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Secretaría de la Comisión Nacional de Bioseguridad

NATIONAL BIOSAFETY COMMISSION REPORT ON THE SITE DIRECTED MUTAGENESIS ("GENE EDITING")

Background

The European Court of Justice ruling of 25th July 2018 established that organisms obtained with the new site directed mutagenesis techniques, including the gene editing technologies, should be considered Genetically Modified Organisms (GMO) and therefore should be regulated by Directive 2001/18/CE of the European Parliament and the Council of 12th of March 2001, on the deliberate release of genetically modified organisms into the environment.

In October 2018, the Interministerial Council for GMOs (CIOMG) requested an expert report to the National Biosafety Commission (CNB) in relation to: 1) the use of these techniques on the different biotechnological sectors, 2) the potential risks to the health and the environment from its use in comparison to other genetic modification techniques that produce GMOs or other mutagenesis techniques used traditionally to modify plants and animals and 3) weigh up, from a scientific and a compliance control points of view, the European Court of Justice sentence on this matter.

In the development of this report have participated not only the National Biosafety Commission experts but also an *Ad-hoc* expert group composed by representatives and researchers from the Biotechnology National Center-CSIC, the University of Lleida, the National Center for Biotechnology and Genomics of Plants (CBGP-UPM-INIA), the Spanish Medicines Agency and Medical Devices (AEMPS), the University of Girona and the National Institute for Agricultural and Food Research and Technology (INIA).

Introduction

The basic gene editing methods developed until now are based on the generation of a cut in one or in the two strands of the DNA double helix as a result of a precise and directed cut in the editing region. This cut is later repaired by one of the cell two alternative mechanisms. (1) The preferential pathway is the non-homologous end-joining. This mechanism consists on the simple joining of the DNA ends and typically introduces additional mutations because it can lead to insertions or deletions during the repair process. (2) In the less frequent pathway, the homologous recombination, the nucleotide sequences are exchanged between two similar or identical molecules of DNA. It can use as template the related region of the homologous genome or an exogenous DNA molecule with sequence homology in the flanking cutting region and suitable to make the correct joint of the terminals. This edited genome is transmitted to the daughter cells.

In the last decades different gene editing methodologies have been developed including methodologies based on nucleases to make site-specific double-stranded DNA breaks (i.e. Transcription Activator-Like Effector Nucleases (TALEN), zinc-finger nucleases), based on genome modifications mediated by nucleic acids (Oligonucleotide Directed Mutagenesis, ODM) or based on a combination of both (CRIPSR/Cas9), allowing specific and precise alterations of the plant, animal or microorganism genome compared to the so called "traditional mutagenesis techniques".

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These gene editing techniques are frequently used in basic research for gene regulation studies, for example in biomedicine, they are used for the generation of new cell and animal disease models. In plant biology these techniques are used for crops quality improvement, the generation of resistance to diseases or herbicides. In gene therapy they can be used to inactivate genes, repair mutations or even insert intact genes. In industrial biotechnology they are used for the biosynthesis of pharmaceutical, chemical or biofuel products, for biosensors development and for bioremediation.

Risks associated to gene editing

The implementation of gene editing techniques nowadays could imply the risk of introducing undesired mutations in other genome regions (*off-target* mutations) [1] and many repaired DNA sequences (*on-target* mutations or genetic mosaicism), including the rearrangement of the edited genome which could be harmful and with unpredictable consequences[2].

Regarding plants, it has to be taken into account that natural genetic alterations can occur. Plants are constantly exposed to environmental stress like UV-B radiation, ozone, desiccation and rehydration and the air and ground pollution that can cause, as a response, the breakdown of one or the two DNA strands. The repair of these mutations by the cell endogenous repair mechanisms described before could introduce errors, some of which could result in direct toxic effects such as reduction of protein synthesis, destruction of the cell membrane, the impair of the plant growth (by altering the photosynthetic protein) or to produce chromosome fusion or genetic changes in the plants that can be transmitted to the next generations. Occasionally, these mutations are a source of natural variation, important for the plant evolution and useful for the crops improvement.

Commonly, mutations in plants have been induced by using chemical substances with mutagenic properties or ionizing radiations. These techniques cannot be controlled or directed against specific genes and also require a long selection process making very likely that the final selected products have additional mutations beyond the desired ones and some of them could be harmful. Many of the common plants for consumption obtained by these techniques have not been submitted to risk assessment and have not been a matter of safety concern; therefore it can be considered that the absence of incidents in their history of use is a proof of their safety.

Gene editing techniques are more specific than traditional techniques and therefore less prone to generate undesired mutations. Overall, the *off-target* mutations in plants are generally less frequent than the somatic mutations that can emerge from tissue cultures[3].

Many efforts have been done to improve the precision and efficiency of these techniques, such as shortening the activity time of the nucleases, avoiding homodimerizations (TALEN and zinc-finger

[1] Li, J.-F., Norville, J.E., Aach, J., McCormack, M., Zhang, D., Bush, J., Church, J.M. and Sheen, J. (2013) Multiplex and homologous recombination mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. Nat. Biotechnol. 31(8), 688–691.
Shan, Q., Wang, Y., Li, J. et al. (2013) Targeted genome modification of crop plants using a CRISPR-Cas system. Nat. Biotechnol. 31(8), 686–688.
Feng, Z., Mao, Y., Xu, N. et al. (2014) Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in Arabidopsis. Proc. Natl Acad. Sci. USA, 111(12), 4632–4637.
[2] Wolt, J.D. (2017). Safety, security, and policy considerations for plan genome editing. Prog. Mol. Transl. Sci. 149, 215-241.
[3] Li, M., Li X., Zhou, Z., Wu, P., Fang, M., Pan, X., Lin, Q (2016). Reassessment of the four yield-related genes Gn1a, DEP1, GS3, and IPA1 in rice using a CRISPR/Cas9 system. Front.Plant Sci. 7,377

proteins), transforming nucleases into nickases and thus originating the breakdown of the DNA single strand instead of breaking the double strand, developing of more specific Cas nucleases, modifying the DNA coupling, developing bioinformatic tools for the desing of these instruments[4] and the design of tools to characterize and detect these *off-target* regions[5].

Regarding their application to animals, the use of these techniques, and more specifically, the CRISPR/Cas9 methodology, has proven to be able, relatively quickly and routinely, to produce discreet genomic changes both in embryonic pluripotent stem cells and in embryos. The previous approaches that tried to produce similar modifications were arduous and often left behind large alterations as the introduction of antibiotic selection genes that required a second step for its removal.

However, this technique has the inconvenience of producing mosaic mutations in a way that some cells carry the desired modification, other cells carry undesired modifications and other cells do not carry any modification. To reduce the formation of mosaic animals, the genome editing must be carried out at an early development point so that all cells of the organisms have the edited sequence. Moreover, although the underlying mechanisms of the mosaic mutations produced by CRISPR/Cas9 are still unknown, the expression and prolonged activity of Cas9 in embryos could contribute to its creation[6]. For this reason Ribonucleoproteins (RNP) (CRISPR/Cas9 that have a limited temporary activity range are currently used. In the current state of knowledge, the use of CRISPR/Cas9 using "*ex vivo*" techniques can be considered safe because the desired clones could be selected while the use of this technique "*in vivo*" is still far from being considered safe.

In human cancer cell lines it was observed more than 50% of *off-target* mutations compared to the mutations in the specific sequence because in these cells, the DNA repair mechanisms are defective[7]. When stem cells were used, the whole-genome sequencing showed an absence of *off-target* mutations[8] or only few events[9]. On the other hand, it has been demonstrated in animals that gene editing could introduce a response in the cells aiming to protect against the DNA damage. This response involves p53 ("the genome keeper") activation and tries to repair the DNA breaks. .It has been found that CRISPR/Cas9 works more efficiently in human pluripotent stem cells with impaired p53 genes. Nevertheless, these cells are the ones that have the highest predisposition to transformation into tumoral cells. Therefore, the insertion in a patient of cells modified with CRISPR/Cas9 tools and with low or

[4] Bortesi L, Zhu C, Zischewski J, Perez L, Bassié L, Nadi R, Forni G, Lade SB, Soto E, Jin X, Medina V, Villorbina G, Muñoz P, Farré G, Fischer R, Twyman RM, Capell T, Christou P, Schillberg S (2016). Patterns of CRISPR/Cas9 activity in plants, animals and microbes. Plant Biotechnol J. 2016 Dec;14(12):2203-2216.

[5] Cameron, P., Fuller, C. K., Donohoue, P.D., Jones, B. N.,Thompson, M.S., Carter, M.M., (2017). Mapping the genomic landscape of CRISPR/Cas9 cleavage. Nat. Methods 14, 600-606.

[6] Tu, Z[1.], Yang, W., Yan, S[.], Yin, A[.], Gao, J[.], Liu, X., Zheng, Y., Zheng, J., Li, Z., Yang, S., Li, S., Guo, X., Li, X. J. Sci Rep. 2017 Feb 3;7:42081. Promoting Cas9 degradation reduces mosaic mutations in non-human primate embryos.

[7] Fu, Y., Foden, J.A., Khayter, C., Maeder, M.L., Reyon, D., Joung, J.K. and Sander, J.D. (2013). High-frequency off-target mutagenesis induced by CRISPR–Cas nucleases in human cells. Nat. Biotechnol. 31, 822–826.

Hsu, P.D., Scott, D.A., Weinstein, J.A., Ran, F.A., Konermann, S., Agarwala, V., Li, Y. (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. Nat. Biotechnol. 31, 827–832.

Kim, D., Bae, S., Park, J., Kim, E., Kim, S., Yu, H.R., Hwang, J. (2015). Digenome-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. Nat. Methods, 12, 237–243.

[8] Smith, C., Gore, A., Yan, W., Abalde-Atristain, L., Li, Z., He, C., Wang, Y. (2014). Whole-genome sequencing analysis reveals high specificity of CRISPR/Cas9 and TALEN-based genome editing in human iPSCs. Cell Stem Cell, 15,12–13.

[9] Veres, A., Gosis, B.S., Ding, Q., Collins, R., Ragavendran, A., Brand, H., Erdin, S. (2014). Low incidence of off-target mutations in individual CRISPR-Cas9 and TALEN targeted human stem cell clones detected by whole-genome sequencing. Cell Stem Cell, 15, 27–30.

null p53 activity could increase the risk that the patient develops a cancer[10], so it must be ensured that the cells administered to the patient have intact p53 activity.

Furthermore, it has been proven that most of the individuals have circulating antibodies directed against the two most common forms of the Cas9 protein used in CRISPR, derived from two Gram positive pathogen bacteria: *Staphylococcus aureus* and *Streptococcus pyogenes* that causes nosocomial infections (in hospitals) or laryngitis/otitis, respectively, and thus our immune system has developed both antibodies and lymphocytes against these bacteria and their components, including the Cas9 nucleases, and this could stop the application of this technology in field trials[11], unless an immune suppression therapy is co-administered. In this sense, work is being done to find *cas* genes from other bacterial and archea species of other ecological niches to which the human population has not been in contact and for which humans are not immunized. Studies are also being done to generate synthetic Cas proteins not recognized by the human immune system.

CONCLUSIONS

As previously stated at the beginning of this report, the European Court of Justice ruling on mutagenesis considers that the organisms obtained from the "traditional mutagenesis techniques" used conventionally and having a long safety record, are still out of the scope of the GMO regulation. However, this verdict assumes that the risk associated with the use of site directed mutagenesis techniques (including gene editing) could be similar to the risks of GMOs obtained by techniques that involve the incorporation of foreign genetic material into an organism (transgenesis). In this sense regarding the latter, it should be noted that after more than 30 years of studies on GMOs to date, no undesirable adverse effects on health or the environment have been detected.

The directed mutagenesis in this aspect is placed within the techniques with minimal risk due to its specificity and selection.

Prior to gene editing techniques, the introduction of novel traits into organisms by genetic engineering was mainly based on the use of techniques with stable insertions but random genetic modifications (for example, conventional mutagenesis) or foreign genetic material (transgenesis). Due to randomness, undesirable alterations in the genome can occur, such as interruption of genes and/or regulatory elements, or the creation of new open reading frames (for example, with a similar sequence to toxins or known allergens), which explains why the processes of selecting GMOs with desirable traits are often complicated and time-consuming. The use of gene editing techniques offers the possibility of reducing the likelihood of these unwanted adverse effects, since it provides a way to address a predefined locus of the genome, together with a rapid subsequent selection to achieve the desired genetic alteration, discarding all the others that may have been generated.

Regarding plants, gene editing can give rise to varieties that are not genetically differentiated from those that carry the same modification generated spontaneously, developed by introgression of the desired gene through successive crosses or induced by traditional mutagenesis and yet, the regulatory requirements for each of these varieties would be different which is difficult to understand from a scientific point of view. For many products it is difficult, if not impossible, to have a detection method.

[10] Leslie K. F. CRISPR, cancer, and p53. *Sci. Signal.* 17 Jul 2018:Vol. 11, Issue 539.

[11] Carsten, T. Ch., Priyanka, S. D., Daniel, P. D., Beruh, D., Natalia, G.-O., Sruthi, M., Mara, P.-D., Joab C., Kenneth, W.,, Matthew, H. P. Identification of Pre-Existing Adaptive Immunity to Cas9 Proteins in Humans, bioRxiv preprint first posted online Jan. 5, 2018.

In the event that the technique used to obtain certain modifications is not reported, it will not be possible to differentiate if they have been obtained by gene editing techniques or any of the non-regulated techniques.

Taking into account the difficulties for detection, identification and quantification, a challenging scenario is presented to also comply with the obligations set out in the directive regarding the traceability and labeling of GMOs, which will be even more difficult for varieties obtained through these techniques that come from countries whose legislation does not consider them as GMOs, and these, definitely, will soon begin to reach our consumer markets.

The National Biosafety Commission already included its opinion in its November 2015 report on new plant breeding techniques (NPBT), as to whether the products obtained through the use of these techniques should be considered as GMOs. This report indicates that in order to determine if an organism falls within the scope of Directive 2001/18/EC, the product, and not the technique with which it is obtained, should be evaluated, and the safety of the product should be established based on its new characteristics, the environment in which it is grown and agricultural practices, for plants, and the human and animal exposure.

For all these reasons and in view of the fast development of these new biotechnological tools, the National Biosafety Commission considers that clarifications are still needed on some implementation issues by European bodies, but also calls for a revision of the current GMO regulation to reflect the latest knowledge and, based on scientific and technical evidence, ensure the health and the environment safety. If the legislation is not updated, important consequences could occur for the EU citizens, the international trade, cooperation with developing countries, and also for European scientific progress.

In this regard, we would agree with the report of the Scientific Advisory Group of the European Commission[12] that "*It is necessary to improve EU legislation on GMOs so that it is clear, evidence-based, applicable, proportionate and sufficiently flexible to face future advances in science and technology in this area. To achieve this, we recommend reviewing the current GMO Directive to reflect current knowledge and scientific evidence, in particular on gene editing and established genetic modification techniques. This must be done in relation to other relevant legislation for food safety and environmental protection*".

Madrid, 14 of January of 2019

[12] https://ec.europa.eu/info/sites/info/files/2018_11_gcsa_statement_gene_editing_2.pdf