Public Consultation on EFSA Scientific Opinion on Synthetic Biology developments in Plants, molecular characterization (MC) and environmental risk assessment (ERA) aspects

GenØk Centre for Biosafety Submission
By Dr. Sarah Agapito

Item	Opinion
1.2 Background and Terms of Reference as provided by the requestor	**EC ToR1 – additional developments in synbio** EFSA was asked to "consider whether and which newer sectors/advances should be considered among SynBio developments, in addition to the six identified by SCs" (ToR1). Clearly, EC was interested in new developments that were not identified in previous expert opinions. In specific, EC requested information on whether there were other applications of SynBio not described by the six previous categories[1]. Instead, EFSA reported on new developments within two out of the six pre-existing SynBio categories. In other words, instead of searching for new developments irrespective to their categorization, EFSA restricted its search to developments that would fall only within categories #1 and #5 identified by the Scientific Committees (SCs) on SynBio in 2014[2].
1.3 Interpretation of the Terms of Reference and Scope	**Developments under categories established in 2014** EFSA has limited the scope of its mandate to the analysis of two out of the six SynBio categories previously identified by the SCs. It is not clear why EFSA decided to restrict the analysis to only categories #1 and #5, namely: Category #1) Genetic part libraries and methods Category #5) DNA synthesis and genome editing It is also not clear why the other categories were excluded from the analysis, namely: Category #2) Minimal cells and designer chassis Category #3) Protocells and artificial cells Category #4) Xenobiology - xenobiology Category #6) Citizen science (Do- It-Yourself biology) EFSA also limited the scope for developments reaching the EU market in the next decade. Considering that the horizon scanning process was based on categories established 6 years ago, EFSA might have missed developments that are already in the market but do not fit into these categories.
2.2 Horizon scanning of SynBio developments	**Horizon scanning restricted to the six SynBio categories** EFSA has carried out a new horizon scanning process via procurement call in 2018 to address the ToRs of its mandate. This process was published separately in July, 2019[3]. The horizon scanning report also only considered "plant SynBio developments moving towards practical applications in the next decade, arising from the previously mentioned SynBio categories"[4].

	Therefore, it can be argued that new developments that do not fit into the two established categories might have been missed. It is relevant to mention that these categories were established based on 350 relevant publications published before February 1st, 2014[5]. **Examples of synbio plant developments already in the market and not considered by EFSA** The limited scope of EFSA in fulfilling ToR1 may have hampered its horizon scanning process and the identification of new SynBio applications in the pipeline. As an example, the use of environmentally applied nucleic acids and proteins for purposes of engineering changes to genes and other genetic material in plants was not considered in this document. Such synthetic biology approach has been identified by the Ad Hoc Technical expert Group on Synthetic Biology (AHTEG) serving the Convention on Biological Diversity in their last report in June 2019[6]. In the review paper of Heinemann and Walker[7], a table containing several plant applications of such environmentally applied nucleic acids, their vehicles and targets is summarized. All of these examples are already patented, as referenced in the above-mentioned table, and shows that commercial uses include biocides for use in agriculture and for trait modification in food and agricultural products (e.g. BioDirect™ from Monsanto Company).
2.3 **Selection of case studies**	**SynBio approaches cannot be interpreted as only engineering complex, multi-gene traits** EFSA focus its analysis of the horizon scanning process on SynBio applications that contain multiple engineered genes and are "more complex then GMPs". In addition, a table provides a summary of the horizon scanning process and groups the applications by "trait". Therefore, it is not described what genetic engineering techniques and approaches were applied. Most relevant, there is no restriction to SynBio definition as to engineering only complex traits and multiple genes. Synthetic Biology, as its own definition by EFSA "is an interdisciplinary field at the interface of engineering and biology aiming to develop new biological systems and impart new functions to living cells". In the SynBio AHTEG report, for instance, the new technological developments in synthetic biology do not even mention "complex and multi-gene traits". The report rather focusses on new trends of genetic modification in the field, the use of artificial intelligence and machine learning, transient modification of organisms, non-canonical molecules, etc[8]. In addition, gene-editing approaches could be applied as a tool for plant synthetic biology by eliminating or adjusting host sequences, inserting non-host genes and regulating the transcription or translation of host or non-host genes. It is unclear why EFSA has narrowed its analysis to SynBio plant applications that have only engineered complex traits. **The three case-studies are not representative** As stated by EFSA, the specific case studies "resemble classical GMPs and were achieved using existing GMO technologies". The three examples have transgene insertions and confer traits for disease resistance and increased nutrition; already commercialized as GMPs.

	Last year, Gao and collaborators have developed an inbred corn and wheat varieties that could be modified by pollenating them with pollen from a haploid inducer line harboring a CRISPR cassette designed to generate a desired agronomic trait[9]. Similarly, gene-drives in plants[10] were not even examined as if close to commercialization in the near future.
3.1.1 Information related to the genetic modification	**CIR 503/2013 only considers nucleic acids (vector) as mutagenic reagent** There is no consideration under CIR 503/2013 for genetic modification of plants via CRISPR/Cas9 gene editing. Whereas EFSA recognizes that, it does not provide any suggestion on how the regulation could be improved to accommodate this technique. EFSA conclusion on "specific requirements [that] may not be needed or may be adapted, depending on the SynBio method used" is not a clear and scientific recommendation to EC. The type of nuclease used and the sequence of guide RNA molecules should be provided under section 1.2.1 of CIR 503/2013. In addition, information on sequences of DNA templates, delivery methods and a comprehensive description of sequencing methods should be described. The outcome results of off-target prediction software for the host genome should also be provided in this section as a guide to the investigation of potential off-target modifications in the following section of the regulation.
3.1.2 Characterization of the modified/ inserted/ deleted sequences	**EFSA suggests no off-target analysis** EFSA concluded that the considerations provided by CIR 503/2013 are adequate and sufficient. The main reason given for coming to this conclusion was that "the analysis of potential off-targets on a regular basis would be of very limited value for the risk analysis". EFSA stated that: (1) "mutations at DNA breaks are the result of the activity of the endogenous repair machinery, the off-target changes introduced by genome editing will be similar to those that occur after the repair of naturally occurring DNA breaks and"; (2) "also similar, but far fewer, than those that occur after using other established mutagenesis techniques that also introduce DNA breaks (e.g. radiation mutagenesis)"; (3) "back crossing steps following DNA modifications may allow removal of most of these potential off-targets from the final product assuming they are not genetically linked to the target site"; (4) "although, several bioinformatic tools are available for off-target prediction (for example, Cas-OFFinder; Bae et al., 2014), the limited availability and completeness of plant reference genomes and the intra-species and intra-varietal variability would not always allow for a reliable prediction of potential off-target mutations". However, EFSA incorrectly concluded that DNA double-stranded breaks (DSB) produced by site direct nucleases (gene editing techniques) are similar to those that occur naturally or by radiation mutagenesis. This error undermines the conclusion that the outcome of both processes is the same and, therefore, should not be investigated. Firstly, kinetics and fidelity studies showed that the repair of Cas9-induced DSBs is not representative for the repair of naturally occurring DSBs indicating that natural processes are bypassed. The data suggest that repair of naturally occurring DSBs is highly precise, e.g. ligation of recombination junctions has been found to have low

	error rates; whereas Cas9-induced DSB have estimated error rates in the range of 20%–100% per break event [11]. Secondly, after exposure to high doses of ionizing radiation that cause hundreds of DSBs per cell, the time courses of such bulk measurements have consistently shown that DSBs are generally repaired with a half-life of 10–60 min. Whereas quantitative modeling of repaired DNA in time series after Cas9 activation reveals variable and often slow repair rates, with half-life times up to 10 hr[11]. Therefore, it is crucial that information requested under item 1.2.2.1 and 1.2.2.2 make clear indication on the need to provide information on the identification of all off-target modifications occurring in the host organism and not only limited to intended modifications. More explicit, this analysis should be done in vivo, in the actual plant subject to approval by EFSA and not only in silico analyses. In addition, EFSA assertions that backcrossing steps following DNA modifications may allow removal of most off-targets from the final product directly contradicts scientific evidence of frequency levels of meiotic recombination and the uneven distribution of crossing overs in the genome of plants. DNA regions with highly repetitive sequences undergo little or no meiotic recombination. Also, in the large genomes of many grass species, including maize, the majority of the chromosomes consist of non-recombining expanses of heterochromatin. Also, when genes are located in close proximity in low or non-recombinogenic regions, the probability of separating them is highly unlikely during breeding[12]. Therefore, it cannot be assumed that off-target DNA modifications will be lost during breeding. The herdability of off-target modification will mostly depend on where in the genome they are located and this can be only known if investigated in the actual GMO.
3.1.3 Information on the expression of the inserted / modified sequences (incl. protein expression)	**EFSA does not address how to analyze numerous newly expressed proteins** EFSA considers challenging to perform protein characterization for numerous newly expressed proteins. However, there is no suggestion on analytical tools and approaches on how it could be done. Still, EFSA concludes that section 1.2.2.3 is adequate for assessing the expression of inserts in the SynBio GMOs. EFSA fails to recognize its own work on omics technologies as valuable addition in some aspects of risk assessment of food and feed products and the environment[13]. Omics approaches, such as proteomics, could help identify thousands of proteins at the same time, without prior knowledge of their identity. Such high throughput techniques could be used to characterize newly expressed proteins at the intended site but also at unintended sites, arising from off-target effects from nucleases, for instance. **EFSA does not include the analysis of unintended protein expression** New SynBio techniques can alter the expression of numerous proteins by modifying DNA sequences at unintended off-target sites. In addition, when entire new metabolic pathways are introduced, these can impact biochemical reactions in cascade. Therefore, CIR 503/2013 section 1.2.2.3 is not adequate or sufficient to require information on unintended protein expression. New guidelines should include information on expression levels of proteins in cascade pathways and also at off-target sites.

3.1.4 Genetic stability of the inserted/modified sequences and phenotypic stability of the GMP	**Genetic stability of gene edits should be included in the CIR text** EFSA recognizes that section 1.2.2.4 cannot be directly applied when SynBio GMP do not contain a transgene. This is because the CIR text refers to the presence of an "insert". EFSA also describes the relevance of demonstrating the genetic stability of the nucleotide change and introduced traits irrespective of an insert introduced. However, EFSA incorrectly concluded that the considerations in section 1.2.2.4 of CIR 503/2013 are adequate. Section 1.2.2.4 is not sufficient in addressing the genetic stability of the DNA modification because it is restricted to testing the transgene. Therefore, the CIR text should be updated to include all DNA changes/modifications present in the GMP.
3.1.5 Bioinformatic analyses	**Horizontal gene transfer can occur in edited genes** In this section, the CIR text is broader than the interpretation of EFSA. CIR does not restrict the assessment of horizontal gene transfer to transgenes. However, EFSA incorrectly considers the assessment of horizontal gene transfer only applicable when transgenes are inserted. The DNA modification of a few nucleotides in the host genome creates a new allele which confers a new trait in the GMP. Regardless of the size of the modification, this allele is still capable of being transferred to other organisms horizontally (HGT).
3.1.7 MC Conclusions and Outlook	The conclusions should reflect the suggested text revision as provided in the above sections.
3.2.2 Plant to micro-organisms gene transfer	Same consideration as section 3.1.5

[1] Mandate for an EFSA opinion on genetically modified organisms developed through synthetic biology and their implications for risk assessment methodologies (Annex). Available at: http://registerofquestions.efsa.europa.eu/roqFrontend/wicket/page?6

[2] SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks), SCHER (Scientific Committee on Health and Environmental Risks), SCCS (Scientific Committee on Consumer Safety), Synthetic Biology II - Risk assessment methodologies and safety aspects, Opinion, May 2015 (page 10).

[3] Katharina Unkel, Doerthe Krause, Thorben Sprink, Frank Hartung, Ralf Wilhelm, 2020. Mapping of plant SynBio developments in the agri-food sector. EFSA supporting publication 2020:EN-1687. 36 pp. doi:10.2903/sp.efsa.2020.EN-1687.

[4] Katharina Unkel, Doerthe Krause, Thorben Sprink, Frank Hartung, Ralf Wilhelm, 2020. Mapping of plant SynBio developments in the agri-food sector. EFSA supporting publication 2020:EN-1687. 36 pp. doi:10.2903/sp.efsa.2020.EN-1687 (pages 5 and 6).

[5] SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks), SCHER (Scientific Committee on Health and Environmental Risks), SCCS (Scientific Committee on Consumer Safety), Synthetic Biology II - Risk assessment methodologies and safety aspects, Opinion, May 2015 (page 10).

[6] AHTEG (2019). *Report of the Ad Hoc Technical Expert Group on Synthetic Biology*. Available at: https://www.cbd.int/doc/c/b2bb/cf58/b09729bb00be6abf72325a1a/synbio-ahteg-2019-01-03-en.pdf

[7] Jack A. Heinemann and Sophie Walker, 2019. Environmentally applied nucleic acids and proteins for purposes of engineering changes to genes and other genetic material. Biosafety and Health 1: 113–123. http://dx.doi.org/10.1016/j.bsheal.2019.09.003.

[8] AHTEG (2019). *Report of the Ad Hoc Technical Expert Group on Synthetic Biology*. Available at: https://www.cbd.int/doc/c/b2bb/cf58/b09729bb00be6abf72325a1a/synbio-ahteg-2019-01-03-en.pdf (page 5)

[9] Chen, K., Wang, Y., Zhang, R., Zhang, H. & Gao, C. CRISPR/Cas genome editing and precision plant breeding in agriculture. Annu. Rev. Plant Biol. 70, 667–697 (2019).

[10] Barrett LG, Legros M, Kumaran N, Glassop D, Raghu S, Gardiner DM. 2019 Gene drives in plants: opportunities and challenges for weed control and engineered resilience. Proc. R. Soc. B 286: 20191515. http://dx.doi.org/10.1098/rspb.2019.1515.

[11] Brinkman et al., 2018. Kinetics and Fidelity of the Repair of Cas9-Induced Double-Strand DNA Breaks. Molecular Cell 70, 801–813. https://doi.org/10.1016/j.molcel.2018.04.016

[12] Kawall K. New Possibilities on the Horizon: Genome Editing Makes the Whole Genome Accessible for Changes. Frontiers in Plant Science. 2019 ;10:525. DOI: 10.3389/fpls.2019.00525.

[13] EFSA (European Food Safety Authority) and Aguilera J, Aguilera-Gomez M, Barrucci F, Cocconcelli PS, Davies H, Denslow N, Dorne JL, Grohmann L, Herman L, Hogstrand C, Kass GEN, Kille P, Kleter G, Nogué F, Plant NJ, Ramon M, Schoonjans R, Waigmann E and Wright MC 2018. EFSA Scientific Colloquium 24 – 'omics in risk assessment: state of the art and next steps. EFSA supporting publication 2018:EN-1512. 30 pp. doi:10.2903/sp.efsa.2018.EN-1512 .