CRISPR/CAS9-NUCLEASE

PAM-SEQUENCE

TARGET SEQUENCE

GENOME

GUIDE RNA

Overview of genome editing applications using SDN-1 and SDN-2 in regard to EU regulatory issues

New methods of genetic engineering (genome editing) and their potential impact on nature protection and the environment

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New methods of genetic engineering (genome editing) and their potential impact on nature protection and the environment

Testbiotech, March 2020

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Table of content

Summary

This report provides overview on possible impacts that new methods of genetic engineering (genome editing) can have on the environment. It is primarily concerned with CRISPR/Cas nucleases classified as 'site directed nucleases' SDN-1 and SDN -2. These applications are not meant to introduce additional gene sequences.

The authors give an overview of the differences between the genome editing of plants with SDN-1 and SDN-2 applications and conventional breeding which are of relevance in the discussion about the regulatory decision-making process:

In the case of conventional breeding, the first step always requires a high degree of genetic diversity that subsequently provides the basis for further crossing and selection. Conventional breeding (including 'random' mutagenesis) can generate biological traits which are desired, complex, distinct and heritable, often based on so-called Quantitative Trait Loci (QTLs) that are, in many cases, not well-defined at the genomic level. Due to the methods used in conventional breeding, some genetic alterations are more frequently observed than others. Inherent natural inheritance mechanisms such as the distance between two genes on a chromosome, recombination hot spots, gene clusters, large genomes, linkage drag, repair mechanisms and epigenetic effects allow some changes and gene combinations to occur more frequently than others, while some have to be considered as unlikely or even very unlikely.

The situation in regard to SDN-1 and SDN-2 applications is very different in at least three aspects: (1) these applications (in most cases) are not meant to increase genetic diversity in a non-targeted way. Therefore, unintended changes in the genome have to be seen as undesirable effects; (2) CRISPR/Cas makes a much larger part of the genome available for genetic change compared to conventional breeding; it allows biological characteristics to be generated that were not previously achievable; (3) complex characteristics cannot be generated with the new methods of genetic engineering if these are not well defined at the genomic level. Thus, in many cases, QTLs might not be so easily achieved by using SDN-1 and SDN-2.

The authors conclude that, in general, higher precision in changing the genome does not necessarily result in greater safety or higher success rates in plant breeding. Imprecise modifications, such as those resulting from 'random' mutagenesis, can be safe as well as beneficial. The authors identified the following aspects as particularly important for regulatory decision-making:

> New patterns of genetic change and resulting genetic combinations are, in many cases, likely to result from the application of SDN-1 and SDN-2.

> The applications of 'old' methods of genetic engineering (such as biolistic methods or *Agrobacterium tumefaciens*) used in most cases to introduce the CRISPR/Cas component into the plant cells can cause a broad range of unintended effects.

> CRISPR/Cas technology itself can cause many specific unintended effects; these would be dependent on the individual process, the surrounding experimental parameters, the chosen target location, on the genome and the specific organism. Therefore, each specific case must be investigated. In many cases, this challenge in risk assessment goes far beyond what is discussed as 'off-target effects'.

The report uses selected examples to provide a greater understanding of regulatory challenges resulting from SDN-1 and SDN-2 applications. The examples are grouped into five categories:

Changes in the composition of plants that may impact the food web

It was shown that changes in plant ingredients such as oil, protein, starch or other biologically active ingredients (such as plant estrogens or vitamins) can have an effect, e.g. on mammalian wildlife species, birds and insects as well as their related food webs. Particularly, if the intended changes in plant composition exceed the range of those in conventionally bred plants, the impact on the food web and the food and feed production chain should be extensively investigated as part of environmental risk assessment. In this context, risk assessment also has to take into account unintended effects that may cause changes in the composition of plants.

Changes in the composition of plants that may impact plant communication and interaction with the environment

The report shows that changes in plant composition can also affect communication and interactions with organisms which do not feed on them but are associated in other ways, e.g. cooperation (such as beneficial insects, e.g. predators or pollinators), or symbionts (such as the plant's microbiome) or also organisms that attack the plants (so-called 'pest' insects). It concludes that the impact on plant communication and interaction with the interconnected environment should undergo detailed environmental risk assessment, especially in cases where the intended changes in plant composition exceed the range of what is known from conventionally bred plants. In this context, risk assessment also has to take unintended effects that may impact plant communication into account.

Changes in the biological characteristics of the GE organisms meant to enhance fitness

A few examples are available of plants where enhanced fitness is intended by the trait, including increased drought tolerance, resistance to pest infestation or to plant diseases caused by viruses or fungi. There are several aspects that are important for risk assessment, e.g. expansion of unsustainable agricultural cultivation in, thus far, near-natural habitats or gene flow to natural populations. The authors highlight the example of rice genetically engineered with CRISPR/Cas that is intended for cultivation on ground with a high salinity. Gene flow could occur to wild rice and become particularly problematic for rice growing due to enhanced fitness of the weedy rice. Consequently, plants generated by SDN-1 or SDN-2 showing enhanced fitness should undergo detailed environmental risk assessment, especially in regard to gene flow and next generation effects. In this context, risk assessment also has to take into account effects that may unintentionally enhance fitness in unexpected ways.

Organisms with the potential to persist and propagate in the environment

One important question with regard to the reliability of the risk assessment for genetically engineered organisms is whether these can spread in the environment. If this cannot be ruled out, the authors show that, in many cases, the uncertainties would be so great that they would outweigh other considerations and render risk assessment inconclusive. This is also because multiplex interrelations with the closer and wider environment pose a real challenge for the risk assessor. While genetic stability over several generations might be demonstrated in domesticated varieties under normal field conditions or green house cultivation, genome x environmental interactions and introgression into heterogeneous genetic backgrounds can still trigger unpredictable next generation effects. Therefore, the authors conclude that organisms derived from SDN-1 or SDN-2 applications able to persist and propagate in the environment should undergo especially detailed environmental risk assessment. In this case, measures to prevent uncontrolled spread must be put in place.

Examples with ethical implications, including animal health and welfare, nature protection and rights of future generations

The authors emphasise that the "radical implications of gene editing (…) for our species and our planet" (Doudna & Sternberg, 2017) not only deserve strict regulatory oversight, but also deserve a broad sociological and ethical debate. The intrusion of GE organisms into native populations would not only raise safety issues, but also fundamentally change our understanding of what is considered 'natural'. Possible consequences could impact all future generations on this planet, including our own species. In this context, the authors recommend the careful consideration of the new concepts, which include strengthening the protection of biodiversity to legally safeguard it as a protected common good for the future.

Further major ethical issues relate to animal welfare and protection. There are already several publications reporting on SDN-1 and SDN-2 and also SDN-3 applications that need mandatory risk assessment. These include applications that can be used on farm animals in order to produce more meat, milk with changed composition, hornless cows, virus-resistant pigs and animals that are adapted to climate change. As discussed in the report, interests in marketing these animals can lead to serious conflicts with well-established social and ethical standards as well as the consensual values of European society.

The authors come to the conclusion that there are several important reasons why organisms derived from applications of SDN-1 and SDN-2 should all have to undergo mandatory risk assessment. In short, the pattern of intended and unintended changes and the resulting new combinations of genetic information arising from genome editing will, in most cases, be different in comparison to those derived from conventional breeding. These differences co-occur with biological characteristics and risks that need to be fully investigated before any conclusions on the safety of the new organisms can be drawn. Detailed examination of an organism's genetic and overall biological characteristics, starting with the process that was used to generate the organism, is needed to decide whether the organism is safe.

The requirements for regulation as foreseen by current GMO law in the EU are mandatory whether or not additional DNA sequences were inserted. In addition, a broad range of ethical and social issues also have to be taken into account by the regulatory decision-makers.

Zusammenfassung

Dieser Bericht befasst sich mit möglichen Auswirkungen der Methoden der neuen Gentechnik (Genome Editing) auf den Schutz der Umwelt. Dabei stehen Anwendungen der Nuklease CRISPR/Cas im Vordergrund, die als 'site directed nucleases' (SDN)-1 und -2 klassifiziert werden. Bei diesen Anwendungen sollen keine zusätzlichen Gene in das Erbgut eingefügt werden.

Die AutorInnen geben einen Überblick über die Unterschiede zwischen Anwendungen des Genome Editing an Pflanzen (SDN-1- und SDN-2) und konventionellen Zuchtmethoden, die in Bezug auf Fragen der Regulierung wichtig sind:

> Demnach ist auf der ersten Stufe der konventionellen Züchtung immer ein hohes Ausmaß an genetischer Vielfalt erwünscht, die dann als Grundlage für weitere Kreuzung und Selektion dient. Dabei können aus konventioneller Züchtung (einschließlich der ,Zufalls-' Mutagenese) biologische Eigenschaften erzielt werden, die erwünscht, komplex und vererbbar sind und oft auf sogenannten Quantitative Trait Loci (QTLs) beruhen, die auf der Ebene der Genetik in vielen Fällen nur unvollständig definiert sind. Zudem werden im Rahmen der konventionellen Züchtung manche genetischen Veränderungen häufiger beobachtet als andere. Inhärente, natürliche Vererbungsmechanismen wie der Abstand zweier Gene auf den Chromosomen, rekombinatorische Hot Spots, Gen-Cluster, die Größe des Genoms, gekoppelte Vererbung oder auch Reparaturmechanismen und epigenetische Effekte führen dazu, dass manche genetische Veränderungen häufiger auftreten, während andere seltener sind oder auch als sehr unwahrscheinlich angesehen werden.

> Diese Situation ist bei SDN-1- und SDN-2-Anwendungen in Bezug auf drei Aspekte grundsätzlich verschieden: (1) Diese Anwendungen zielen (in der Regel) nicht auf eine unspezifische Erhöhung der genetischen Vielfalt. Daher müssen nicht beabsichtigte Veränderungen des Erbgutes als unerwünschte Effekte angesehen werden. (2) Das Werkzeug CRISPR/Cas macht wesentlich mehr Abschnitte des Genoms für genetische Veränderungen verfügbar und ermöglicht so die Generierung biologischer Merkmale, die zuvor nicht möglich waren. (3) Mit Hilfe von neuen Gentechnikverfahren können komplexe Merkmale nur dann erzielt werden, wenn ihre genetischen Grundlagen vollständig bekannt sind.

Daraus folgt, dass eine höhere Präzision der Erbgutveränderung nicht unbedingt mit einer höheren Sicherheit oder einer höheren Züchtungseffizienz einhergeht. Auch unpräzise genetische Veränderungen, beispielsweise durch die Zufallsmutagenese, können sowohl sicher als auch vorteilhaft sein. Die folgenden Aspekte werden in Bezug auf die Regulierung als besonders wichtig identifiziert:

In vielen Fällen resultieren Anwendungen von SDN-1- und SDN-2-Verfahren in neuen Mustern von genetischen Veränderungen und neuen genetischen Kombinationen.

In den meisten Fällen werden dabei alte Gentechnikverfahren, wie biolistische Methoden („Genkanone") oder auch der Gentransfer via *Agrobacterium tumefaciens* eingesetzt, um den CRISPR/Cas-Komplex in die Zellen einzuführen. Dadurch kann eine große Bandbreite ungewollter genetischer Effekte ausgelöst werden.

Die CRISPR/Cas-Methode kann auch selbst viele unerwünschte Effekte verursachen, deren Auftreten vom individuellen Verfahren, den experimentellen Rahmenbedingungen, den ausgewählten Zielregionen im Erbgut und den spezifischen Organismen abhängig sein kann und die daher fallspezifisch untersucht werden müssen. Das geht weit über die vielfach diskutierten off-target-Effekte hinaus.

Anhand von ausgewählten Beispielen gibt der Bericht Einblick in konkrete regulatorische Herausforderungen, die sich bei Anwendungen von SDN-1- und SDN-2-Verfahren ergeben. Diese Beispiele sind in fünf Gruppen kategorisiert:

Veränderungen in den Inhaltsstoffen von Pflanzen, die Auswirkungen auf die Nahrungsnetze haben können

Es wird gezeigt, dass die Veränderung von pflanzlichen Inhaltsstoffen wie Öl, Eiweiß, Stärke und anderen biologisch aktiven Stoffen (wie pflanzliche Östrogene oder Vitamine) auch Auswirkungen auf Wildtiere wie Säugetiere, Vögel oder Insekten und deren Nahrungsnetze haben kann. Insbesondere dann, wenn derartige Veränderungen ein Ausmaß überschreiten, das mit konventioneller Züchtung erreicht wird, müssen deren Auswirkungen auf die Nahrungsnetze und auch die Futter- und Lebensmittelerzeugung eingehend untersucht werden. In diesem Zusammenhang müssen auch unerwünschte Effekte berücksichtigt werden, die zu Veränderungen der Inhaltsstoffe führen können.

Veränderungen in den Inhaltsstoffen von Pflanzen, die Auswirkungen auf deren Interaktion und Kommunikation mit der Umwelt haben können

Wie der Bericht zeigt, können Veränderungen der pflanzlichen Inhaltsstoffe auch Auswirkungen auf die Kommunikation und die Interaktion mit Organismen haben, denen diese Pflanzen nicht als Futter dienen, sondern auf andere Weise mit ihnen in enger Wechselwirkung stehen. Dies betrifft beispielsweise Insekten (Bestäuber oder Nützlinge) oder Symbionten (wie assoziierte Mikroorganismen). Daraus folgt, dass Auswirkungen auf die Kommunikation und Interaktionen von Pflanzen mit ihrer Umwelt insbesondere dann eingehend untersucht werden müssen, wenn diese Veränderungen ein Ausmaß überschreiten, das mit konventioneller Züchtung erreicht wird. In diesem Zusammenhang müssen auch unerwünschte Effekte berücksichtigt werden, die zu Veränderungen der Inhaltsstoffe führen können.

Veränderungen der Eigenschaften von Pflanzen, die geeignet sind, deren Fitness zu erhöhen

Es gibt einige Beispiele für Pflanzen, bei denen es beabsichtigt ist, die Fitness zu erhöhen, wie beispielsweise verbesserte Toleranz gegenüber Trockenheit, Befall von Schädlingen oder Pflanzenkrankheiten. Es gibt verschiedene Aspekte, die für die Risikobewertung wichtig sind, wie bspw. die Ausweitung einer nicht nachhaltigen Landwirtschaft auf bisher naturnahe Habitate oder auch Genübertragungen auf natürliche Populationen. Als Beispiel wird ein gentechnisch veränderter (GV) Reis angeführt, der auf Böden mit erhöhtem Salzgehalt wachsen soll und mit Hilfe von CRISPR/Cas generiert wurde. Diese genetische Veranlagung kann auch auf Wildreis übertragen werden, wodurch der für den Reisanbau besonders problematische unkrautartige Reis eine erhöhte Fitness erwerben wird. Daraus folgt, dass GV Pflanzen, die eine erhöhte Fitness aufweisen, einer eingehenden Risikobewertung unterzogen werden müssen. Dabei müssen insbesondere Auskreuzung und Hybridisierungseffekte mit besonderer Sorgfalt untersucht werden. In diesem Zusammenhang müssen auch unerwünschte Effekte berücksichtigt werden, die unerwarteterweise zu einer Erhöhung der Fitness führen können.

GV Organismen, die in der Umwelt überdauern und sich fortpflanzen können

Eine wichtige Frage im Hinblick auf die Verlässlichkeit einer Risikoabschätzung von GV Organismen ist, ob sich diese in der Umwelt ausbreiten können. Falls dies nicht ausgeschlossen werden kann, bleiben in vielen Fällen so große Unsicherheiten, dass die Ungewissheiten überwiegen und die Risikobewertung nicht belastbar ist. Der Grund dafür: Die vielfältigen und komplexen Wechselwirkungen mit der Umwelt stellen unter diesen Bedingungen eine besonders große Herausforderung für die Risikobewertung dar. Auch wenn sich domestizierte Pflanzensorten über mehrere Generationen im Labor, im Gewächshaus oder unter normalen Feldbe-

dingungen genetisch stabil zeigen, können veränderte Umweltbedingungen oder Kreuzungen mit genetisch heterogenen Populationen zu unerwünschten Effekten bei den Nachkommen führen. Deswegen müssen GV Organismen, die aus SDN-1- oder SDN-2-Anwendungen stammen und in der Umwelt überdauern und sich ausbreiten können, einer besonders gründlichen Risikoprüfung unterzogen werden. Es müssen Maßnahmen gegen ihre unkontrollierte Ausbreitung ergriffen werden.

Beispiele für ethische Implikationen, einschließlich Tiergesundheit und Tierschutz, Erhalt der Natur und die Rechte künftiger Generationen

Die AutorInnen betonen, dass die "Tatsache, dass das Redigieren von Genen für unsere Spezies und unseren Planeten so radikale Folgen haben wird" (Doudna & Sternberg, 2018, Seite 251) nicht nur einer strikten Regulierung bedürfen, sondern auch einer breiten öffentlichen Debatte über deren soziale und ethische Folgen erfordern. Die Einführung von gentechnisch veränderten Organismen in natürliche Populationen werden nicht nur neue Risiken verursachen, sondern auch unser Verständnis darüber grundlegend verändern, was wir als ,natürlich' ansehen. Mögliche Folgen können alle nachfolgenden Generationen auf diesem Planeten betreffen, einschließlich unserer eigenen. In diesem Zusammenhang plädieren die AutorInnen des Berichts dafür, neue Konzepte zu prüfen, wie die natürliche und gewachsene biologische Vielfalt in Zukunft besser geschützt und auch für nachkommende Generationen als gemeinsames Gut gesichert werden kann.

Weitere ethische Probleme betreffen den Tierschutz. Mehrere Publikationen berichten über Anwendungen von SDN-1, -2 und auch -3 bei Nutztieren mit dem Ziel, mehr Fleisch, Milch mit veränderten Inhaltsstoffen, Rinder ohne Hörner, virusresistente Schweine und an den Klimawandel angepasste Tiere zu erhalten. Wie im Bericht diskutiert wird, können kommerzielle Interessen zur Vermarktung dieser Tiere tiefgreifende Konflikte mit allgemein akzeptierten sozialen und ethischen Standards der europäischen Wertegemeinschaft verursachen.

Die AutorInnen kommen zu der Schlussfolgerung, dass es mehrere wichtige Gründe gibt, warum alle Organismen, die aus Anwendungen von SDN-1- und SDN-2-Verfahren stammen, einer verpflichtenden Risikobewertung unterzogen werden müssen. Kurz zusammengefasst sind in den meisten Fällen die Muster der beabsichtigten und unbeabsichtigten genetischen Veränderungen und die daraus hervorgehenden Genkombinationen deutlich von denen zu unterscheiden, die aus konventioneller Züchtung resultieren. Diese Unterschiede gehen mit biologischen Eigenschaften einher, die eingehend untersucht werden müssen, bevor eine Aussage über die Sicherheit der neuen Organismen getroffen werden kann.

Die Voraussetzungen für eine Regulierung, wie nach den Gesetzen der EU vorgesehen, sind daher gegeben, unabhängig davon, ob zusätzliche Gene eingeführt werden oder nicht. Zudem müssen bei der politischen Entscheidungsfindung eine große Bandbreite an ethischen und sozialen Fragen berücksichtigt werden.

1. Introduction

Several new techniques to generate genetically engineered (GE) organisms have been developed in the past decade. In particular, the so-called 'genome editing' technologies have been much discussed. They include oligonucleotide-directed mutagenesis (ODM), zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), meganucleases and CRISPR (clustered regularly interspaced palindromic repeats) techniques, with CRISPR/Cas becoming the predominant 'genome editing' technology today. Genome editing tools can be applied in genetic engineering for various purposes: to produce cisgenic, intragenic and transgenic organisms, to build synthetic genomics, to induce RNA-directed DNA methylation (RdDM) and to generate gene drive organisms.

Compared to the first generation of GE organisms, which are predominantly herbicide tolerant and insect resistant, these new methods can give rise to a broader spectrum of novel traits and new genetic combinations. They are also readily applicable to a broader range of species.

This condensed overview deals primarily with EU regulatory issues, especially applications involving site directed nuclease-1 (SDN-1) and site directed nuclease-2 (SDN-2). SDN-1 and SDN-2 are the most frequently applied genome editing applications, whereby CRISPR technology is the most important (Modrzejewski et al., 2019).

CRISPR/Cas, ZFN, TALEN and meganucleases all use site directed nucleases (SDNs) to introduce a DNA double strand break at specific sites. Depending on whether a repair template is used or not, these methods can induce either non-specific changes to one or more base pairs (termed SDN-1) via a non-homologous end joining repair mechanism or specific changes to nucleotide sequences (termed SDN-2) via homologous recombination mediated by homology directed repair. The induced changes at or around the target site can be substitutions, deletions or insertions of one or more base pairs. Depending on the specific SDN-1 or SDN-2 application, more extensive overall changes are possible and involve, for example, multiplexing which targets several genes at once, or repeated applications of SDN-1 or SDN-2 (Zetsche et al., 2017; Raitskin and Patron, 2016; Wang et al., 2016a). Changes involving the insertion of whole genes (including gene-stacking) are also possible and mediated by the use of donor DNA, termed site directed nuclease-3 (SDN-3) (Eckerstorfer et al., 2019; Lusser et al., 2012; Sander and Joung, 2014).

In the following, we use specific examples of SDN-1 and SDN-2 applications to compare the new genome editing technologies to methods used in conventional breeding; we also draw conclusions regarding regulatory requirements.

Our overview will be – coincidentally – published together with some other reports or statements on the same or a similar topic (e.g. Leopoldina, 2019; SAM, 2018) that come to different conclusions. The differences can be explained by the perspective from which these other reports or statements are written, i.e. the other reports (e.g. Leopoldina, 2019; SAM, 2018) are, to a large extent, driven by the perspective of those developing and applying the technology. This overview, on the other hand, follows the perspective of protected goods, such as nature protection, the environment and the health of plants, animals and humans.

Overview of genome editing applications using SDN-1 and SDN-2 in regard to EU regulatory issues | 11

2. Differences between genetic engineering and conventional breeding (including 'random' mutagenesis) with relevance to EU regulation

2. Differences between genetic engineering and conventional breeding (including 'random' mutagenesis) with relevance to EU regulation

Essentially, conventional breeding is always drawn from a range of genetic and biological diversity found in natural populations as well as in previously bred plant and animal varieties and breeds. In addition, spontaneous new mutations occur and specific triggers can increase the mutation rate. In particular with plants, additional 'tricks' can be used to increase genetic diversity, e.g. by exposing the seeds to specific chemicals to increase the natural rate of mutations. This process is known as mutation breeding ('conventional' mutagenesis or 'random' mutagenesis), which, in a first step, can enhance genetic diversity (see, for example, Oladosu et al., 2016). Plant cells can also react to non-specific external stress factors such as sunlight or chemicals in the environment. The process of conventional mutagenesis has been used in plant breeding since the mid-twentieth century (see, for example, Mba, 2013). It is important to understand that, taken as a whole, the results of mutagenesis are not predictable but, at the same time, cannot be considered to be totally random. Recent findings show there are many differences between spontaneously occurring mutations or induced genetic modifications and the pattern of genetic changes arising from genetic engineering.

There has been an increase in our knowledge about factors and aspects impacting the mechanisms and processes of mutations. For example, the chromatin structure has an impact on the frequency of mutations (Makova & Hardison, 2015). Repair mechanisms are also of major relevance. These come into play in the repair of DNA mismatches during replication (Belfield et al., 2018). The fact that genes can be present in several copies can be thought of as a backup (see, for example, Sanchez-Leon et al., 2018; Kannan et al., 2018). Furthermore, there are regions within the genome which mutate or recombine more frequently than others. These so-called 'hot-spots' favour the emergence of new genetic combinations (Choi et al., 2018; Si et al., 2015; Rogozin et al., 2003). It should also not be overlooked that genetic linkage (linkage drag) can lead to substantial restrictions in the new combination of traits (Lin et al., 2014).

All these cellular factors are in place and operate when a mutation occurs spontaneously or when it is induced in conventional mutagenesis breeding, for example, to ensure that some genomic locations are more frequently changed than others. Genome editing opens up new possibilities by making the whole genome accessible for changes (Kawall, 2019), but conventional mutagenesis does not bypass the named natural factors and mechanisms. Similarly, the new technical potential of genome editing is also expressed in the COGEM (2019) report as follows:

> *"Now that we are sequencing genomes, we know that the genes that breeders select and cross out are located at the ends of the plant chromosomes. But the genes in the middle, that is about 30 percent of the genes, are not accessible to breeders. With gene editing we are now able to change genes we have never had access to before."*

Similarly, Duensing et al., (2018) state:

> *"One important difference is that some crop genes lie in low or non-recombinogenic regions of the chromosome. (…) Genome editing ensures all genes are amenable to allele replacement." Duensing et al., (2018) specifically refer to the possibility of changing all gene copies by applying methods such as SDN1: "(…) genome editing can be targeted to a specific gene. However, few plant genes are found as single genes. (…) genome editing is adept at knocking out genes present in multiple copies. Thus, whenever a crop is found with multiple copies of the same gene knocked out, it will be almost certain that genome editing was used."*

More generally, breeding based on conventional mutagenesis speeds up evolutionary processes that might also occur naturally. In short, the methods and mechanisms used in what is known as 'conventional' breeding:

> ❯ make use of genetic diversity as a starting point;

> ❯ are applied to the whole cell or organisms;

> ❯ do not insert or delete genetic information using direct technical interventions.

Overview of genome editing applications using SDN-1 and SDN-2 in regard to EU regulatory issues |　　　　12

2. Differences between genetic engineering and conventional breeding (including 'random' mutagenesis) with relevance to EU regulation

Ultimately, breeding through mutagenesis increases genetic diversity without direct technological interventions. It is only through crossing and selection that plants and animals exhibiting beneficial traits can emerge as new varieties. The process is time-consuming and requires careful choice and repeated testing by breeders. Some organisms (products) resulting from conventional breeding might even require risk assessment in regard to health and the environment.

On the other hand, genetic engineering directly intervenes at the molecular level, i.e. inserting biological material that was prepared outside of the cells to achieve targeted changes in the genome or epigenome. Such interventions must undergo a mandatory risk assessment as required by Directive 2001/18/EC.

Genome editing and related techniques:

> are not based on natural biodiversity and the use of its large genetic pool. They aim to achieve quite distinct changes in the genome;

> are able to bypass mechanisms of natural heredity and gene regulation;

> enable the generation of genetic combinations that do not occur naturally, e.g. plants with specific patterns of change in the genome.

Conventional breeding always starts with a broad genetic diversity which is then followed by further steps of crossing and selection. Therefore the distinction between 'unintended' and 'intended' mutations is not really applicable, because, at least initially, a high rate of mutation is desired in the plants: it contributes to greater genetic diversity and allows choice through crossing and selection. Therefore, mutations are favourable or unfavourable, but not 'intended' or 'unintended'.

Contrary to conventional breeding, genetic engineering is not based on, or aiming to use, a large pool of genetic diversity. Rather, the goal of a technical intervention is a targeted, directed change in the genome. Therefore, only under these circumstances can the term 'unintended effect' be used in a meaningful way.

In general, greater precision at the molecular level does not directly result in greater safety or higher success rates in plant development. Imprecise modifications, such as those resulting from conventional mutagenesis, can be both safe and beneficial. For example, the desired traits derived from conventional breeding are often based on so-called quantitative trait loci (QTLs) which reveal certain traits only when properly combined. In many cases, the right combination and the contribution of single QTLs for a desired trait remain unknown. The possibility to make use of a pool of combinations of naturally evolved QTLs can be a huge advantage in conventional breeding compared to genetic engineering techniques, which mostly work with 'building bricks' of defined genetic information in isolation (see, for example, Fleury et al., 2012).

Overview: three specific differences between conventional breeding and new methods of genetic engineering (SDN-1 and SDN-2). The following three specific differences between conventional breeding and genome editing are all relevant in terms of EU regulation, the first one is probably the most fundamental.

(1) The first difference concerns the patterns of genetic change and the resulting new combinations of genetic information: it is not about quantity, but rather the quality and specific pattern of genetic change. Due to the methods used in genetic engineering, the resulting patterns of genetic change as well as biological characteristics and associated risks can be substantially different compared to those derived from conventional breeding. The intended genetic alterations of SDN-1 interventions often show specific patterns because the applied nucleases will, in most cases, cut all (or at least many) copies of the target gene throughout the genome.

Overview of genome editing applications using SDN-1 and SDN-2 in regard to EU regulatory issues | 13

2. Differences between genetic engineering and conventional breeding (including 'random' mutagenesis) with relevance to EU regulation

For example, TALENs was used in sugar cane to change 107 out of 109 gene copies of one gene to improve its quality as agro-fuel (Kannan et al., 2018). Furthermore, so-called multiplexing might be applied, which means that not just one, but several genes will be affected (Shen et al., 2017). These examples illustrate the high potential of SDN1 processes to penetrate the genome and cause profound alterations in the biological characteristics of plants without introducing any additional DNA sequences.

Another example from basic research, the so-called 'monarch-fly', shows that changes of just a few nucleotides in a specific combination can trigger major biological effects and associated risks: a particular gene in fruit flies (*Drosophila melanogaster*) was adjusted to resemble a similar gene in the monarch butterfly by applying SDN-2. Just three tiny changes in individual base pairs within that gene can make the fruit flies resistant to toxins produced by specific plants. As a consequence, the larvae ingest the toxin and may thereby become toxic to other animals feeding on them. The toxin still can be detected in adults. Releasing the flies into the environment may have detrimental effects on the food web and interconnected ecosystems (Karageorgi et al., 2019). If such GE organisms are not strictly regulated, they might be released unnoticed into the environment.

To decide whether organisms from the aforementioned examples are safe, detailed examination of their specific combination of genetic information and their overall biological characteristics is needed. Thus, there is a need for regulation as foreseen by the current EU GMO regulation, even if no additional DNA sequences are inserted as is the case with SDN-1 or SDN-2 applications. The reason being that, as explained, traits introduced via a specific pattern of genetic change can cause new combinations of genetic information in the organisms, which are different compared to the ones derived from conventional breeding or occurring in nature and may cause biological traits conferring specific risks.

(2) The second difference concerns the process of introducing the components needed to establish the new trait in the cells. This process requires in one way or another the introduction of material that was prepared outside of the cells to achieve targeted changes in the genome or epigenome. In many cases, the 'old', non-targeted techniques of genetic engineering (such as transformation by *Agrobacterium* and biolistic methods) are used in a first step (for overview, see: Testbiotech, 2019) to insert the SDN components into the cells. It is only in a second step that the nuclease is produced by the cells and starts to 'search and cut' the target site(s). It is known from current literature that these first steps give rise to many unintended effects, such as deletions and rearrangements, that can include unintended insertion of additional DNA and impact gene expression (see, for example, Forsbach et al., 2003; Kim et al., 2003; Latham et al., 2006; Makarevitch et al., 2003; Rang et al., 2005; Windels et al., 2003). It is also known that the first step can impact epigenetic regulation (Jupe et al., 2019). Therefore, not only the new trait, but also the process for introducing the trait is decisive for risk assessment. This is set out below in more detail.

(3) The third difference concerns on-target and off-target effects specifically caused by the activity of the SDN components (such as the Cas nuclease) that are introduced. The extent of specific on-target and off-target effects of SDN-1 and SDN-2 interventions largely depends on various experimental parameters such as (i) the specific nuclease(s) used; (ii) the target organism and its tissue, respectively; (iii) the targeted gene(s); (iv) the way in which the components are introduced into the cells; (v) the dosage of the nuclease(s); (vi) with CRISPR/Cas, the guide RNA used and (vii) duration of the intervention (for overview, see Eckerstorfer et al., 2019; Agapito-Tenfen et al., 2019; see also below). All these technical details determine the precision as well as the efficiency of an intervention. They need to be taken into account by competent authorities in order to identify potential unintended effects specifically caused by a specific genome editing intervention.

3. Overview of some examples of specific applications with relevance for EU regulation

In the following section, we give an overview of some examples of specific applications (mostly SDN-1 and SDN-2) in plants and animals used for food production or in natural populations. All examples have potential implications for nature conservation and environmental protection (also including aspects of human health). Relevant criteria for the selection of these examples with relevance for risk assessment are:

> changes in the composition of plants, e.g. protein, starch or constituents with specific biological activity that may impact the food web ('the wider environment');

> changes in the composition of plants that may have an effect on biological characteristics with relevance for plant communication, including symbionts such as microorganisms and pollinators ('the closely interconnected environment');

> changes in the biological characteristics of the GE organisms affecting their reaction to environmental stressors (biotic and abiotic) that may also alter their impact on ecosystems, especially if they potentially enhance fitness;

> the potential of GE organisms to persist and propagate in the environment, become invasive or cause disturbance and disruptive long-term effects.

Further, we include some examples with ethical implications, including animal health and welfare, naturalness and rights of future generations.

The examples are presented in a condensed form to provide information about the specific regulatory aspects.

3.1 Changes in the composition of plants that may impact the food web

If plant components such as oil, protein, starch or other constituents with specific biological activity (such as plant estrogens or vitamins) are changed, this may impact wild life species such as insects or birds feeding on those plants and related food webs. For example, Colombo et al. (2018) indicate potential hazards for food webs that result from the extensive cultivation of GE plants, such as (transgenic) oilseed rape producing long-chain omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are not generally produced by terrestrial plants. In other words, novel plant components with no putative evolutionary adaption may enter ecosystems and cause complex risks: the omega-3 fatty acids in the plants can, for instance, change the growth and fecundity of the organisms that feed on them, since they are normally not present in terrestrial diets. If such GE oilseed rape were to be grown in Europe, relevant characteristics might spread by gene flow to other domesticated or wild populations, and resulting effects could be carried forward into the food chain. Hixson et al. (2016) tested what impact artificial EPA and DHA has on a terrestrial crop pest of brassicaceae plants, the cabbage white butterfly (*Pieris rapae)*. When larvae of the butterfly were fed a diet containing EPA and DHA, the adults were heavier and had smaller wings compared to larvae that were fed on normal canola oil, i.e. without EPA and DHA (Hixson et al., 2016). This study indicates that an altered fatty acid composition in plants can have an impact on the associated food web, showing the necessity for an adequate risk assessment of these plants.

The case study shows that the impact of GE plants on the food web should undergo specific environmental risk assessment whenever the intended change in plant composition exceeds the range of conventionally breed plants. There are several examples of plants derived from SDN-1 and SDN-2 that are substantially changed in their composition.

For example, Morineau et al. (2017) targeted CRISPR/Cas9 to conserved regions in the subgenomes of *Camelina sativa* to alter the three CsFAD2 genes. The three alleles were altered in different combinations which allowed the evaluation of the contribution of each allele to the level of polyunsaturated fatty acids (PUFAs) and the effect thereof on the overall development of the plants (see also Jiang et al., 2017). According to USDA / APHIS[1], the company Yield 10 intends to change three genes (18 genome locations in total) in camelina to specifically change its oil composition. For this the gene scissor CRISPR/Cas is used together with two guide RNAs in one go, which is known as multiplexing. It allows the simultaneous change of several genes and the introduction of gene combinations which are difficult or impossible to achieve through conventional breeding. Similar outcomes can be reached if the steps involved are carried out one after the other. The probability that these gene locations would all be coincidentally changed simultaneously through conventional mutagenesis is very low indeed.

Other examples of plants derived from SDN-1 (or SDN-2) with intended compositional changes are soybeans (Haun et al., 2014; Demorest et al., 2016) with altered oil composition, potato (Andersson et al., 2018), rice (Sun et al., 2017), maize (DuPont & Pioneer, 2015) and sugar cane (Kannan et al., 2018) with altered carbohydrate composition, wheat (Sanchez-Leon et al., 2018) with altered protein composition and tomatoes (Zsögön et al., 2018) with changes in content of carotenoids. All these plants may pose specific risks for the related food web or the human food chain while several of them are not intended for any food uses at all. For example, the changes in oil content as performed in *Camelina sativa* (Jiang et al., 2017; Morineau et al., 2017) are meant for the production of agrofuels.

Consequently, the impact on the food web and the food and feed production chain should be extensively investigated in environmental risk assessment if the intended changes in plant composition exceed the range of that in conventionally bred plants. There are several examples of plants derived from SDN-1 and SDN-2 applications which are substantially different in their composition (see above). If they are released into the environment without sufficient regulatory oversight, they may disturb or interrupt related food webs. They may also pose risks for human and animal health if they enter the food and feed chain.

Furthermore, it has to be considered that drastic changes in the composition of plants may not only affect the intended metabolic pathway, but also other interconnected pathways. Unintended changes derived thereof may be influenced by the specific environmental conditions under which the plants are grown or other environmental interactions. Therefore, the possibility of any unintended changes in plant composition will require close attention and detailed investigation during risk assessment.

Finally, it has to be considered that SDN-1 (and SDN-2) applications as used, for example, in *Camelina sativa* by Yield 10 are a multistep process that involves the application of 'old' non-targeted methods of genetic engineering, such as biolistic methods or transformation by *Agrobacterium tumefaciens* in the first step (for overview, see Testbiotech, 2019 and Table 2). These methods of genetic engineering can result in complex genetic insertions containing multiple copies of the transgene and/or rearrangements of both the DNA intended to be inserted and the host plant DNA, and can also result in other unintended effects such as epigenetic alterations in the vicinity of the integration site (Forsbach et al., 2003; Jupe et al., 2019; Kim et al., 2003; Latham et al., 2006; Makarevitch et al., 2003; Rang et al., 2005; Windels et al., 2003). Therefore, GE plants need to be carefully assessed, including in regard to these associated risks.

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1   www.aphis.usda.gov/aphis/ourfocus/biotechnology/am-i-regulated/Regulated_Article_Letters_of_Inquiry

3.2 Changes in the composition of plants that may impact plant communication and interaction with the environment

Changes in plant composition can also affect communication and interactions with organisms that do not feed on them but are associated in other ways, e.g. in cooperation or synergism (such as the plant microbiome or beneficial insects, e.g. pollinators) or attacking the plants (so-called 'pest' insects).

Plants can 'communicate' with their environments via multiple biochemical pathways. These pathways include exchange of information with other plants, microorganisms and insects (see Schaefer & Ruxton, 2011). Various compounds are involved, e.g. volatile substances, secondary metabolites and biologically active compounds. For example, the phytohormone jasmonate and its derivates thereof have important roles as signaling molecules in plant defence, particularly against insect herbivores (Glazebrook, 2005; Howe and Jander, 2008). Linolenic acid is a precursor molecule of jasmonic acid (Gfeller et al., 2010). Its concentration is intentionally changed in several genetically engineered plants to alter their fatty acid composition. (Do et al., 2019; Okuzaki et al., 2018; Abe et al., 2018). Linolenic acid is threefold unsaturated and is produced in plants in two enzymatic reactions from the monounsaturated oleic acid and the twofold unsaturated linoleic acid as intermediate, involving FAD2 (Fatty Acid Desaturase 2) and FAD3 (Fatty Acid Desaturase 3).

SDN-1 using TALENs was applied in soybeans to lower the expression of FAD2 (Haun et al., 2014) and FAD3 (Demorest et al., 2016). Thereby, the content of oleic acid was increased from 20% to 80% and linoleic acid was reduced from 55% to less than 4%. The engineered soybeans, meant for food and feed, are already being grown by Calyxt in the US[2].

There are not many publications on the consequences of a reduced content of linoleic and linolenic acid in plants, and for their interconnected organisms. However, there are findings which indicate a substantial impact on polyunsaturated fatty acids (PUFAs) and defence mechanisms of soybeans: if soybeans enhance the release of jasmonic acid, ladybird beetles (*Coccinella septempunctata*), which are the natural predators of aphids (such as *Aphis glycines*) are attracted (Zhu and Park, 2005). If aphids (*Aphis glycines*) attack plants, they seem to be able to reduce the content of PUFAs (such as linoleic acid and linolenic acid) and downregulate the plant defence mechanism which is based on elevated levels of jasmonic acid; this is beneficial for the aphids, but detrimental to the soybeans (Kanobe et al., 2015). Similar effects may be caused by intervention in the genome as described above (Haun et al., 2014; Demorest et al., 2016).

Consequently, the impact on plant communication and interaction with its interconnected environment should undergo detailed environmental risk assessment if the intended changes in plant composition exceed the range of what is known for plants derived from conventional breeding. If these plants were to be released into the environment without sufficient regulatory oversight, they could disturb or interrupt the ecological, or agri-ecologic networks, or pose risks to plant health, the ecosystem services or wild life.

Furthermore, as mentioned under 3.1., it has to be considered that drastic changes in the composition of plants may not only concern the intended metabolic pathway but also those which are interconnected. These unintended changes may also be influenced by the specific environmental conditions under which the plants will be grown or other interactions with the environment. Therefore, the unintended changes in plant composition will also require close attention and detailed investigation during risk assessment.

2　　https://www.businesswire.com/news/home/20190122005964/en/Calyxt-Doubles-2018-High-Oleic-Soybean-Acres

Finally, applications of SDN-1 (and SDN-2) as performed for the Calyxt soybean are – in most cases – a multistep process which involves the application of 'old' non-targeted methods of genetic engineering, such as biolistic methods or transformation by *Agrobacterium tumefaciens* in the first step (for overview, see Testbiotech, 2019 and Table 2). These methods of genetic engineering are also known to go along with many unintended effects (see above) which have to be carefully assessed in regard to associated risks.

3.3 Changes in the biological characteristics of the GE organisms intended to enhance fitness

Fitness is a term used in evolutionary biology. In populations of species with sexual reproduction, fitness can be quantified by comparing the reproductive success of specific individuals carrying specific properties compared to the rest of the population. If the reproductive success of the former is higher than that of the rest, the specific properties are likely to confer enhanced fitness. In general, fitness is dependent on the environment rather than a fixed characteristic of a given property. The consequence is that some individuals might be better suited to survive under specific environmental stressors than others.

In the context of risk assessment of GE plants, there are a few examples where enhanced fitness is intended by the trait. One example is the transgenic maize MON 87460, which is supposed to have higher tolerance to drought, but which apparently failed to show the desired effects in field trials in South Africa[3]. In other cases, GE plants show enhanced fitness that might derive from the insertion of traits rendering them more tolerant to pest infestation or plant diseases caused by viruses or fungi, or herbicide resistant crops which when grown in rural areas are treated with the complementary herbicide for managing weeds.

There are several aspects that have to be considered in the context of GE plants with enhanced fitness, such as expansion of unsustainable agricultural cultivation to more natural habitats or gene flow to weedy species. For example, a recent publication reported on rice with improved salinity tolerance engineered with CRISPR/Cas9, targeting the OsRR22 gene (Zhang et al., 2019). Rice has a high potential for hybridisation with wild relatives. Domesticated grasses (*Poaceae*) still have a high potential for persistence and invasiveness. Rice provides a useful example here since it has a history of double domestication (or re-domestication) with periods in between of "de-domestication", or reversion to a wild form (Vigueira et al., 2013; Kanapeckas et al., 2016). Consequently, gene flow is very often observed between weedy rice (which is also known to occur in Europe) and cultivated rice forms growing in vicinity (Chen et al., 2004). Gene flow between fields and weedy rice can also be circular, reiterative and repetitive (see also Lu and Snow, 2005). Unexpected effects in hybrid generations resulting from GE rice and weedy rice are repeatedly reported: for example, seeds from F1 hybrids resulting from rice producing Bt toxins had higher germination rates and produced more seedlings than their weedy parents (Cao et al., 2012).

Consequently, plants generated by SDN-1 or SDN-2 which might show enhanced fitness should be subjected to detailed environmental risk assessment, especially in regard to gene flow and next generation effects. If such plants are released into the environment without sufficient regulatory oversight, they may disturb or interrupt the ecological or agri-ecologic networks, or even become invasive and replace natural populations.

Furthermore, there are some common issues relating to GE organisms described in chapters 3.1 and 3.2: Changes in the composition of plants that may impact the food web, the communication of the plants with

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3   https://www.acbio.org.za/sites/default/files/duments/Minister's_final_decision_on_Monsanto_appeal.pdf

other species or changes in the biological characteristics of the GE organisms may not only concern the respective intended metabolic pathways, but also other interconnected pathways. Unintended changes derived thereof may be influenced by the specific environmental conditions under which the plants are grown or other environmental interactions (e.g. interactions with other species). Therefore, the possibility of any unintended changes in plant composition will require close attention and detailed investigation during risk assessment.

Finally, it has to be considered that SDN-1 (and SDN-2) applications to improve salinity-tolerance in rice is, in most cases, a multistep process involving the application of 'old' non-targeted methods of genetic engineering, such as biolistic methods or *Agrobacterium tumefaciens* in the first step (for overview, see Testbiotech, 2019 and Table 2). These methods of genetic engineering are also known to go along with many unintended effects (see above) which have to be carefully assessed in regard to associated risks.

3.4 Organisms with the potential to persist and propagate in the environment

It is known that the robustness and reliability of environmental risk assessment of GE organisms is largely influenced by the question of whether the GE organisms can spread in the environment. Very generally stated, the risk assessment will be less certain and face complex questions, if engineered or synthetically formed organisms can persist in the environment and if gene flow to wild relatives with viable offspring can be established (Bauer-Panskus et al., 2020).

In many cases, significant uncertainties remain, and some unknowns might prevail that make the risk assessment inconclusive. This is because multiplex interrelations with the closer and wider environment pose a real challenge for the risk assessor. While genetic stability over several generations might be demonstrated in domesticated varieties under normal field conditions or green house cultivation, genome x environmental interactions and introgression into heterogeneous genetic backgrounds can still trigger unpredictable next generation effects.

Whatever the case, the biological characteristics of the original GE organisms produced in the lab and grown under controlled conditions, cannot be regarded as sufficient to predict all relevant effects that can emerge in next generations and in interaction with those receiving environments where the organisms might occur. For example, several publications report that unintended genomic effects can be triggered by changing environmental conditions or biotic and abiotic stressors (Fang et al., 2018; Matthews et al., 2005; Meyer et al., 1992; Trtikova et al., 2015; Zeller et al., 2010; Zhu et al., 2018). So far these observations are based on experiments with transgenic plants. It remains to be investigated to what extent next generation effects (triggered by heterogeneous genetic backgrounds or changes in the environment) can be excluded, predicted or observed in organisms derived from SDN-1 or SDN-2 applications.

Very generally, if the problem of spatio-temporal controllability is not solved, the uncertainties and unknowns in risk assessment will sooner or later dominate the available knowledge, affecting the ability to conclude on the safety of GE organisms (Bauer-Panskus et al., 2020). This problem also concerns long-term effects on the food web, interaction and signaling pathways, intra- and interspecies communication, invasiveness, as well as human, animal and plant health.

There are several plant species engineered with SDN-1 or SDN-2 applications that have the potential to persist and propagate in the environment, such as camelina, penny cress, green foxtail, rice, arabidopsis, oilseed rape and poplar trees, as well as insects (such as honey bees and *drosophila*).

Therefore, organisms derived from SDN-1 or SDN-2 applications should undergo detailed environmental risk assessment, especially in terms of gene flow and next generation effects. If such organisms are released into the environment without sufficient spatio-temporal control, they may disturb or interrupt the ecological or agri-ecologic networks, or even become invasive and replace natural populations.

3.5 Examples with ethical implications, including animal health and welfare, naturalness and rights of future generations

Jennifer Doudna, in her book "A Crack in Creation" (2017) explains that the new methods of genetic engineering, and especially the CRISPR technology, can be used to bring to an end the natural processes of evolution that have emerged over nearly four billion years:

> *"Gone are the days when life was shaped exclusively by the plodding forces of evolution. We are standing on the cusp of a new area, one in which we will have primary authority over life's makeup and all its vibrant and varied outputs. Indeed, we are already supplanting the deaf, dumb, and blind system that has shaped genetic material on our planet for eons and replacing it with a conscious, intentional system of human-directed evolution."* (Page 243/244).

Similarly, George Church, another leading expert in the field of genome editing, states in his book "Regenesis" (Church & Regis, 2012):

> *"Synthetic genomics has the potential to recapitulate the course of natural genomic evolution, with the difference that the course of synthetic genomics will be under our own conscious deliberation and control instead of being directed by the blind and opportunistic processes of natural selection." (*Page 13)

The *"radical implications of gene editing (…) for our species and our planet"* (Doudna & Sternberg, 2017, page 243) not only deserve strict regulatory oversight, but also deserve a broad sociological and ethical debate. It has to be emphasized that besides GE organisms, so far, all organisms can be considered 'natural offsprings' of the 'first cell' and are not technically designed by mankind: natural mechanisms, such as gene regulation and patterns of reproduction, still work no matter, whether an organism is domesticated or not. This situation which has lasted for about 4 billion years and is called evolution might now come to an end.

Applications of SDN-1 and SDN-2 and also other genetic engineering techniques are proposed for use in the conservation of biodiversity, amongst others, in an IUCN report (2019). Proposed usage includes intervention in natural populations of corals, trees, insects and mammals. Currently, this includes discussion of the introduction of so-called gene drives for the eradication of species regarded as problematic for humans (CSS, 2019).

The intrusion of GE organisms into native populations will not only raise safety issues, it would also fundamentally change our understanding of what is considered natural and what the consequences would be for all future life on this planet, including our own species. In this context, there are some suggestions that must be carefully considered, such as strengthening the protection of biodiversity, which implies that the "nature of life" should be legally safeguarded for the future as a protected common good (see Chapron et al., 2019).

Whatever the case, when researchers reflect on the prospect of taking control of our natural legacy, the socio-ethical dimension has to be given high priority, including questions such as: (i) do humans have a right to change their legacy?; (ii) who can make decisions?; (iii) on what grounds?; (iv) who will be involved?; (v) how should the precautionary principle be applied?; and (vi) how can future generations be given sufficient freedom of choice to make their own decisions? These issues also include more recent problems such as freedom of

choice in regard to food and obstacles in the coexistence of agricultural practices, such as organic agriculture.

Further major issues for ethical debate include animal welfare. There are several applications of SDN-1 and SDN-2 and also SDN-3 on farm animals, to produce more meat, milk with changed composition, hornless cows, virus-resistant pigs and animals that are adapted to conditions of climate. Patents have already been filed for several such animals (Table 1), reflecting the considerable interest in the marketing of GE animals.

Table 1: Examples of patents filed by Recombinetics (USA) for livestock genetically engineered with nucleases, such as CRISPR/Cas, (Testbiotech, 2018)

Application Number	Claims
WO 2012116274	Methods using nucleases to increase muscle growth in cattle and pigs.
WO 2013192316	Methods using nucleases to increase muscle mass in certain cattle; and produce hornless cattle.
WO 2014070887	Livestock that do not reach sexual maturity and can be fattened for longer. Farmers cannot use these animals for breeding.
WO 2014110552	Hornless cattle for natural and synthetic genetic applications.
WO 2015168125	Animals with multiple genetic changes.
WO2055030881	Applications of nucleases (TALEN) and resulting animals. Amongst others, pigs, cattle, horses, fish, dogs, cats and primates are claimed.
WO 2017062756	Male sterility in livestock as well as wild populations.
WO2017040695	Selection of genetic variants in cattle such as polled, climate adaptation and fertility and other related usages.

As requested by Directive 2001/18/EC, the risk manager also has to take ethical and social implications into account. In many cases, the intended traits, such as higher production in muscle mass (meat), are likely to face adverse consumer reactions. In addition, the application of genome editing in mammals is a multistep process, in many cases involving cloning (Tan et al., 2016) which raises additional ethical concerns (EGE, 2008). The process of cloning is known to cause side effects with severe implications for animal health; this has been shown to be relevant, e.g. for the genome editing of pigs (Wang et al., 2016b). Another example in this context is bacterial DNA that was unintentionally inserted in the genome of GE cattle (SDN-3 via TALENs) (Norris et al., 2020) which raises questions concerning safety and also ethics, since the cattle had to be killed after the additional DNA was detected.[4]

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4   www.technologyreview.com/s/614235/recombinetics-gene-edited-hornless-cattle-major-dna-screwup/

4. How to regulate SDN-1 and SDN-2?

According to EU law (Directive 2001/18/EC), all organisms derived from processes of genetic engineering generally require specific, case-by-case and step-by-step risk assessment before they are released into the environment or allowed for use in food products. These requirements also concern organisms generated through SDN-1 and SDN-2 applications. They need to be applied during the mandatory risk assessment and approval process before they are released into the environment or placed onto the market. The situation was confirmed by the EU Court in its decision C-528/16, stating:

"As the referring court states in essence, the risks linked to the use of those new techniques/methods of mutagenesis might prove to be similar to those which result from the production and release of a GMO through transgenesis. It thus follows from the material before the Court, first, that the direct modification of the genetic material of an organism through mutagenesis makes it possible to obtain the same effects as the introduction of a foreign gene into that organism and, secondly, that the development of those new techniques/methods makes it possible to produce genetically modified varieties at a rate and in quantities quite unlike those resulting from the application of conventional methods of random mutagenesis."

The need to regulate SDN-1 and SDN-2 applications, as also expressed in the court decision, is backed by evidence relating to the substantial differences between conventional breeding and the new methods of genetic engineering:

SDN-1 and SDN-2 applications, often carried out simultaneously in a multiplexing approach or in series, cause typical patterns of genetic change and new combinations of genetic information that can also be used for identification and traceability (see also Duensing et al., 2018). As far as the deregulation of products in the US is concerned and as far as the relevant data are made available, (see Testbiotech, 2019 and Table 2) this finding seems to be applicable to all GE organisms derived from methods of genome editing. For example, this is also the case with so-called 'non-browning mushrooms' which was the first CRISPR organism to be deregulated by the USDA: here several copies of one gene were changed to block the production of a specific enzyme (Waltz, 2016; Gartland et al., 2017). It is unlikely that a similar mushroom could ever develop spontaneously. Table 2 also shows (as far as the data are made available) that 'old' methods of genetic engineering (such as biolistic methods, Agrobacterium tumefaciens or polyethylene glycol (PEG)-mediated transformation) were always used as a first step to introduce the CRISPR/Cas components into the cells.

Table 2: Overview of organisms genetically engineered with nucleases and classified as non-regulated by USDA / APHIS (CBI: Confidential Business Information; PEG: polyethylene glycol (PEG)-mediated transformation method)[5]

	Date of decision	Organism	Applicant	Methods/steps	Intended trait
1	16.12.2011	Unknown	Cellectis (now Calyxt)	(1) not specified (2) meganucleases	Not specified
2	08.03.2012	Maize	Dow AgroSciences (Corteva Agriscience)	(1) not specified (2) zincfinger nuclease	Reduced phytate level
3	28.08.2014	Potato	Cellectis (now Calyxt)	(1) PEG (2) TALEN	Not specified / CBI

5   source: www.aphis.usda.gov/aphis/ourfocus/biotechnology/am-i-regulated/Regulated_Article_Letters_of_Inquiry

	Date of decision	Organism	Applicant	Methods/steps	Intended trait
4	05.05.2015	Soybean	Cellectis (now Calyxt)	(1) CBI (2) TALEN	Changed fatty acid composition
5	20.05.2015	Soybean	Cellectis (now Calyxt)	(1) CBI (2) TALEN	Changed fatty acid composition
6	22.05.2015	Rice	Iowa State University	(1) not specified (2) TALEN	Improved resistance to bacterial blight
7	12.11.2015	Maize	Agrivida	(1) CBI (2) meganucleases	Changed starch composition
8	15.04.2016	Mushroom	Penn State University	(1) PEG (2) CRISPR	Non browning / improved storage
9	18.04.2016	Maize	DuPont Pioneer (Corteva Agriscience)	(1) biolistic transformation (2) CRISPR	Changed starch composition („waxy corn") (no precise information, CBI)
10	15.09.2016	Potato	Calyxt	1) PEG (2) TALEN	For improved processing (no precise information, CBI)
11	02.11.2016	Wheat	Calyxt	(1) biolistic transformation (2) TALEN	Improved resistance to powdery mildew
12	02.12.2016	Potato	Simplot	1) Agrobact. tumefaciens (2) TALEN	Improved storage
13	07.04.2016	Green foxtail	Danforth Center	(1) Agrobact. tumefaciens (2) CRISPR	Change in timing of flowering for higher yield
14	29.08.2017	Camelina	Yield 10	(1) Agrobact. tumefaciens (2) CRISPR	Changed oil composition (no precise information, CBI)
15	25.09.2017	Alfalfa	Calyxt	(1) not specified (2) TALEN	For improved digestibility (no precise information, CBI)
16	16.10.2017	Soybean	USDA	(1) Agrobact. tumefaciens (2) CRISPR	Knock-out of two genes that are assumed to affect stress and salt tolerance
17	29.12.2017	Tobacco	North Carolina State University	(1) Agrobact. tumefaciens (2) meganucleases	Reduced nicotine content
18	16.01.2018	Maize	Pioneer (Corteva Agriscience)	(1) biolistic transformation (2) CRISPR	Improved resistance to northern leaf blight (NLB), with insertion of a repair template DNA (SDN2)

	Date of decision	Organism	Applicant	Methods/steps	Intended trait
19	20.03.2018	Wheat	Calyxt	(1) not specified (2) TALEN	For improved nutritional quality (exact gene and phenotype claimed as CBI).
20	14.05.2018	Tomato	University of Florida	(1) Agrobact. tumefaciens (2) CRISPR	For easier harvesting
21	8.06.2018	Pennycress	Illinois State University	(1) Agrobact. tumefaciens (2) CRISPR	Changed oil composition (increased number of changed genes) (exact gene and phenotype CBI)
22	7.09.2018	Camelina	Yield 10	(1) Agrobact. tumefaciens (2) CRISPR	Changed oil composition (nine target genes changed), exact gene and phenotype claimed as CBI
23	08.02.2019	Lettuce	Intrexon	(1) CBI (2) ‚gene edited'	Gene and phenotype claimed as CBI
24	25.02.2019	Nicotiana attenuata (coyote tobacco)	Max Planck Institute for Chemical Ecology	(1) Agrobact. tumefaciens (2) CRISPR	Modified nectar composition
25	19.04.2019	Pennycress	Illinois State University	(1) Agrobact. tumefaciens (2) CRISPR	Gene and phenotype claimed as CBI
26	17.06.2019	Soybean	University of Minnesota	1) Agrobacterium rhizogenes (2) CRISPR	Changes in petiole length
27	17.06.2019	Soybean	University of Minnesota	(1) Agrobacterium rhizogenes (2) CRISPR	Changed seed composition
29	31.07.2019	Tobacco	Altria Client Services LLC	(1) PEG (2) CRISPR	Gene and phenotype claimed as CBI
30	29.01.2020	Pennycress	Illinois State University	(1) Agrobact. tumefaciens (2) CRISPR	Gene and phenotype claimed as CBI
31	29.01.2020	Pennycress	CoverCress Inc.	(1) Agrobact. tumefaciens (2) CRISPR	Gene and phenotype claimed as CBI
32	29.01.2020	Citrus	Soilcea	(1) PEG (2) CRISPR	Tolerance to citrus canker caused by Xanthomonas citri ssp. citri (Xcc) bacterium
33	14.02.2020	Tomato	Michigan State University	(1) Agrobact. tumefaciens (2) CRISPR	Changes in acylsugar specialized metabolites

Since SDN-1 and SDN-2 applications can bring about new combinations of genetic information and genetic changes with a unique pattern, it is not unlikely that the resulting effects are of a specific biological quality that may generate new risks compared to those resulting from conventional breeding (including conventional mutagenesis).

Further, in many cases, the process of genetic engineering as well as genome editing involves methods such as 'gene canon' or agrobacterium transformation, which can cause a wide range of unintended effects (see above). There are additional specific unintended effects caused by SDN-1 and SDN-2, such as off-target as well as on-target effects.

Specific off-target effects caused by SDN-1 or SDN-2 have been detected and reported during experiments with several crop plants, including rice, soy and barley (Modrzejewski et al., 2019; Wolt et al., 2016; Zhu et al., 2017; Eckersdorfer et al., 2019). Braatz et al. (2017), for example, found through whole-genome sequencing that transformation of oilseed rape with a CRISPR/Cas9 expression construct resulted in at least five independent insertions of vector backbone sequences in the genome of the modified plant. Further findings on unintended effects are reported in farm animals such as pigs (Ryu et al., 2018), as well as model animals such as rats and mice (Anderson et al., 2018; Shin et al., 2017).

There are also examples of unintended on-target-effects: for example, a general problem with DNA-based CRISPR/Cas9 is the unintended insertion of the DNA or partial DNA-fragments encoding the CRISPR/Cas9 complex itself into the genome of the plant (see Liang et al., 2017). Further, large deletions and complex rearrangements have been reported during the CRISPR/Cas9 process (Kosicki et al., 2018; Hahn & Nekrasov, 2019). In addition, large deletions induced by a single guide RNA were found to delete whole exons causing exon skipping in cell lines (Mou et al., 2017; Sharpe and Cooper, 2017; Kapahnke et al., 2016; Tuladhar et al., 2019). Exon skipping can produce mRNAs with intact reading frames that encode altered, partially functional proteins which have to be assessed within risk assessment.

In order to detect such unintended effects it is essential to apply the available methods diligently for genome analysis, but also that the specific methods which were applied during the genetic engineering of an organism are known. Thus, the current EU GMO Regulation correctly requests that all organisms derived from processes of genetic engineering generally require a specific and case-by-case risk assessment.

As more experience is gathered with gene editing, it will become necessary to establish more specific guidance or implementing regulations to ensure that risk assessment meets the new challenges. For example, current risk assessment practices in the EU may need to be expanded in order to assess the additional unintended effects that genome editing can cause: since the goal of genome editing, in many cases, is to deeply change plant metabolism, the current 'comparative risk assessment' approach (adopted by EFSA) might have reached its limits, since in many cases it can be very difficult or impossible to find comparative organisms.

Therefore, the molecular characterisation element of the risk assessment will need to be expanded to include analysis for unintended changes at the genomic level, including off-target effects, unintended on-target effects and effects on genomic regulation. There are several techniques that can be used to detect and assess unintended effects generated by the genome editing process. These are collectively summarised as 'omics' approaches and include analysis of the RNA profile (transcriptomics), the protein profile (proteomics) and the metabolite profile (metabolomics). These techniques allow the identification of specific unintended effects (i.e. off-target effects, unintended on-target effects, effects on genomic regulation) arising from the application of 'old' and 'new' methods of genetic engineering. In this context, the development of new standards in applying molecular analytic tools is indispensable.

On the other hand, if a company can show evidence through the use of adequate methods (such as whole genome sequencing) that their organisms are identical to other organisms already on the market, it is likely to reduce the number of further examinations needed. However, to show the potential equality of the respective products and organisms to comparators derived from conventional breeding or natural populations, a basic set of data still has to be provided within the mandatory approval process to come to a reliable decision-making.

Therefore, it is likely that EFSA and / or the Commission will present some guidance or implementing regulation which will incorporate some flexibility in the step by step procedure. It should be completely unproblematic to devise such a scheme within current regulation.

In general, risk assessment of organisms developed using new methods of genetic engineering, such as SDN-1 and SDN-2 applications, should take the following criteria into account:

> the whole pattern of genetic changes and their effects need to be considered, including their impact on cells and organisms;

> if, in specific cases, it is assumed that the results of genome editing cannot be distinguished from those of conventional breeding, comparative data must be provided for confirmation, including whole genome sequencing data;

> data from whole genome sequencing must also be provided to assess unintended changes in the genome that might have been caused by older genetic engineering techniques such as 'gene gun' methods (biolistic methods) or agrobacterium transformation;

> 'omics' data are necessary to assess changes in the transcriptome, the proteome and the metabolome in order to assess the effects of gene changes on the organism;

> the GE organisms should be exposed to a wide range of defined environmental stress conditions to specifically test their response to climate change or pathogens;

> the risk assessment of human and animal health in relation to the respective products should also take the microbiome of the gastrointestinal tract into account, e.g. by using metagenomics or metabolomics data;

> if the application is for plant cultivation, the effects on the food web have to be taken into account;

> likewise, potential adverse effects on pollinators, beneficial and protected species;

> likewise, effects on the associated microbiome (in particular soil organisms) must be taken into consideration;

> effective measures need to be implemented and prohibitions imposed to prevent the uncontrolled spread of GE organisms into the environment.

In addition:

> all relevant genomic data providing information on the exact genetic changes should be collected and made publicly available in data bases, amongst others, to allow for independent research;

> labelling should be mandatory and measures should be taken to protect conventional production of seeds, food and feed in order to enable freedom of choice for breeders, farmers and consumers.

5. Final considerations and conclusions

There are several published reports and statements from scientific institutions (such as the SAM Statement, 2018; Leopoldina, 2019) that do not investigate the differences between conventional breeding and the new methods of genetic engineering or its specificity in more detail such as, e.g. publications by Eckerstorfer et al. (2019) or Kawall (2019).

The aforementioned statements also disregard other decisive aspects, such as the multistep process being applied to plants and the risks of specific examples of organisms that have already been generated by SDN-1 or SDN-2. Instead, for example, Leopoldina (2019) proposes excluding applications of SDN-1 and SND-2 as well as uses of 'dead' nucleases for 'base editing', which have not so far been a major factor in applications of the technology (see Modrzejewski et al., 2019), but which nevertheless raise new safety issues (see Grunewald et al., 2019; Jin et al., 2019; Zuo et al., 2019).

As shown in this condensed overview, there are several decisive reasons why organisms derived from applications of SDN-1 and SDN-2 should all have to undergo mandatory risk assessment:

> In most cases, the old techniques of genetic engineering (such as *Agrobacterium tumefaciens* and biolistic methods) are used in a first step; these can cause a wide range of unintended effects.

> The processes of SDN-1 and SDN-2 are known to cause specific unintended changes at the genomic level, including off-target effects, unintended on-target effects and also effects on genomic regulation.

> The intended effects often exhibit specific patterns of genetic alteration because in most cases the nucleases will act on all (or at least many) copies of the target gene throughout the genome.

> The intended genetic changes can also include targeted sites which rarely undergo spontaneous or induced mutations as seen in conventional breeding methods.

In short, the pattern of intended and unintended changes and the resulting new combinations of genetic information arising from genome editing will, in most cases, be different in comparison to those derived from conventional breeding. These differences co-occur with biological characteristics and risks that need to be fully investigated before any conclusions on the safety of the new organisms can be drawn. Detailed examination of an organism's genetic and overall biological characteristics, starting with the process that was used to generate the organism, is needed to decide whether the organism is safe. The requirements for regulation as foreseen by current GMO law in the EU is given, no matter whether additional DNA sequences were inserted or not.

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