Submission of information on synthetic biology and supporting documentation
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We would like to share our observation that when reflecting on the provided six topics we realised there is a need for an open and ongoing list of the different techniques and tools that are components and contributors to synthetic biology to facilitate further work.

Without clarity of what is included and needs to be addressed it is difficult to provide answers or input to topics such as topic (a), sub-paragraph (a), which is looking at 'benefits and adverse effects of organisms, components and products of synthetic biology on biodiversity, with a view to filling knowledge gaps and identifying how those effects relate to the objectives of the Convention and its Protocols'.

It is widely understood, that genome editing tools and techniques and in particular CRISPR/Cas are tools of synthetic biology. Puchta for example published under the topic of Synthetic Biology the article "Using CRISPR/Cas in three dimensions: towards synthetic plant genomes, transcriptomes and epigenomes" (Puchta H, *The Plant Journal* (2016), doi: 10.1111/tpj.13100) where he states in the abstract:

> "It is possible to target individual sequence motives within genomes by using synthetic DNA-binding domains. This one-dimensional approach has been used successfully in plants to induce mutations or for the transcriptional regulation of single genes. When the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system was discovered, a tool became available allowing the extension of this approach from one to three dimensions and to construct at least partly synthetic entities on the genome, epigenome and transcriptome levels. The second dimension can be obtained by targeting the Cas9 protein to multiple unique genomic sites by applying multiple different single guiding (sg) RNAs, each defining a different DNA-binding site. Finally, the simultaneous use of phylogenetically different Cas9 proteins or sgRNAs that harbour different types of protein binding motives, allows for a third dimension of control. Thus, different types of enzyme activities – fused either to one type of Cas9 orthologue or to one type of RNA-binding domain specific to one type of sgRNA – can be targeted to multiple different genomic sites simultaneously. Thus, it should be possible to induce quantitatively different levels of expression of certain sets of genes and at the same time to repress other genes, redefining the nuclear transcriptome. Likewise, by the use of different types of histonemodifying and/or DNA (de)methylating activities, the epigenome of plants should be reprogrammable. On our way to synthetic plant genomes, the next steps will be to use complex genome engineering approaches within or between species borders to restructure and recombine natural or artificial chromosomes."

Genome editing tools, esp. the CRISPR/Cas system, have gained prominence for readily altering genomes at predetermined sites to make small alterations such as small deletions or insertions (indels) or small substitutions (eg point mutations). There is an unfortunate tendency to suggest the exclusion of such LMOs (GMOs) from the LMO (GMO) regulations, based on the claim and assumption that CRISPR/Cas is 'precise' and off-target effects would

only occur at sites of high sequence homology to the target site, if at all, and that such sites can easily be determined by the use of specific algorithms and hence checked (and ruled out) by various sequencing methodologies.

Yet this assumption is not holding true. Instead, Schaefer et al. (2017) recently found about 1900 small genome wide mutations when using CRISPR/Cas in mice. They reported:

> "However, concerns persist regarding secondary mutations in regions not targeted by the single guide RNA (sgRNA). Algorithms generate likely off-target sites for a given gRNA, but these algorithms may miss mutations. Whole-genome sequencing (WGS) has been used to assess the presence of small insertions and deletions (indels) but not to probe for single-nucleotide variants (SNVs) in a whole organism. We performed WGS on a CRISPR–Cas9-edited mouse to identify all off-target mutations and **found an unexpectedly high number of SNVs compared with the widely accepted assumption that CRISPR causes mostly indels at regions homologous to the sgRNA**."

It is of particular interest, that none of the mutations were found in those sites predicted to be the likely off-target sites. Instead, looking at two mice in parallel, they found the same 117 indels and 1,397 SNVs were detected in both of the CRISPR-treated mice, which indicated nonrandom targeting.

Concerning the consequences the authors stated: "Although our CRISPR-treated mice did not display obvious extraocular phenotypes, it is possible the mice may reveal phenotypes in time, when they are challenged or bred to homozygosity."

Whilst there might be differences between plants and animals regarding perhaps degree or location of process-induced mutations, at present such research data is only availble from animal models. The testing for off-target effects in plants has vastly been carried out on expected off-target sites, not on the whole genome and not for SNVs.

Another recent publication, Shin et al. (2017), reported complex deletions and insertions at 17 sites in the mouse genome.

The claim of precision was also given for the early LMOs, claiming that the only difference was the inserted DNA. Yet research could show that the process of genetic modification also gave rise to hundreds of genome wide mutations (Wilson et al 2006).

It will be important when looking at adverse effects and potential adverse effects, including both direct and indirect effects, to refrain from working with assumptions, especially when these provide an assumed sense of safety and predicatability. Thus **uncertainty assessement** will have to be strongly integrated into any regulations and risk assessments.

Equally important is to allow for sufficient time for data and evidence to emerge, as well as to design experiments in a way that can reveil problems more readily. The story of thalidamide in the US for example shows the importance of enforcing delays and insisting on proof, even against the given regulations at the time (Paul and Bereano 2015). Other cases that highlight the need to strictly apply the precautionary principle can be found in 'Late lessons from early warnings: the precautionary principle 1896-2000', (EEA 2001).

For more information on the discussion regarding the lack of clarity of what constitutes LMOs, GMOs, "New Breeding Techniques" (NBTs) or synthetic biology, please see Paul et al. (2017).

Gene Drives

An important point touching a number of the listed six topics is that of gene drives, including the topic of public dialogues. The need for an open and critical public debate has been identified by scientists working on Gene Drives (Esvelt et al. 2014).

Whilst the Committee on Gene Drive Research in Non-Human Organisms (National Academies of Science, USA) published a consensus report and Recommendations for Responsible Conduct in 2016 on the basis of wide consultations, it offers valuable information and advice but cannot substitute for a public dialogue. To our knowledge no open and critical public dialogue has taken place in any country thus far.

It will be important not to overstate the potential benefits, as there are still substantial technical hurdles, including the build up of 'resistance' to CRISPR/Cas, eg via mutation of sequence recognition sites. It is also important not to overstate the potential benefits to allow for a measured deliberation, including ethical and ecological aspects.

References put forward as extra information (and submitted as pdfs where available)

Esvelt KM, Smidler AL, Catteruccia F and Church GM, "Concerning RNA-guided gene drives for the alteration of wild populations", *eLife* 2014 Jul 17;3. pii: e03401. doi: 10.7554/eLife.03401.

The National Academies of Sciences (USA), *Gene Drives on the Horizon: Advancing Science, Navigating Uncertainty, and Aligning Research with Public Values*, report (Washington DC, USA, 2016) http://www.nap.edu/23405

Paul H, Buecking E and Steinbrecher RA. 'New Breeding Techniques' and synthetic biology - genetic engineering by another name," *The Ecologist,* 4 April 2017, http://www.theecologist.org/News/news_analysis/2988799/new_breeding_techniques_and_synthetic_biology_genetic_engineering_by_another_name.html

Puchta H. Using CRISPR/Cas in three dimensions: towards synthetic plant genomes, transcriptomes and epigenomes. *The Plant Journal* (2016), doi: 10.1111/tpj.13100

Puchta H and Fauser F. Synthetic nucleases for genome engineering in plants: prospects for a bright future. *The Plant Journal* (2014) 78, 727–741, doi: 10.1111/tpj.12338

Schaefer KA et al. Unexpected mutations after CRISPR–Cas9 editing *in vivo. Nature Methods* (2017) May 30;14(6):547-548. doi: 10.1038/nmeth.4293

see also: https://phys.org/news/2017-05-crispr-gene-hundreds-unintended-mutations.html

Shin HY et al. CRISPR/Cas9 targeting events cause complex deletions and insertions at 17 sites in the mouse genome. *Nature Communications*, 31 May 2017, DOI: 10.1038/ncomms15464

Wilson AK, Latham JR and Steinbrecher RA. Transformation-induced mutations in transgenic plants: analysis and biosafety implications, *Biotechnology and Genetic Engineering Reviews* 23 (2006): 209–237 http://econexus.info/publication/transformation-induced-mutations-transgenic-plants