of this Opinion, documents in Table 3 were screened for their adequacy and sufficiency for the food and feed risk assessment of GMPs obtained through current and near future SynBio developments.

[10] Up to December 2021.

Table 3: Documents relevant for the food and feed risk assessment of Synbio GMPs

	Reference	Title
1	Directive 2001/18/EC	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
2	Commission Directive 2018/350	Commission Directive (EU) 2018/350 of 8 March 2018 amending Directive 2001/18/EC of the European Parliament and of the Council as regards the environmental risk assessment of genetically modified organisms
3	Regulation (EC) No 1829/2003	Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed
4	EC Regulation No 503/2013	Commission Implementing Regulation (EU) No 503/2013 of 3 April 2013 on applications for Authorisation 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
5	EFSA GMO Panel (2011a)	Guidance for risk assessment of food and feed from genetically modified plants
6	EFSA GMO Panel (2011b)	Guidance on selection of comparators for the risk assessment of GM plants
7	EFSA Scientific Committee (2011)	Guidance on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed
8	EFSA GMO Panel (2015)	Guidance on the agronomic and phenotypic characterisation of GMPs
9	EFSA GMO Panel (2017)	Guidance on allergenicity assessment of genetically modified plants
10	EFSA (2019b)	Statement on human dietary exposure to GM food
11	EFSA (2014)	Explanatory statement for the applicability of the Guidance of the EFSA Scientific Committee on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed for GMO risk assessment

2.4. Consultation

In line with its policy on openness and transparency, EFSA consulted EU Member States and its stakeholders by an online public consultation. Between January 2022 and March 2022, all stakeholders were invited to submit their comments on the draft GMO Panel Opinion. Following this consultation process, the document has been revised by the GMO Panel and the experts of its ad hoc expert WG and comments received have been incorporated whenever appropriate. The outcome of the online public consultation is annexed to this Opinion (Annex B).

3. Assessment

3.1. General outline to food and feed risk assessment of GMPs

Regulation (EC) No 1829/2003 sets out the legal basis for the risk assessment of GM food and feed in the EU, providing for:

'a scientific evaluation to be carried out on the risks that the genetically modified food or feed may present for human and animal health'.

It also sets out that:

'a genetically modified food or feed must not differ from the food or feed that it is intended to replace to such an extent that its normal consumption would be nutritionally disadvantageous for humans or animals'.

To address the above, the EFSA GMO Panel developed a risk assessment strategy, which formed the basis of Regulation (EU) No 503/2013. This strategy is based on the comparison of GMPs (and derived food and feed) with closely related traditionally cultivated crops that have a 'history of safe use for consumers and/or domesticated animals' (EFSA GMO Panel, 2011a).

The starting points to structure and conduct the risk assessment of GMPs are a comprehensive **molecular characterisation** of the GM plant in question along with a **comparative analysis** of the relevant characteristics of the GM plant with respect to a comparator,[11] considering the natural variability defined by a set of non-GM reference varieties. Requirements for the MC and ERA of SynBio GMPs were previously assessed using the three selected case studies (EFSA GMO Panel, 2021a; see also Section 1.4 of this Opinion). Intended and unintended effects identified and characterised by MC and comparative analysis are assessed with regard to their relevance for food and feed safety and nutrition as below detailed:

- A toxicological and allergenicity assessment is conducted on newly expressed proteins, and on new compounds/altered levels of constituents identified by the comparative compositional analysis. In addition, existing guidelines require to investigate the potential adverse effects on the whole food and feed by a 90-day feeding study on single-event GMPs [Regulation (EU) No 503/2013, Annex II, II, 1.4 and 1.5 and relevant EFSA documents, see Table 3].
- A nutritional assessment is conducted to demonstrate that the intended/unintended effects of the genetic modification on the levels of endogenous constituents and/or the presence of new constituents identified by comparative compositional analysis do not represent a nutritional concern [Regulation (EU) No 503/2013, Annex II, 1.6].
- An estimate of the expected intake of the GMP products is conducted on the basis of representative consumption data for products obtained from conventional plants. Expected intake of endogenous constituents and/or new constituents shall be estimated. [Regulation (EU) No 503/2013, Annex II, 2, and EFSA documents, Table 3].

The risk assessment of GMPs as above described should be carried out on a case-by-case basis taking into account the type of genetic modification, and the different issues considered in the hazard identification and characterisation and in the exposure assessment (EFSA GMO Panel, 2011a).

The WG notes that the GMP risk assessment strategy (EFSA GMO Panel, 2011a,b; Regulation (EU) No 503/2013 – see Table 3) has been originally set for GMP applications bringing new traits (such as insect resistance, herbicide tolerance or compositional changes) – individually or in limited combinations – into crops commonly used as food and feed (e.g. maize, soybean, oilseed rape) for which representative consumption data are available. The food and feed risk assessment of such GMPs aims at identifying risks for humans and animals and confirming their 'substantial equivalence' to the conventional crops that they are intended to replace. A key element in addressing these risk assessment questions is the comparison of the GMP with an appropriate comparator (i.e. a conventional counterpart genetically similar and with a history of safe use).

3.2. Evaluation of the adequacy and sufficiency of the guidelines for the food and feed risk assessment of SynBio GMPs

To address ToR2, ToR3 and ToR4 of this mandate (Sections 1.2 and 1.3) the GMO Panel verified whether the existing guidelines on comparative analysis, toxicological, allergenicity, nutritional and dietary exposure assessment of GMPs were adequate and sufficient to address the risk assessment of food and feed from SynBio GMPs, or whether updates are needed, using the four identified case studies (Table 2) as examples.

3.2.1. Case study 1 – vitamin B12-producing maize

Case study 1 consists of a sweet maize engineered to produce vitamin B12 (cobalamin) by the transgenic insertion of a molecular stack containing multiple engineered genes from one or more bacterial vitamin B12 biosynthesis pathways (EFSA GMO Panel, 2021a). This test case is relevant to evaluate the adequacy and sufficiency of the food and feed risk assessment guidelines considering: the multiplicity of newly introduced genes and consequently the number of proteins (enzymes) newly expressed; the complexity of the introduced metabolic pathway and the potential impact of such enzymes (and their combination) on the plant metabolic pathways; and the *de novo* presence of vitamin B12 and possible metabolic by-products compared with conventional maize.

[11] Regulation (EC) No 1829/2003, Annex II, II, 1.3.1 and EFSA GMO Panel (2011b).

3.2.1.1. Comparative analysis

The GMO Panel previously concluded on the adequacy and sufficiency of the comparative analysis guidelines for this case study (EFSA GMO Panel, 2021a). This WG elaborated further on comparative analysis elements of relevance for the food and feed risk assessment (compositional analysis). The intended trait for this SynBio maize is the production of vitamin B12, a novel compound with respect to conventional maize. The addition of the endpoint 'vitamin B12' to the maize compositional endpoint list (OECD, 2002) would allow the identification and characterisation of the intended trait properly informing the food and feed risk assessment. The large standard range of endpoints recommended for maize by OECD (2002) would allow the identification of possible unintended compositional effects related to the genetic modification of relevance for the food and feed risk assessment. Information from the SynBio product design and optimisation could effectively anticipate the expected compositional characteristics of the SynBio product and inform risk assessors of the selection of appropriate analytes that might be relevant for food and feed safety.

In conclusion, the existing guidelines on comparative analysis would be **adequate and sufficient** for the food and feed risk assessment of case study 1.

3.2.1.2. Toxicology

The GMO Panel verified the adequacy and sufficiency of the existing guidelines for the toxicological assessment of newly expressed proteins, new compounds, altered levels of constituents and the whole food/feed.

Several proteins are newly expressed in this maize (EFSA GMO Panel, 2021a). According to the existing guidelines, a toxicological assessment would be required for all these new proteins individually. If the HoSU of the genes' sources and of the newly expressed proteins is not demonstrable, animal studies on each individual protein would be needed (primarily 28-day repeated-dose toxicity studies). MC, bioinformatics analyses and *in vitro* studies (stability to heat and pH; digestibility) on the new proteins are needed to further inform the assessment. It would also be necessary to evaluate if there are potential synergistic or antagonistic interactions of possible safety concerns between the numerous proteins newly expressed, and to conduct combination studies if necessary. On the basis of the available information for case study 1, there are insufficient elements supporting a HoSU of both the genes' sources and the newly expressed proteins. The introduced genes in this maize derive from bacteria (e.g. *Pseudomonas denitrificans*, *Rhodobacter capsulatus*) found primarily in soil and/or surface water. There is no evidence that humans or animals relevant for the food and feed safety assessment have any significant exposures to these organisms through the diet or other routes. No pathway for vitamin B12 synthesis is present in non-modified plant or animal cells. In ruminants, and other mammals that have intestinal fermentation, vitamin B12 is produced by bacteria in the gut and subsequently absorbed and stored. The principal sources of vitamin B12 in the diet are animal products such as meat, liver, dairy products and fish. It is therefore expected that the applicant would need to provide 28-day repeated-dose toxicity studies on each individual protein newly expressed in this maize. It would also be anticipated that the applicant would have to provide molecular characterisation, bioinformatics analyses and *in vitro* studies on each new protein and an assessment of potential interactions among them.

This scenario would challenge the current strategy for the toxicological assessment of new proteins with:

- the need for numerous animal studies, implying the use of hundreds of animals in contrast with the EU aim to replace, reduce and refine the use of animals for scientific purposes[12];
- limitations in the amounts of test material of sufficient quality for safety studies on all the newly expressed proteins, particularly if some proteins are intractable (Bushey et al., 2014; Hurley et al., 2016; Eaton et al., 2017; EFSA GMO Panel, 2021b, 2022).

The GMO Panel considers that new approach methodologies (NAMs) would have to be developed and validated to overcome the above issues and strengthen the safety assessment. These could include, for example *in silico* toxicity predictive tools and *in vitro* studies. In addition, the SynBio approach itself could help in strengthening the toxicological assessment of newly expressed proteins. For example, the new sequences to be introduced should be selected in the design phase from source

[12] Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

organisms having a HoSU if feasible, therefore waiving the need for *in vivo* toxicological studies. Also, the *in silico* tools and *in vitro* studies conducted in the modelling and validation phases could be designed to include considerations on the safety aspects of the new proteins. In conclusion, the existing guidelines are **adequate** for the toxicological assessment of proteins newly expressed in this case study. Because of the high number of new proteins to test, the GMO Panel sees the need for the **development of alternative strategies** to overcome practical challenges.

Regarding new compounds other than proteins, vitamin B12 is not present in conventional maize and therefore is considered as a new compound for this crop to be assessed. According to the existing guidelines, an evaluation of the toxic potency of the new compound, considerations on the need of toxicological testing and a determination of its concentration in the GM food and feed would be needed. If the new compound did not have a HoSU for consumption in food and feed, the applicant shall provide additional information.[13] Vitamin B12 is comprehensively described in its dietary sources, biological role and effects of excess intake (EFSA NDA Panel, 2015). This vitamin is commonly present in the human diet from animal -derived foods. No adverse effects of vitamin B12 were identified that could be used as a basis for deriving a tolerable upper intake level (UL). Therefore, no further toxicological studies on vitamin B12 are required. Further considerations on the safety of vitamin B12 will be discussed in the section on nutritional assessment. In conclusion, the existing guidelines would be **adequate and sufficient** for the toxicological assessment of new compounds in case study 1.

The assessment of unintended altered levels of constituents in this maize would be driven by the outcome of comparative compositional assessment. For changes of the levels of constituents beyond the natural variation identified by the comparative compositional analysis, a detailed risk assessment would be conducted based on the knowledge of the physiological function and/or toxic properties of these constituents, and their history of consumption. If needed, additional toxicological tests should be provided. In conclusion, the existing guidelines would be **adequate and sufficient** for the toxicological assessment of altered levels of endogenous constituents in case study 1.

Regulation (EU) No 503/2013 requires a 90-day toxicity study in rodents on the whole food and feed from GMPs. Other studies testing the whole food and feed on animals may be required, on a case-by-case basis and triggered by MC and comparative analysis/compositional studies. The GMO Panel is of the opinion that a 90-day study on the whole food and feed would not provide any added value for the safety assessment of this maize considering the intended trait, because there are no toxicological concerns associated with excess vitamin B12 and no ULs have been set (EFSA NDA Panel, 2015). Potential hypothesis triggering a 90-day feeding study on the whole food and feed, as well as other animal feeding studies, would depend upon the outcome of comparative analysis. In conclusion, the existing guidelines would be **adequate and sufficient** for the conduction and assessment of a 90-day toxicity study on the whole food and feed in case study 1.

3.2.1.3. Allergenicity

The GMO Panel evaluated the adequacy and sufficiency of the existing guidelines on allergenicity assessment. The specific allergy risk of GMPs is associated with: (1) exposure to newly expressed protein(s) that can be present in edible parts of the plants or in the pollen; this point is related to the biological source of the transgene; and (2) changes to the allergenicity of the whole plant and its products, for example due to overexpression of natural endogenous allergens as an unintended effect of the genetic modification; this point is related to the biology of the recipient plant itself.

As regards the newly expressed proteins, the existing guidelines require a weight-of-evidence approach primarily based on (1) information on the source of the transgene and the protein itself relevant for allergenicity; (2) an amino acid sequence homology comparison between the newly expressed protein and known allergens; (3) pepsin resistance and *in vitro* digestibility tests; and (4) specific serum screening (if necessary) (Codex Alimentarius, 2009; EFSA GMO Panel, 2011a). Furthermore, the possible role of the newly expressed proteins as adjuvants should be assessed when known functional aspects or structural similarities may indicate a possible adjuvant activity. Finally, an assessment of newly expressed proteins in relation to their potential to cause celiac disease is also performed (EFSA GMO Panel, 2017). In conclusion, the existing guidelines are **adequate** for the

[13] Additional information would be analogous to that described in the 'Guidance for submissions for food additive evaluations by the EFSA Panel on Food Additives and Nutrient Sources added to Food' of 16 August 2012 and Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives [Regulation (EU) 503/2013 Annex II, II].

allergenicity assessment of proteins newly expressed in case study 1. This scenario would challenge the approach currently followed (see also Section 3.2.1.2 Toxicology above) and there is a need to consider whether studies that were actually developed for assessing GMPs expressing a limited number of new individual proteins (e.g. *in vitro* digestibility studies) could be practically used for assessing SynBio GMPs expressing a high number of new proteins as in this case study. The GMO Panel has taken a step forward and recently published an opinion on **development needs for the allergenicity** and protein safety assessment of food and feed products derived from biotechnology (EFSA GMO Panel, 2022).

As regards the allergenicity assessment of the whole plant, maize is not considered a common allergenic food (OECD, 2002). Therefore, based on the existing guidelines, experimental data to analyse the allergen repertoire of this maize and to compare it with that of its conventional counterpart is not necessary on a routine basis (EFSA GMO Panel, 2011a). However, depending on the outcome of the composition and MC, specific experimental data targeting endogenous allergens in maize may be requested. A guidance on the selection of allergens for assessment, and the interpretation of results can be found in the EFSA GMO Panel guidance on (2017). In conclusion, the existing guidelines would be **adequate and sufficient** for the allergenicity assessment of the case study 1.

3.2.1.4. Nutritional assessment

The nutritional assessment per se always starts after the completion of the comparative compositional assessment that will inform on changes in composition (intended and unintended). The relevance of these changes from a nutritional point of view will be assessed following a weight-of-evidence approach that compiles information on the biological relevance of the compounds under assessment, the role of the crop as contributor to the total intake of the compounds, and the magnitude and direction of the observed changes. The GMO Panel evaluated the adequacy and sufficiency of existing guidelines for the nutritional assessment of case study 1 involving the presence of vitamin B12 and any other additional endpoint that might be identified during the comparative compositional analysis.

In the context of the human nutritional assessment, vitamin B12 is a compound with biological relevance as its deficiency leads to megaloblastic anaemia and neurological disfunction among other symptoms (Ralapanawa et al., 2015). In 2015, the EFSA NDA Panel set Adequate Intakes (AI) across different population groups, from 1.5 μg/day in infants aged 7–11 months to 4 μg/day for children aged 15–17 years old and adults, with higher requirements for pregnant women (4.5 μg/day) and lactating women (5 μg/day) (EFSA NDA Panel, 2015). However, in case study 1, vitamin B12 is a new constituent and it is expected to be at relatively high levels to convert maize into a relevant source of vitamin B12. Therefore, a higher intake of this vitamin is expected in the population consuming the GM maize (and its products) compared with those consuming conventional maize (and its products). This information indicates that the nutritional assessment of this GM maize should focus on safety concerns linked to excess vitamin B12 intake rather than to deficiency. As, currently, toxicological evidence indicates that an excess of vitamin B12 is not adverse and no UL exists for vitamin B12 (EFSA NDA Panel, 2015), it can be concluded that the consumption of vitamin B12 from this maize and its products does not represent a safety concern. Should the current toxicological information on vitamin B12 change in the future a new nutritional assessment might be required. This new assessment might need to include an estimation of the dietary intake of vitamin B12 considering the predicted consumption of the GM maize (and its products), and a comparison with reference doses if available (e.g. UL).

As regards animal nutritional assessment, vitamin B12 is a compound of biological relevance for farmed (food producing and non-food producing) and companion animals. Essential for DNA synthesis, its deficiency causes the inhibition of cell division, which might severely affect animal health (MacDonald, 2011). Studies on mice suggest that levels of vitamin B12 in the diet of at least several hundred times the requirement are well tolerated (NRC, 1987). Vitamin B12 is listed in the EU Register of Feed Additives, pursuant to Regulation EC No 1831/2003, under the subclassification of vitamins and provitamins, but no maximum content in complete feed or daily ration is set. It is important to consider that sweet corn is not a commonly used variety for animal feed. Moreover, it is unlikely that farm and companion animals fed balanced rations or commercial diets will receive excessive doses of vitamin B12. Therefore, the consumption of the maize described as case study 1 and its products does not represent a nutritional concern in farmed and companion animals.

In conclusion, the existing guidelines would be **adequate and sufficient** for the nutritional assessment of the case study 1 in humans and animals.

3.2.1.5. Dietary exposure

Several proteins are newly expressed in this SynBio case (EFSA GMO Panel, 2021a). In line with the existing guidelines, dietary exposure to the proteins newly expressed in the GMP should be estimated for humans and animals. Dietary exposure should be based on the expression levels reported for these enzymes, the current available consumption data and feed practices, the conventional foods and feeds (and their products) currently available in the market and the described processing conditions. Further details on human and animal dietary exposure are described in EFSA (2019a,b). The existing guidelines would be **adequate and sufficient** for dietary exposure assessments of case study 1.

3.2.1.6. Conclusions case study 1

Based on the scrutiny of the existing guidelines, the GMO Panel concludes that these would be **adequate** for the food and feed risk assessment of SynBio case study 1 but would need **further developments** as regards the safety assessment of new proteins. The current strategy might face practical challenges because of the large number of newly expressed proteins in this type of SynBio product. The GMO Panel sees the need to review the guidelines as new approaches are becoming available for use in risk assessment to strengthen and streamline the protein safety assessment (e.g. based on NAMs such as *in silico* prediction tools and in vitro testing for protein toxicity and allergenicity, see EFSA GMO Panel, 2022).

3.2.2. Case study 2 – low-gluten wheat

SynBio case study 2 is a low-gluten wheat obtained by targeted disruptive mutations of multiple α-gliadin genes using CRISPR/Cas9 genome editing. This is intended to decrease α-gliadin concentration and therefore decrease the gluten content. No new proteins are expressed in this SynBio GMP. The GMO Panel previously concluded that this test case differs, from the MC viewpoint, from the GMP applications assessed to date, being obtained by genome editing (EFSA GMO Panel, 2021a). The genetic intervention is intended to target the plant composition acting on multiple endogenous genes; the consequent compositional modifications (intended and unintended) make this case study of relevance to evaluate the adequacy and sufficiency of the food and feed risk assessment guidelines.

3.2.2.1. Comparative analysis

The GMO Panel had previously concluded on the adequacy and sufficiency of the comparative analysis guidelines for this case study (EFSA GMO Panel, 2021a). Compositional endpoints for food and feed risk assessment should be selected to identify and characterise the intended effect of the genetic intervention (i.e. α-gliadin decreased levels) as well as possible variations in the levels of other constituents and/or *de novo* synthesised or produced compounds occurring as a compensatory effect. For example, other gluten proteins, such as glutenins, or other biologically inactive storage proteins in wheat (OECD, 2003) should be evaluated (see Section 3.2.2.3 below for further details). The knowledge available on the SynBio design and modelling could effectively support an informed decision on the selection of endpoints to properly characterise the composition of this SynBio GMP as to intended and unintended effects.

The GMO Panel concludes that the existing guidelines on comparative analysis would be **adequate and sufficient** for the food and feed risk assessment of the case study 2.

3.2.2.2. Toxicology

The GMO Panel evaluated the adequacy and sufficiency of the existing guidelines with regards to the toxicology assessment as previously described (see Section 3.2.1.2).

No new proteins are intended to be expressed in this SynBio GMP (EFSA GMO Panel, 2021a) and therefore the toxicological assessment of new proteins does not apply.

For new compounds/altered levels of endogenous constituents, an alteration of the levels of gliadins beyond the natural variation is expected and would be identified by comparative compositional analysis; no toxicological hypothesis requiring further investigation would be identified based on the knowledge of the physiological function and/or toxic properties of these constituents. Potential unintended effects leading to the expression or production of new compounds or altered levels of

constituents (e.g. due to compensatory effects on other glutenins, see also Section 3.2.2.3) would be identified in the comparative analysis and would need toxicological assessment.

For the toxicological assessment of the whole food and feed, if the only changes in composition of this SynBio crop are reduced α-gliadin levels (intended trait) and/or in compensatory effects on other glutenins (unintended effects), these would not translate into an *a priori* hypothesis to be tested in a 90-day feeding study in rodents. In fact, the rat model is not sensitive to capture glutenin/gliadin-related effects of relevance for human and animal target populations. The outcome of comparative analysis would allow the identification of possible unintended effects (other than glutenins) as possible toxicological hypotheses to be explored in a targeted 90-day feeding study.

In conclusion, the existing guidelines would be **adequate and sufficient** for the toxicological assessment of this case study.

3.2.2.3. Allergenicity

The GMO Panel evaluated the adequacy and sufficiency of the existing guidelines with regards to the allergenicity assessment as described above (see Section 3.2.1.3).

No new proteins are intended to be expressed in this SynBio plant (EFSA GMO Panel, 2021a) and therefore the allergenicity assessment of new proteins does not apply.

The allergenicity assessment of new compounds or altered levels of constituents will be conducted on the basis of the results of the comparative compositional analysis. As above indicated, compositional analysis could be strengthened by the knowledge on the design and modelling, allowing the identification of unintended effects of relevance for allergenicity assessment. Wheat is listed in Commission Directive 2007/68/EC24 and subsequently in the Annex II of the EU Regulation 1169/2011 on food information to consumers,[14] which include a list of most common allergenic foods. According to Regulation (EU) 503/2013:

> 'when the recipient plant is known to be allergenic, the applicant shall assess any potential change in the allergenicity of the genetically modified food or feed by comparison of the allergen repertoire with that of its conventional counterpart. The potential overexpression of natural endogenous allergen(s) in the genetically modified plant shall, in particular, be investigated'.

The identification of proteins relevant for the endogenous allergenicity assessment of wheat should be conducted according to the principles previously described in the EFSA guidance on allergenicity of GMP (EFSA GMO Panel, 2017). The wheat gluten proteins (i.e. gliadins and glutenins) are active in celiac disease (non-Immunoglobulin E (IgE)-mediated reactions) and in IgE-mediated food allergy reactions. In addition, wheat non-gluten proteins, such as an alpha-amylase/trypsin inhibitor, lipid transfer proteins (LTPs), cupins and profilins, have been identified as important B-cell epitopes in wheat allergy (including baker's asthma) (EFSA NDA Panel, 2014). In this context, it has been described that the reduction in the gliadin content in wheat engineered with RNA interference (RNAi)-mediated gene silencing (Gil-Humanes et al., 2014) or CRISPR/Cas9 technology (Sánchez-León et al., 2018) may promote a compensatory response in glutenins, increasing their high molecular weight fraction. The methodology for quantification of endogenous allergens and gluten proteins, as well as the data interpretation for the risk assessment should be also performed in line with the relevant EFSA GMO Panel guidance document (2017).

In conclusion, the existing guidelines would be **adequate and sufficient** for the allergenicity assessment of this case study.

3.2.2.4. Nutritional assessment

Based on the weight-of-evidence-approach as described in case 1 (Section 3.2.1.4), the GMO Panel evaluated the adequacy and sufficiency of the existing guidelines for the nutritional assessment of case study 2, a low-gluten wheat obtained by targeted disruptive mutations of multiple α-gliadin genes using genome editing. Beyond the consideration on non-IgE- and IgE-mediated reactions, the changes in the levels of gluten proteins should not represent a nutritional concern, although attention will be paid to possible effects on the proteins in wheat grains.

[14] 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 608/2004.

In conclusion, the existing guidelines would be **adequate and sufficient** for the nutritional assessment of this case study.

3.2.2.5. Dietary exposure

The GMO Panel evaluated the adequacy and sufficiency of the existing guidelines for this case study as described in case study 1 (Section 3.2.1.5.) and concludes that they would be **adequate and sufficient** for the dietary exposure assessment in case study 2.

3.2.2.6. Conclusions case study 2

Based on the scrutiny of the existing guidelines, the GMO Panel concludes that these would be **adequate and sufficient** for the food and feed risk assessment of SynBio case study 2.

3.2.3. Case study 3 – fungal-resistant oilseed rape

Case study 3 is a fungal-resistant oilseed rape obtained by transgenic insertion of a plant resistance gene and deletion through genome engineering of 'susceptibility' genes, which promote the infection processes by mediating host recognition by the pathogen in the early stages (EFSA GMO Panel, 2021a). This case study is considered relevant to evaluate the adequacy and sufficiency of existing guidelines for the food and feed risk assessment of SynBio GMP with agronomic intended traits and potential compensatory effects.

3.2.3.1. Comparative analysis

The GMO Panel previously concluded on the adequacy and sufficiency of comparative analysis guidelines for this case study (EFSA GMO Panel, 2021a). There are no intended compositional changes in this case study. Considering the intended trait, the compositional endpoints described in OECD 2011 are adequate to investigate possible unintended effects related to the genetic modification, and no additional endpoints are needed. Information from the SynBio product design and optimisation could effectively anticipate the expected compositional characteristics of the SynBio product and inform risk assessors of the selection of appropriate analytes that might be relevant for food and feed safety. In conclusion, the existing guidelines on comparative analysis would be **adequate and sufficient** for the food and feed risk assessment of case study 3.

3.2.3.2. Toxicology

The GMO Panel evaluated the adequacy and sufficiency of the existing guidelines with regard to the toxicological assessment as previously described (see Section 3.2.1.2).

The genetic modification in this case study aims at introducing a new protein conferring an agronomic trait. The toxicological assessment of this new protein will take into account the HoSU of the gene's source and of the protein, MC, bioinformatics analyses and *in vitro* studies (stability to heat and pH; digestibility). If the HoSU of the gene's source and of the newly expressed protein is not demonstrable, a 28-day repeated-dose toxicity study would be needed.

No new compounds or altered levels of endogenous constituents are expected considering the intended trait. Potential unintended effects leading to the expression or production of new compounds or altered levels of constituents would be identified in the compositional analysis and would need toxicological assessment.

As regards the whole food and feed, the intended trait is based on an enhancement of the plant's natural immune system such that no new molecules would be produced, there is no hypothesis to trigger a 90-day feeding study on whole food/feed. A potential hypothesis triggering a 90-day feeding study would be driven by the outcome of the comparative analysis.

In conclusion, the existing guidelines would be **adequate and sufficient** for the toxicological assessment of case study 3.

3.2.3.3. Allergenicity

The GMO Panel evaluated the adequacy and sufficiency of the existing guidelines with regards to the allergenicity assessment as described above (Section 3.2.1.3).

For the allergenicity assessment of proteins newly expressed in case study 3, the GMO Panel concludes that the existing guidelines would be **adequate and sufficient**.

For the allergenicity assessment of the genetically modified food or feed, oilseed rape is not considered a common allergenic food (OECD, 2011). Therefore, experimental data to analyse the

allergen repertoire of this SynBio oilseed rape and to compare it with that of its conventional counterpart is not necessary on a routine basis (EFSA GMO Panel, 2011a). However, depending on the outcome of the compositional analysis and MC, specific experimental data targeting endogenous allergens in oilseed rape would be requested. A guidance on the selection of allergens for assessment, and the interpretation of results can be found in the EFSA GMO guidance on allergenicity (2017).

In conclusion, the requirements of existing guidelines would be **adequate and sufficient** for the allergenicity assessment of case study 3.

3.2.3.4. Nutritional assessment

Based on the weight-of-evidence-approach as described in case 1 (Section 3.2.1.4), the GMO Panel evaluated the adequacy and sufficiency of the existing guidelines for the nutritional assessment of case study 3, a fungus-resistant GM oilseed rape. In conclusion, the existing guidelines would be **adequate and sufficient** for the nutritional assessment of this case study.

3.2.3.5. Dietary exposure

The GMO Panel evaluated the adequacy and sufficiency of the existing guidelines for this case study (as described in case study 1, Section 3.2.1.5) and concludes that they would be **adequate and sufficient** for the dietary exposure assessment in case study 3.

3.2.3.6. Conclusions case study 3

Based on the scrutiny of the existing guidelines, the GMO Panel concludes that these would be **adequate and sufficient** for the food and feed risk assessment of the SynBio case study 3.

3.2.4. Case study 4 – *De novo* domesticated tomato

This case study has not been analysed in the previously published opinion covering the MC and ERA of GMPs developed by SynBio approaches (EFSA GMO Panel, 2021a). It is a *de novo* domestication product in which multiplex editing is used to introduce into *Solanum pimpinellifolium* (a wild species closely related to domesticated tomato) nutritional and high yield characteristics of commercially cultivated tomato varieties (*Solanum lycopersicum*), for an analogous consumption as food and feed (see Section 2.2).

3.2.4.1. Comparative analysis

This case study highlighted potential issues for the applicability of the existing comparative analysis guidelines with respect to the availability of the conventional counterpart and non-GM reference varieties. The parental line used to obtain this SynBio product (*S. pimpinellifolium*) is not commonly consumed; sporadic consumption of this plant can be considered in the context of the food and feed risk assessment but would not be sufficient to support a solid HoSU of this product. The selection of reference varieties would also be challenging: wild tomato varieties of commercial use as food and feed might not be available. Tomatoes cultivated for food and feed purposes could be of interest for comparison, considering the intended use of the SynBio tomato, but would be genetically far from the SynBio plant. As a consequence of the lack of an appropriate comparator (EFSA GMO Panel, 2011a,b,c), the comparative analysis for this SynBio case may not be carried out as described in the existing guidelines.

The GMO Panel explored how to address the food and feed risk assessment of Synbio GMPs challenging the current comparative analysis, and identified two possible approaches, i.e. stand-alone assessment or the use of multiple comparators.

Stand-alone assessment

The GMO Panel has previously identified cases in which the selection of the comparator is challenging, namely GMPs with complex modifications (e.g. with major compositional changes, or with new agronomic traits) or where the GMP and derived food and/or feed 'is not closely related to a food and/or a feed with a history of safe use' (EFSA GMO Panel, 2011a,b) . For such cases, the GMO Panel recognised that a comparative risk assessment cannot be conducted and identified as an alternative approach the stand-alone safety and nutritional assessment of the GMP and derived food and feed (EFSA GMO Panel, 2011a,b). The stand-alone assessment would be focused on the characteristics of the genetic modification, on constituents of the GMP and, if needed, on the whole GM food and feed. This approach is in line with the assessment of novel foods, and as such taken up

by Regulation (EU) No 503/2013.[15] In particular, the GMO Panel had also recognised and recommended that the safety and nutritional assessment strategy for such cases would need to be further developed (EFSA GMO Panel, 2011b).The GMO Panel notes that a stand-alone assessment would focus on a specific SynBio product and its intended use/s (e.g. as commercial tomato for consumption in this case study). As a consequence, it would not be possible to conclude that the GMP is as safe and nutritious as a conventional crop and might imply a limitation of the general scope of the authorisation of GMPs as described in Regulation (EC) No 1829/2003.[16]

Multiple comparators

The GMO Panel also explored the use of multiple comparators as an alternative strategy to maintain elements of the comparative analysis for this Synbio GMP. In the current guidelines, additional comparators can be introduced on a case-by-case basis where agronomic conditions of relevance have to be represented in the comparative analysis (EFSA GMO Panel, 2011b, Regulation 503/2013). Extending this concept, a possible scenario for case study 4 would include multiple comparators. This would allow for the identification of effects related to the genetic modification and put them in the context of the food and feed risk assessment:

- a comparator with a similar genetic background, i.e. *S. pimpinellifolium*; this would allow the identification and characterisation of the intended effects of the SynBio approach and drive towards the selection of (additional) appropriate endpoints;
- a comparator/comparators having a HoSU could serve to put the findings into the food and feed risk assessment perspective. This/these comparator/s should be chosen taking into account the intended use of the SynBio product (e.g. commercial tomato).

Reference varieties should be chosen taking into considerations the intended use.

In conclusion, the GMO Panel considers that the existing guidelines would be **adequate** for SynBio GMPs such as the *de novo* domesticated plants. However, for such cases it may not be possible to carry out a comparative analysis as for GMP applications assessed to date, because essential items (a comparator and reference varieties in line with the existing guidelines) would not be available. The existing guidelines consider these cases, recognise the need to deviate from the classical comparative analysis, and propose general strategies on how to proceed for the food and feed risk assessment (i.e. stand-alone assessment of the composition; comparative analysis with multiple non-GM counterparts). The GMO Panel considers that elements of the comparative analysis could offer an added value and should be incorporated in the assessment of such SynBio GMPs. In general, current guidelines should be revised to support the comparative analysis of SynBIO GMPs against multiple comparators or for their stand-alone assessment.

3.2.4.2. Toxicology

The GMO Panel evaluated the adequacy and sufficiency of the existing guidelines with regards to the toxicology assessment as previously described (Section 3.2.1.2).

No new proteins are intended to be expressed in this SynBio GMP and therefore the toxicological assessment of new proteins does not apply. The identification and characterisation of new compounds and altered levels of endogenous constituents would be driven by the information from comparative analysis and/or compositional assessment, discussed above. The hypothesis to be investigated in a 90-day toxicity study on the whole food and feed would derive from comparative analysis/compositional assessment. The strategy to select an appropriate comparator should be developed, in line with the above.

In conclusion, existing guidelines would be **adequate and sufficient** for the toxicological assessment of this case study.

3.2.4.3. Allergenicity

The GMO Panel evaluated the adequacy and sufficiency of the existing guidelines with regards to the allergenicity assessment as described above (Section 3.2.1.3).

[15] Where no appropriate conventional counterpart can be identified, a comparative safety assessment cannot be made and consequently a safety and nutritional assessment of the genetically modified food or feed shall be carried out as for novel foods falling within the scope of Regulation (EC) No 258/97 of the European Parliament and of the Council (Regulation 503/2013, Annex II, II).

[16] GMPs for food and feed uses, food and feed containing and consisting of GMPs and food/feed produced from or containing ingredients produced from GMPs.

No new proteins are intended to be expressed in this SynBio GMP and therefore the allergenicity assessment of new proteins does not apply. The identification and characterisation of new compounds and of altered levels of endogenous constituents of relevance for the allergenicity assessment would be driven by the information from comparative analysis and/or compositional assessment, above discussed.

In conclusions, existing guidelines would be **adequate and sufficient** for the allergenicity assessment of this case study.

3.2.4.4. Nutritional assessment

Based on the weight-of-evidence-approach as described in case 1 (Section 3.2.1.4), the GMO Panel evaluated the adequacy and sufficiency of the existing guidelines for the nutritional assessment of case study 4. New compounds and altered levels of endogenous constituents of relevance for the nutritional assessment will be properly identified and characterised from the comparative analysis and/or the compositional assessment above discussed. Depending on the changes identified, the nutritional assessment might include dietary intake estimations considering the predicted consumption of the SynBio product, and a comparison with reference doses if available (e.g. UL, maximum doses without adverse effects, etc.). In principle, existing guidelines would be **adequate and sufficient** for the nutritional assessment of this case study.

3.2.4.5. Dietary exposure

The GMO Panel evaluated the adequacy and sufficiency of the existing guidelines for this case study (as described in case study 1, Section 3.2.1.5) and concludes that they would be **adequate and sufficient** for the dietary exposure assessment in case study 4.

3.2.4.6. Conclusions case study 4

The GMO Panel concludes that existing guidelines these would be **adequate** but would need **further developments** as regards the comparative analysis of the SynBio case study 4. Current guidelines should be further developed to provide more detailed procedures for the comparative analysis with multiple comparators or for stand-alone assessments.

4. Conclusions and Recommendations

4.1. Conclusions on ToR1

Previous conclusions regarding whether and which newer sectors/advances should be considered among SynBio developments (in addition to the six identified by the Scientific Committees) remain applicable (EFSA GMO Panel, 2021a).

4.2. Conclusions on ToR2

Based on Synbio plant products expected to be developed for the market in the next decade, the GMO Panel did not identify novel potential hazards and risks for humans and animals (farmed and companion animals) that could be posed by food and feed from GM plants obtained through current and near future SynBio approaches.

4.3. Conclusions on ToR3

Based on selected hypothetical case studies representing Synbio plant products likely to be developed for the market in the next decade, the GMO Panel considers that the existing guidelines are adequate and sufficient for the food and feed risk assessment of some SynBio GMP applications. However, in other cases the existing guidelines are adequate but need updating. Considering the range of GMPs that can be obtained by SynBio, the GMO Panel highlights that a case-by-case approach is pivotal to complete the risk assessment.

4.4. Conclusions on ToR4

Areas where the existing guidelines may need updating are those related to the **safety assessment of new proteins** and the **comparative analysis**.

The potential for a large number of new proteins expressed in SynBio GMPs would imply practical challenges. The GMO Panel sees the need to review the guidelines as new approaches are becoming available for use in risk assessment to streamline and strengthen the protein safety assessment (e.g. based on NAMs such as *in silico* prediction tools and *in vitro* testing for protein toxicity and allergenicity, see EFSA GMO Panel, 2022).

The GMO Panel confirms that the comparative analysis may not always be applicable to some SynBio GMPs for which a comparator cannot be identified (EFSA GMO Panel, 2021a). Such situations are described by the existing guidelines, which however provide only general strategies on how to proceed for the food and feed risk assessment. The GMO Panel recommends that the comparative strategy remains the preferred approach for the risk assessment of SynBio GMP food and feed, though updated guidance documents are needed for the comparative analysis in cases where a comparator cannot be identified, focusing on:

- the choice of the comparator and the identification of multiple comparators;
- the comparative analysis protocol and related statistical analysis.

In case a stand-alone assessment of the SynBio GMP is conducted, an appropriate procedure should also be developed. For that purpose, potential integration of risk assessment approaches for the safety and nutritional assessment of the SynBio GMP and derived food and feed as for other novel foods, as already indicated by the GMO Panel (EFSA GMO Panel, 2011a,b) and Regulation (EU) No 503/2013 should be considered.

4.5. Recommendations

The knowledge available from the SynBio design and modelling could effectively anticipate the expected characteristics of the SynBio product and inform risk assessors of the selection of appropriate analytes that might be relevant for food and feed risk assessment. The reporting and use of such knowledge for the risk assessment should be integrated into future guidance documents.

Future guidance will need to encourage applicants to select plants to be modified and SynBio approaches based not only on practicability and interoperability, but also to support risk assessment, for example through a documented record of safe use and consumption of genes that provide the source of newly expressed proteins. As a way to facilitate the risk assessment of SynBio GMPs and their products and reduce the amount of data required, applicants should consider food and feed risk assessment aspects throughout the SynBio design process.

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Abbreviations

AI	adequate intake
CIR	Commission Implementing Regulation
ERA	Environmental risk assessment
GMP	genetically modified plants
GMO	genetically modified organism
HGT	horizontal gene transfer
HoSU	History of safe use
IgE	Immunoglobulin E
LTP	lipid transfer protein
MC	molecular characterisation
NAM	new approach methodologies
ORF	open reading frame
PMEM	post-market environmental monitoring
RA	risk assessment
SC	Scientific Committee
SCCS	Scientific Committee on Consumer Safety
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SCHER	Scientific Committee on Health and Environmental Risks
SDN	site-directed nucleases
SynBio	Synthetic Biology
SAM	Scientific Advice Mechanism
ToR	Term of Reference
UL	upper intake level
WG	Working Group
WP	Work package

Appendix A – Engineering cycle of synthetic biology (from EFSA GMO Panel, 2021a)

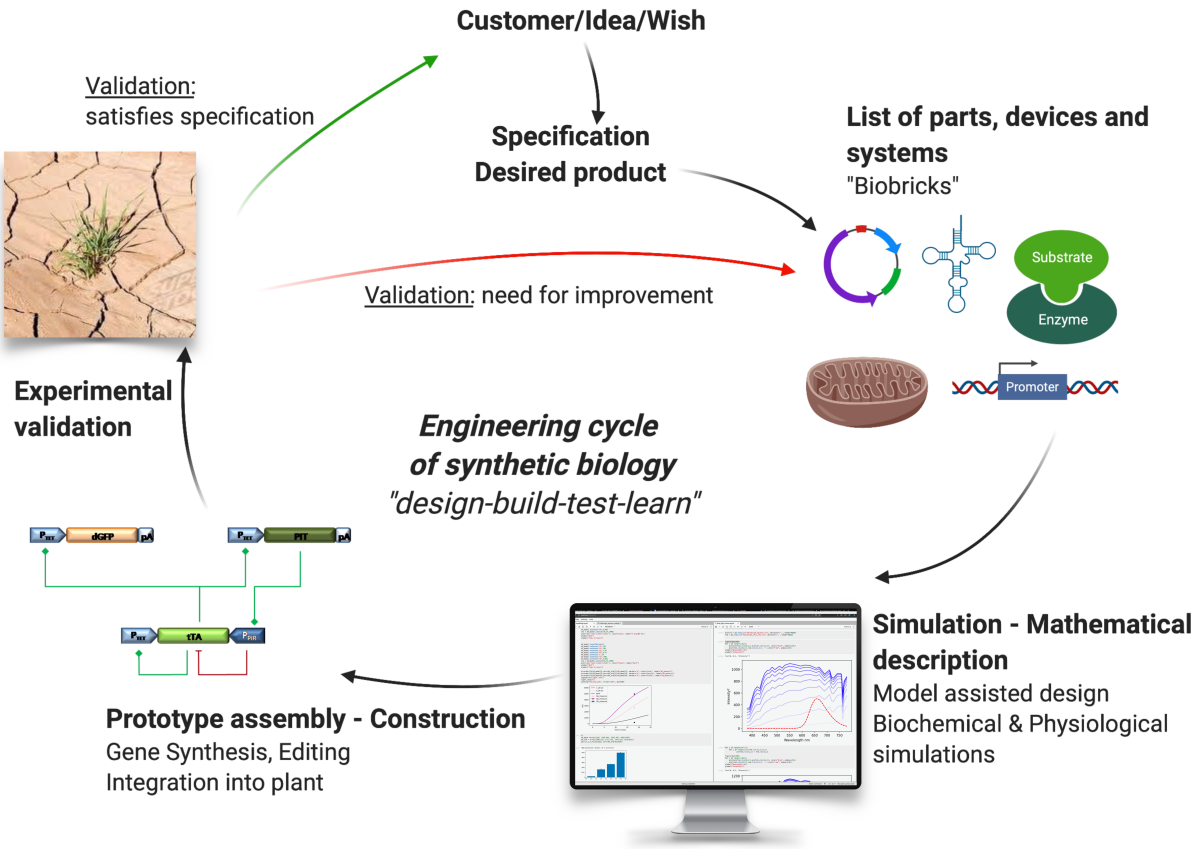

Figure A.1: Synthetic biology is a new discipline bridging engineering with life sciences. It applies basic engineering principles for the rational modular, combinatorial assembly of biological parts into higher order complex signaling and metabolic structures with novel, desired functionalities. Key to the strategy is the implantation of the engineering cycle in biology relying in mathematical modeling for the design and quantitative functional characterization of the molecular parts and for guiding the assembly, implementation, and optimization of the individual modules and networks. A prototype is assembled and experimentally tested. If the products satisfies the specifications a priori established, then it is considered finished. If not, the product reenters the cycle for improvement, the process continuing until it fulfils the specifications

Appendix B – Outcome of the public consultation on the draft scientific opinion on the Evaluation of existing guidelines for their adequacy for the food and feed risk assessment of genetically modified plants obtained through synthetic biology

Appendix B can be found in the online version of this output ('Supporting information' section): https://doi.org/10.2903/j.efsa.2022.7410