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Proving That a Genome-edited Organism Is Not GMO.

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Abstract

A genome-edited agricultural product that is proven to contain no exogenous DNA is not subject to genetically-modified organism (GMO) regulation in some countries. However, whether such proof is definitive is often disputed. We discuss the approaches to substantially proving that a genome-edited organism is not GMO, while considering social aspects.

Key words

genome-editing; agriculture; GMO; regulation; negative proof; society.

Emerging issue in agricultural genome-editing

The advent of genome-editing technologies, such as ZFN, TALEN, CRISPR-Cas9, and base-editing, has facilitated targeted mutagenesis to breed crops and livestock, making them safer and more nutritious, as well as higher in yield and tolerance to biotic and abiotic stress [1]. At least seven countries exempt genome-edited agricultural products from GMO regulation, if producers can demonstrate that such organisms have no exogenous genetic materials in the genome (Table 1). The USA is one of the countries that actively promotes this policy. The US Department of Agriculture confirmed that a soy variety mutated with TALEN is not a GMO, and its oil product has already been sold (https://calyxt.com/first-commercial-sale-of-calyxt-high-oleic-soybean-oil-on-the-u-s-

market/) . In Japan, a tomato variety mutated with CRISPR-Cas9 was classified as a non-GMO product, and its seedlings have been distributed (https://sanatech-seed.com/en/products-en/) . However, the US Food and Drug Administration (FDA) recently discovered plasmid DNA, containing bacterial genes, in the genome of edited cattle regarded as non-GMO in Brazil [2], underscoring the importance of in-depth consideration of the exclusion of genome-edited organisms from GMO regulation. Even in cases like this, where the captured exogenous DNA is not necessarily hazardous to other organisms, the unexpected finding of GMOs among deregulated genome-edited agricultural products may cause an uproar in society [3]. To begin with, proof of the nonexistence of something is highly debatable.

We explore here approaches to proving that a genome-edited organism is not a GMO, while considering the social aspects, such as the cost-effectiveness of the approach and the morals status of the plants and animals produced.

Mechanism of genomic capture of exogenous DNA

It is pseudological to claim that proving the nonexistence of something is impossible, because one can prove a negative as much as one can prove anything at all [4]. Investigating the trigger and process of genome-editing is expected to uncover approaches that can cogently prove the nonexistence of exogenous DNA in a genome-edited organism to some extent.

In the genome, DNA double-strand breaks (DSBs) rarely occur due to DNA replications or active oxygen species in the cell culture, in addition to extrinsic factors such as UV (spontaneous DSBs). Genome-editing begins by intracellularly introducing artificial enzymes, those genes or transcripts. Nuclease-based genome-editing efficiently causes a DSB at an intended, target site in the genome. The intended DSB undergoes error-prone repair via non-homologous end joining (NHEJ) that can result in insertion or deletion (indel) mutations or the capture of another DNA fragment between the DNA ends, or a repair along a DNA template via homology-directed repair (HDR). However, the introduced nucleases might unintentionally cause DSBs at off-target sites (unintended DSBs) Meanwhile, spontaneous DSBs could occur during nuclease-based genome-editing, as well as during base-editing that employs other enzymes that efficiently induce

a specific base change at a target site, without creating DSBs [1, 5]. Those intended, unintended and spontaneous DSBs might also result in unwanted captures of exogenous DNA via NHEJ or HDR.

In addition to the case of genome-edited cattle [2], other reports showed exogenous DNA contained in a genome-editing reagent can be unwantedly captured at intended and/or unintended DSB sites in animal and plant genomes primarily via NHEJ [3, 6, 7] Remarkably, it was also revealed that CRISPR-Cas9-induced DSBs in mouse embryos captured DNA sequences derived from retrotransposons, in which guide RNA of CRISPR-Cas9 was reverse-transcribed and captured in the genome [8]. Furthermore, it was shown that exosome can mediate the genomic capture of bovine DNA derived from cell culture medium in mouse cells treated with CRISPR-Cas9 [9].

Therefore, if exogenous nucleic acid is contained in a genome-editing reagent or culture medium, intended, unintended, and spontaneous DSB sites could become hot spots for genomic capture of exogenous DNA. However, such unwanted genomic captures might be under reported or overlooked unless producers or regulators systematically analyze the process of, and products by genome-editing.

Combining mutually complementary assays with limitations

If developers wish their genome-edited agricultural products to be exempted from GMO regulation, they must carefully demonstrate that those products have no exogenous DNA in the genome.

In the hornless cattle developed with TALEN, whole genome sequencing (WGS) overlooked the capture of a template plasmid at the target (intended DSB) site [2], underscoring the importance of sufficient recognition that assays have different limitations, such as analytical bias and different levels of effectiveness, accuracy and sensitivity. To avoid a similar failure and enhance scientific rigor, it is appropriate to combine multiple assays with different limitations that will complement each other, even for intended DSB sites; in other words, multiple complementary methods are needed, in addition to requisite, targeted sequencing (or WGS) (Figure 1).

Although unintended DSB sites are not defined as such, potential off-target DSB sites can be reliably predicted by combining an *in silico* search to determine a target

sequence and empirical assays involving nucleases *in vitro* or *in vivo*, which have different benefits and limitations [5]. Although there is currently no consensus regarding the assessment of the off-target effect of genome-editing [10], using an *in vivo* nuclease-based assay that better reflects intracellular factors, together with an *in vitro* nuclease-based assay with higher accuracy, in addition to an *in silico* search, can determine the DSB sites worthy of subsequent genetic analysis among predicted potential off-target sites. Then, the determined DSB sites can be investigated by combining multiple genetic analyses, such as the previously-described analyses for intended DSB sites (Figure 1). If such mutually complementary assays identify hundreds or thousands of potential off-target sites, an appropriate threshold level, which is the number of base mismatches with a target sequence in an *in silico* search and which has a certain level of sensitivity and accuracy in nuclease-based assays, will likely be required.

Because spontaneous DSB sites are unpredictable, locus-specific genetic analyses are inapplicable. In this case, analyses such as quantitative PCR (qPCR) and genomic Southern blotting that use primers and probes based on the exogenous nucleic acid are applicable (Figure 1). However, such assays are unlikely to detect all small insertions of up to several hundred base pairs. In particular, spontaneous DSBs occur *in vitro* only about once in 10^8 bp (approximate DNA content of the average human chromosome) per cell cycle [11]. Given that the risk of genomic capture at spontaneous DSB sites is extremely low, data obtained by combining two different assays, such as qPCR and Southern blotting (or WGS), can be considered reasonable evidence for the absence of exogenous DNA in the host genome. Scientific evidence gained through the above-mentioned mutually-complementary analyses can substantially prove the nonexistence of exogenous DNA in a genome-edited organism.

Deregulation policy based on social consensus

Should the risks of genomic capture at intended, unintended, and spontaneous DSB sites have equal weight? Should approaches to proving that a genome-edited organism is not a GMO be applied to any species? These approaches require substantial labor, time and cost. Meanwhile, there have been few GM animals approved for food consumption, compared with GM plants [12].

Principle 15 of the UN Rio Declaration (https://www.un.org/en/development/desa/population/migration/generalassembly/docs/globalcompact/A_CONF.151_26_Vol.I_Declaration.pdf) allows states to take cost-effective measures to protect the environment [13]. If a country deliberately weighs the benefits of agricultural genome-editing against the potential environmental and health risks, as well as the substantial administrative burden, it might reach a consensus to take somewhat simplified approaches to proving that there is no exogenous DNA in genome-edited organisms. For example, it could be proven by combining only two specific genetic assays at intended and unintended sites, such as a PCR-based locus-specific assay or genomic Southern blotting, in addition to targeted sequencing (or WGS); setting a moderate threshold level to determine potential off-target sites for further investigation in well-studied crops (such as rice); and eliminating the requirement to investigate the possible genomic capture at spontaneous DSB sites. To ensure consumer acceptance of genome-edited agricultural products, the deregulation policy should be stipulated in GMO regulation amended through sufficient public consultation, as in Australia [14] (Table 1).

Unlike crops, livestock are generally reared and managed within facilities. Therefore, the environmental risk of genome-edited livestock appears to be low, and demonstrating the absence of exogenous DNA in genome-edited animals seems unnecessary. However, the US FDA has required mandatory premarket new animal drug regulatory evaluation for all genome-edited animals even those to be consumed as food (Table 1). Psychological studies have suggested that many people view GM animals as less acceptable than GM plants primarily because animals are closer to humans than plants in terms of moral status [12]. The FDA's strict policy might reflect a tacit agreement that reviewing genetically-engineered animals should progress, considering the social situation. In countries where further exploitation of animals is a growing concern, the exemption of genome-edited animals from GMO regulation may be shelved.

Concluding Remarks

Some countries have deregulated genome-edited organisms that do not have exogenous DNA. Our consideration suggests that developers can substantially prove that a genome-

edited organism is not a GMO using mutually-complementary assays. However, the deregulation should be based on legislation founded on a social consensus reached through scientific, economic and moral considerations, and through public consultation.

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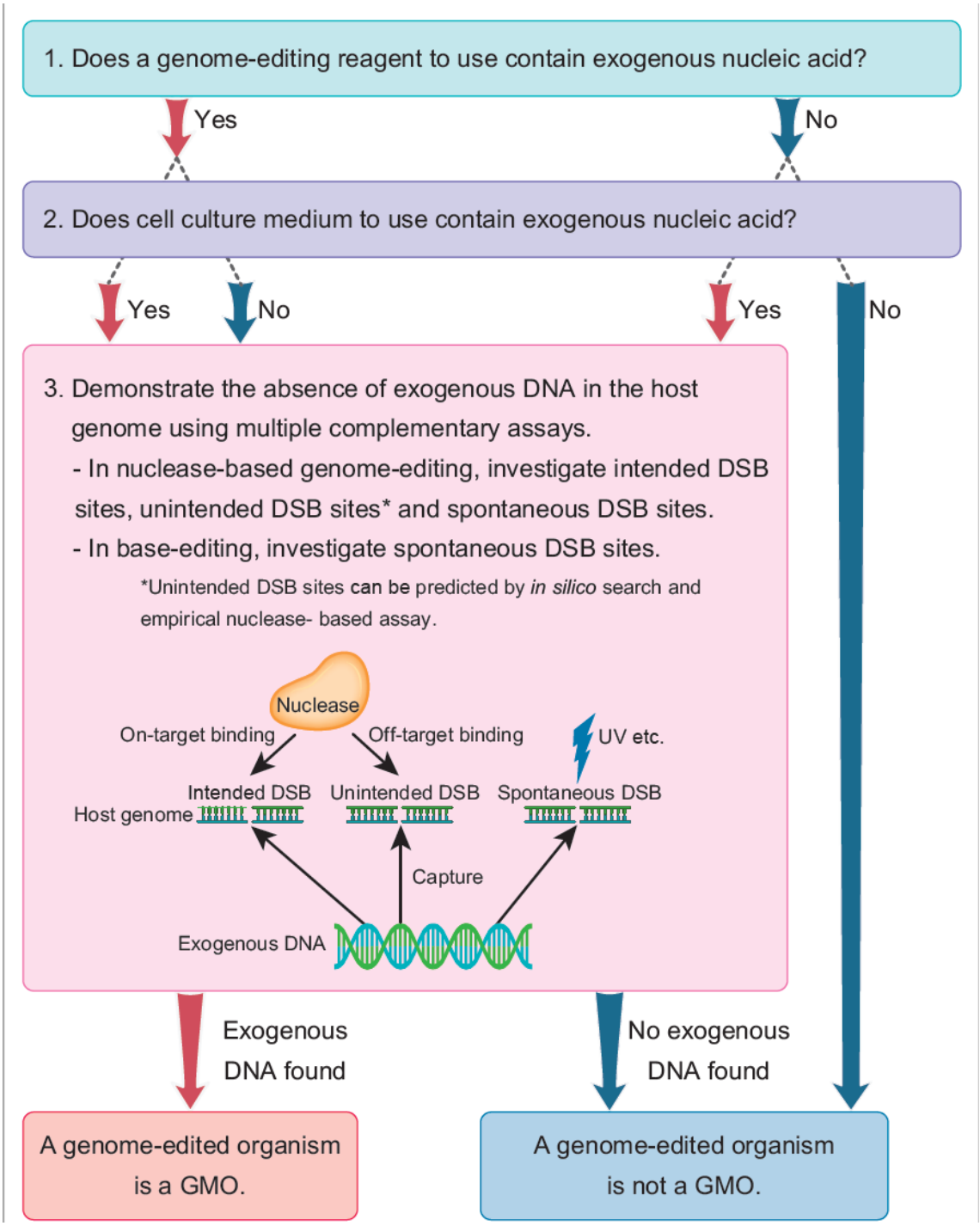

Figure 1. Approaches to substantially proving that a genome-edited organism is not a GMO.

First, developers should confirm whether or not their genome-editing reagent and

components of cell culture medium contain exogenous nucleic acid. If such materials do not contain exogenous nucleic acid, the resultant genome-edited organisms are not GMO. If their genome-editing system does contain exogenous nucleic acid, they should investigate the presence of exogenous DNA at intended double-strand break (DSB) sites, unintended DSB sites and spontaneous DSB sites, by combining multiple complementary assays. Specifically, in use of nuclease-based genome-editing techniques,

intended DSB and unintended DSB sites are investigated using locus-specific PCR assay(s) and/or genomic Southern blotting, in addition to targeted sequencing, (or WGS). Unintended DSB sites to be analyzed may be determined using an *in vitro* nuclease-based assay, together with an in vivo nuclease-based assay in addition to in silico search of a target sequence in the genome. Spontaneous DSB sites are investigated by assays such as qPCR and genomic Southern blotting (or WGS). In use of base-editing, spontaneous DSB sites are examined as described above.

Table 1. National policies that can exempt genome-edited agricultural products from GMO regulation.

Jurisdiction	Relevant policy	Conditions for exemption or organisms to be exempted
Argentina	MAGYP (Ministry of Agriculture, Livestock, and Fisheries) Resolution No. 173/2015.	A novel combination of genetic material is not created in a plant.
Australia	Gene Technology Amendment (2019 Measures No. 1) Regulations 2019.	In an organism, transgenes and expressed products have degraded, or no site-directed nuclease transgenes are inherited.
Brazil	CTNBio (National Technical Commission on Biosafety) Normative Resolution No.16/2018.	An organism has at least one of the following characteristics. I. Product with proven absence of recombinant DNA/RNA, obtained by a technique employing GMOs as a parent; II. Product obtained by a technique using DNA/RNA that will not multiply in living cells; III. Product obtained by a technique that introduces targeted site mutations, causing gain or loss of gene function, with the proven absence of recombinant DNA/in the product. IV. Product obtained by a technique where there is a temporary or permanent expression of recombinant DNA/RNA molecules, with the presence or introgression of these molecules in the product. and

		V. Product where techniques employing DNA/RNA molecules are used which, whether absorbed or not systemically, do not cause permanent modification of the genome.
Chile	Applicability of Resolution No. 1,523 / 2001 in propagation material developed by new breeding techniques 2017.	A propagation material has no novel combinations of genetic material.
Colombia	ICA (Colombian Agricultural & Farming Institute) Resolution No. 00029299/2018.	A final product does not contain any foreign genetic material.
Japan	Ministry of the Environment, Natural Conservation Bureau Notification No.1902081, 2019.	A resultant organism contains no nucleic acid or replicated product thereof, obtained through use of technologies for the processing of nucleic acid extracellularly.

USA*	Article 1, 7 Code of Federal Regulations § 340 Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which are Plant Pests or Which There is Reason to Believe are Plant Pests.	I(b) The regulations do not apply to plants that have been modified such that they contain either a single modification of a type listed in paragraphs (b)(1) through (3) of this section, or additional modifications as determined by the Administrator, and described in paragraph (b)(4) of this section. (1) The genetic modification is a change resulting from cellular repair of a targeted DNA break in the absence of an externally provided repair template; or (2) The genetic modification is a targeted single base pair substitution; or (3) The genetic modification introduces a gene known to occur in the plant's gene pool, or makes changes in a targeted sequence to correspond to a known allele of such a gene or to a known structural variation present in the gene pool.

*The use of genome-editing for animal breeding is regulated under 21 Code of Federal Regulations § 321(v), which demands the application of 'New Animal Drugs' that are not generally recognized as safe and effective for its use in animals.