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Biotechnology

Why 'New GE' needs to be regulated

Frequently Asked Questions on 'New Genetic Engineering' and technical backgrounds for CRISPR & Co

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A Testbiotech Briefing

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Summary

This briefing gives a condensed overview of scientific information decisive for the regulation of 'New Genetic Engineering' (New GE) techniques.

New GE - or 'new genomic techniques', or 'genome editing' - opens up new possibilities which go beyond conventional breeding and previous methods of genetic engineering. One of the most important tools in this scenario are CRISPR/Cas gene scissors (nuclease). Gene scissors can be synthesized in the laboratory, programmed to specific sites of the target genome and introduced into the cells of plants and animals by various technical processes.

In contrast to chemical or physical mutagens, e.g. radiation, tools such as CRISPR/Cas can directly interfere with biological mechanisms in the cells. During evolution, mechanisms emerged which, for example, can protect specific genomic regions against too frequent mutations. They are, as it were, the flexible safety barriers of evolution. These mechanisms seem to be mostly relevant to genes that are of special importance to the survival of a species. New GE is designed to circumvent these mechanisms. In particular, the nuclease CRISPR/Cas for the first time makes the whole genome available for technical interventions and alterations. Genomic conditions, which until now could hardly be influenced via breeding, can now be accessed. Organisms generated with New GE may contain profound changes in their genomes and show new biological characteristics, even if no additional genes are inserted.

The technical potential of New GE can be used to make changes in the biological characteristics of plants without introducing any additional DNA sequences. These changes can exceed the range of characteristics developed gradually through evolution or previous breeding methods. Therefore, risks associated with the release or usage of the genetically engineered organisms for food production need to be thoroughly examined.

There are plans to introduce into agriculture and forestry many plants and animals edited with new GE and showing new biological characteristics within short periods of time. Examples include plants with changes in their composition, which often also impact their interactions with the environment (such as pollinators, wild species and soil organisms). Other organisms show enhanced fitness, and can therefore become invasive and replace natural species. Further examples are pesticide resistant honeybees displaying behavioural changes during pollination. Another goal of New GE is to enhance the profits from forestry. In addition, there are ongoing projects to genetically engineer wild (non-domesticated) species such as corals, rodents, flies and wild plant species. However, if a large number of these New GE organisms are released within short periods of time, they may become disruptive to ecosystems and severely endanger biodiversity.

Besides the intended new biological characteristics, there are further risks linked to the introduction of New GE organisms into the ecosystems and agriculture: a wide range of specific unintended effects have been observed when New GE is applied. These effects arise for example from the multi-step process of the genetic intervention, which in many cases also implies the use of Old GE methods. Further causes of unintended effects include the lack of precision in the CRISPR/Cas gene scissors. There have also been reports of rearrangements of the genome, including the unintended insertion of additional genes. In many cases, the genome is unintentionally cut in regions which are similar to the target DNA, i.e. the gene scissors can cut these by mistake. These unintended effects can be significantly different to those caused by conventional breeding. Therefore, risk assessment has to take all effects arising from the multi-step process of New GE into account.

If the regulation of New GE is insufficient, problems similar to those known in the US are likely to emerge: by October 2020, around 80 New GE organisms were already exempt from regulation. At the same time, there is no access to detailed data and information on the process and target of the genetic intervention used in those cases.

Therefore, the necessary information for monitoring, control and independent risk assessment are not available.

Without sufficient regulation of New GE

- severe damage to biological diversity is likely;
- risks to food production may be introduced and accumulate unnoticed;
- access to data needed for risk assessment by independent experts is not made available;
- no measures can be taken against the uncontrolled spread of the organisms in the environment;
- no data are available to track and trace the New GE organisms and products derived thereof;
- agriculture and food production relying on GE free sources can no longer be protected.

Therefore, a detailed examination of all organisms derived from New GE has to be mandatory and must start with the specific process that was used. The need for detailed risk assessment cannot be limited to organisms with additionally inserted gene sequences. Without strict regulation of New GE, the uncontrolled release of large numbers of organisms with characteristics not gradually developed through evolution can be expected. This would result in the substantial likelihood of damage to ecosystems, agriculture, forestry and food production.

About Testbiotech

Testbiotech is an independent institute for the impact assessment of biotechnology and GE organisms. Our work is strictly based on science and the interpretation of the information available; our focus is on the protection of health, the environment and nature. Testbiotech is free from any financial or other interest in the development and application of genetically engineered (GE) organisms. We also have extensive experience regarding the risk assessment of genetically engineered organisms carried out in the European Union. More information about our activities in this field can be found in our publications (www.testbiotech.org/en/publikationen).

For several years, Testbiotech has focused on the new techniques in genetic engineering ('genome editing'). We were involved in a publicly funded scientific project on so-called gene drives (www.genetip.de) and are currently conducting a project on the horizon scanning of 'synthetic biology' and 'genome editing' (www.fachstelle-gentechnik-umwelt.de) funded by the German Federal Ministry of the Environment, Nature Conservation and Nuclear Safety.

Fig 1: The model plant Arabidopsis has been used for many scientific experiments on the mechanisms of heredity, gene regulation, genome organisation. It has become evident that there are several natural mechanisms which protect important genomic regions against too many changes (see infobox 'flexible safety barriers'). The CRISPR/Cas gene scissors are designed to circumvent these mechanisms (Kawall, 2019).

The purpose of this briefing

The debate on New GE – in official Commission terminology also called "genome editing" or "new genomic techniques" - and its regulation is gaining momentum on the agenda of EU policy makers. It is becoming increasingly important for policy makers to understand the potential, but also the challenges the new technology presents for agriculture, the environment, health and society at large.

In 2012, the discovery of CRISPR/Cas technology led to new developments in genetic engineering. CRISPR/Cas is the most powerful in a series of new genetic engineering techniques that enable far-reaching interventions into the genetic code, even if no new genes are inserted (Testbiotech, 2020a).

According to a 2018 European Court of Justice ruling, all organisms derived from New GE are subject to current GMO regulation. The ruling states that the risks associated with the new techniques might prove to be similar to those resulting from the production and release of GE organisms derived from transgenesis and methods of 'old' genetic engineering. Therefore, New GE organisms should only be authorised following an assessment of the risks they present for human health and the environment.

Many stakeholders involved in the development and application of New GE or who have an interest in its marketing deny the need for detailed and mandatory risk assessment.

This briefing provides an overview of both the potential and the challenges of New GE in 10 short chapters, based on the available scientific literature. Some content, such as info-graphics 2 to 6, are sourced from the Project Genetic Engineering and the Environment (Fachstelle Gentechnik und Umwelt, FGU: https://fachstelle-gentechnik-umwelt.de). To improve readability, we do not refer to all relevant publications. However, all further relevant publications can be found through the references mentioned in the text. There is one exception in regard to genome organisation and gene regulation: there are many new publications on this issue, we have therefore provided specific references to this topic in the infobox on 'flexible safety barriers'. Furthermore, at the end of the briefing, a glossary has been added to explain technical terms underlined in the text. Some repetitions cannot be avoided because the contents of the specific chapters are closely interconnected. Otherwise, this briefing is strictly directed at the regulatory issues. Only in chapter 10 do we widen the perspective to more general aspects.

The first part (Chapters 1-5) gives some technical background. The second part (Chapters 6-9) gives an overview of four key questions for policy makers to consider when regulating New GE:

- What are the differences between New GE and conventional breeding?
- Are there specific risks associated with New GE?
- How should the risks of New GE organisms be assessed within the approval process?
- Can New GE organisms be identified and traced?

We hope that the interested public will find the information useful and helpful in coming to their own conclusions in this contentious debate on New GE.

Some Background on New GE

1. How CRISPR & Co works

New GE uses tools such as the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 system, Transcription activator-like effector nucleases (TALENs) or zinc-finger nucleases (ZNF) to enable the rapid introduction of targeted genetic modifications in genomes. These nucleases or gene scissors can be applied in a wide range of organisms such as plants, animals and humans (Doudna & Sternberg, 2017).

Unlike chemical or physical mutagens (such as radiation), New GE can directly interfere with the biological mechanisms in the cells and circumvent gene regulation and genome organisation established by evolution. The 'gene scissors' consist of enzymes (proteins) which can 'cut' DNA. These enzymes are combined with guiding molecules which bind at the target site in the genome before the cut is accomplished.

CRISPR/Cas also follows this basic design: it is composed of two elements, the cutting enzyme and the guiding molecule (see Figure 2). The guide RNA guides the enzyme (in this case the gene scissors) to the target site to be cut in the genome. For this purpose, the guide RNA is synthesized to mirror the target DNA sequence and enable a perfect match.

After being introduced into the cells, the nucleases can impair specific genetic information (knock out) or also insert new gene functions. There are also applications which are not meant to alter the DNA, but to alter gene expression (activity) by interfering with epigenetic mechanisms (Kawall et al., 2020).

Since the advent of the CRISPR/Cas technique (first applied in 2012), there has been a significant rise in the number of applications (Modrzejewski et al., 2019). This type of nuclease is more flexible and easier to apply than most of the previous biotechnological mutagens, such as TALENs or ZNF.

There are several categories of processes enacted by the gene scissor: (1) SDN-1 known as a site-directed nuclease, where no additional DNA is inserted. So far, most of the applications in plants and animals belong to this category. (2) SDN-2 where only some elements (base pairs) are exchanged to change the function of the original gene; (3) SDN-3 where additional genes are inserted. Most applications in plants and animals published so far are derived from SDN-1 methods.

The new techniques, such as SDN-1, are often used in a multiplex fashion, enabling the simultaneous or successive combination of multiple alterations (also called multiplexing, see Testbiotech 2020a and Figure 4).

Typically, the techniques cause specific patterns of change in the genome which result in new combinations of genetic information (Figure 4), previously not achievable. As a result, organisms generated using these techniques may contain pervasive changes in their genomes, even if no additional genes are inserted.

Fig. 2: The CRISPR/Cas gene scissors at work

Cas9 is guided by a specific guiding molecule (guide RNA) to its target site, where the nuclease can cut both strands of DNA. The PAM (Protospacer Adjacent Motif)-sequence serves as an initial site of recognition for the CRISPR/Cas system. If the site in front of the PAM sequence is congruent to the guide RNA, Cas9 is activated and starts cutting.

2. The gene scissors circumvent the natural mechanisms of genome regulation and genome organisation

The nuclease searches within the whole genome to find its target site. Once it reaches the target site, the nuclease binds to the DNA sequence that the guide molecule is designed for and can then make a cut.

The cell will subsequently activate its repair mechanism to mend the damaged DNA (Kawall, 2019). Under natural conditions, when DNA damage occurs, the original sequence can in many cases be restored and mutation avoided. However, this natural mechanism can be hampered or prevented because the gene scissors will again 'recognise' the repaired DNA sequence and cut again.

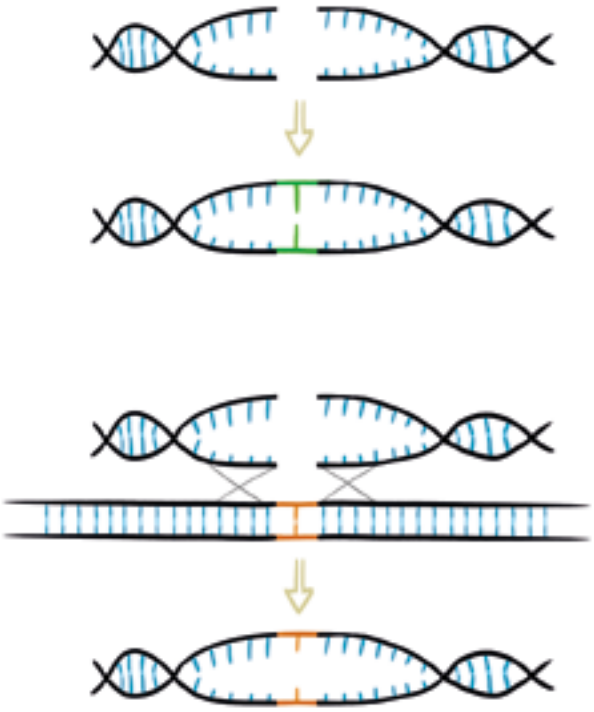
The cycle of 'recognition', cutting and repair will only come to an end if a change in the DNA sequence is reached which can no longer be recognised as the target site. Thus, typically, the gene sequence will be altered in a way that its original function is impaired (knock-out). The result is that the nucleases make it nearly impossible to restore the original function of the target gene – contrary to what would occur naturally.

There are further differences compared to natural mechanisms: as recent research shows, the occurrence of mutations in the genome is not completely random (see infobox 'flexible safety barriers'). Mutations which are permanent alterations of the DNA can occur spontaneously and, in the case of plants, also be induced by physical and chemical mutagens. However, within these processes, some genes can be altered at a much higher frequency compared to others. Mechanisms which influence the rate of mutation are for example the repair mechanisms: some genes (due to epigenetic markers) are repaired more often compared to others (Kawall, 2019). Furthermore, in many cases, there are greater distances within the genome between the regions with important gene functions. The distances between the regions can influence the likelihood of spontaneous mutations (see infobox 'flexible safety barriers') which cause the mutations not to be distributed equally throughout the genome. In addition, especially in plants, there are very often many copies of one gene which make their genome more robust: if one copy is lost, the others still fulfill the natural function.

It is argued that these mechanisms of natural gene regulation and genome organisation can help to protect those genomic regions against too frequent mutations, which is especially important for the survival of the species (see infobox 'flexible safety barriers'). New GE is designed/made to overcome these mechanisms. Tools such as CRISPR/Cas make the whole genome available for technical interventions and alterations (Kawall, 2019). Organisms generated using these methods may have profound changes in their genomes, even if no additional genes are inserted.

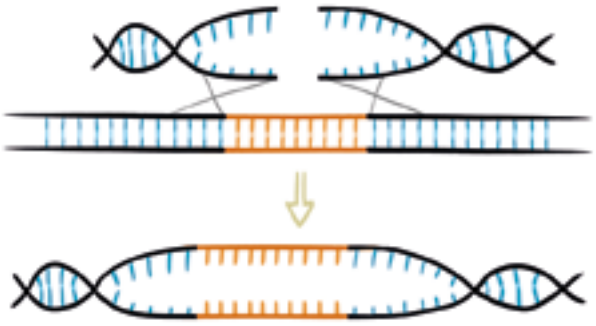
CRISPR/Cas can circumvent the natural mechanisms of gene regulation and genome organisation that protect genomic regions with important gene functions against being mutated too frequently. For the first time, New GE has made the whole genome available for technical interventions and alterations. This is a major difference in comparison to previous techniques of genetic engineering and methods of conventional breeding.

Fig. 3. Three different types of 'gene scissor' applications (site directed nucleases, SDN) for targeted DNA cuts



SDN (1): the DNA is cut but no additional DNA is inserted. The cell repairs the strands of DNA, but the gene scissors prevent the recovery of the original sequence. If the cell can manage to fully repair the original gene, the guiding mechanism of the nuclease will again recognise this DNA sequence and the gene scissors will cut it again. As a result, the gene-function can be lost and the gene is 'knocked out'. This is the technique most frequently used in New GE. See for example 'Genetically Engineered Wheat', 'CRISPR Mushrooms' and 'CRISPR Camelina' (Glossary).

SDN (2): The DNA is cut and the gene function changed: the technique allows the targeted exchange of some DNA letters in the DNA sequence. The target genes will gain a new function. See 'Monarch-Fly'



SDN (3): The DNA is cut and additional gene sequences are introduced: additional genes can be inserted into the genome with the help of the gene scissors – these might be derived from other species.

3. Technical potential

The potential of New GE can be exemplified by its applications in plants: many plant species have complex genomes exhibiting considerable diversity in both size and structure. Challenges to plant breeding include high numbers of chromosomes, many copies of genes and combinations of genes undesirable for the breeder but always inherited together with the desirable genes.

For example, major relevant food crops, such as rapeseed, wheat, potato, cotton and sugarcane, are polyploid and inherit more than two paired sets of chromosomes. Additionally, plant genomes often contain highly repetitive genomic regions, exhibiting large genome sizes in many species. The complexity of plant genomes poses a serious challenge to making genetic alterations requiring the targeting of multiple genes or several copies of one gene in combination. In this regard, traditional breeding and mutagenesis techniques using chemicals or radiation to introduce mutations in plants are known to have specific limitations: these physico-chemical mutagenes will trigger many mutations, but in most cases will not be successful in establishing specific patterns of genetic change and producing the desired new combination of genes (the new genotype).

Tools such as CRISPR/Cas enable complex alterations of the genome in ways that until now were not possible. Multiplexing approaches (parallel alterations of several genes) or repeated, serial applications allow the alteration of multiple genes, the targeting of all copies of a gene family and also several genes with different functions. Multiplex 'genome editing' applications have been used to change many major crop plants (Eckerstorfer et al., 2019).

One example: CRISPR/Cas enabled the knock-out of 35 variants of one gene family responsible for producing specific proteins (gliadins) - see 'CRISPR Wheat' and Sanchez-Leon et al., 2018). Even though no additional genes were inserted, the result was a pervasive alteration of the plant genome.

New GE techniques can also overcome limitations of the genetic linkage between different traits sometimes present in conventional breeding of plants. If a desired gene is linked to a gene with adverse effects on, e.g. yield or fruit shape, 'genome editing' can be used to break this linkage by knocking out the undesirable gene (Kawall, 2019).

The specific genetic combinations arising from CRIPR/Cas applications very often result in complex patterns of genetic change which cannot be achieved by conventional breeding. Consequently, there is also a lack of regulatory experience with the respective plants and animals. Methods of risk assessment to assess the new phenotypes, including complex alteration of metabolic pathways and plant components, still need to be developed.

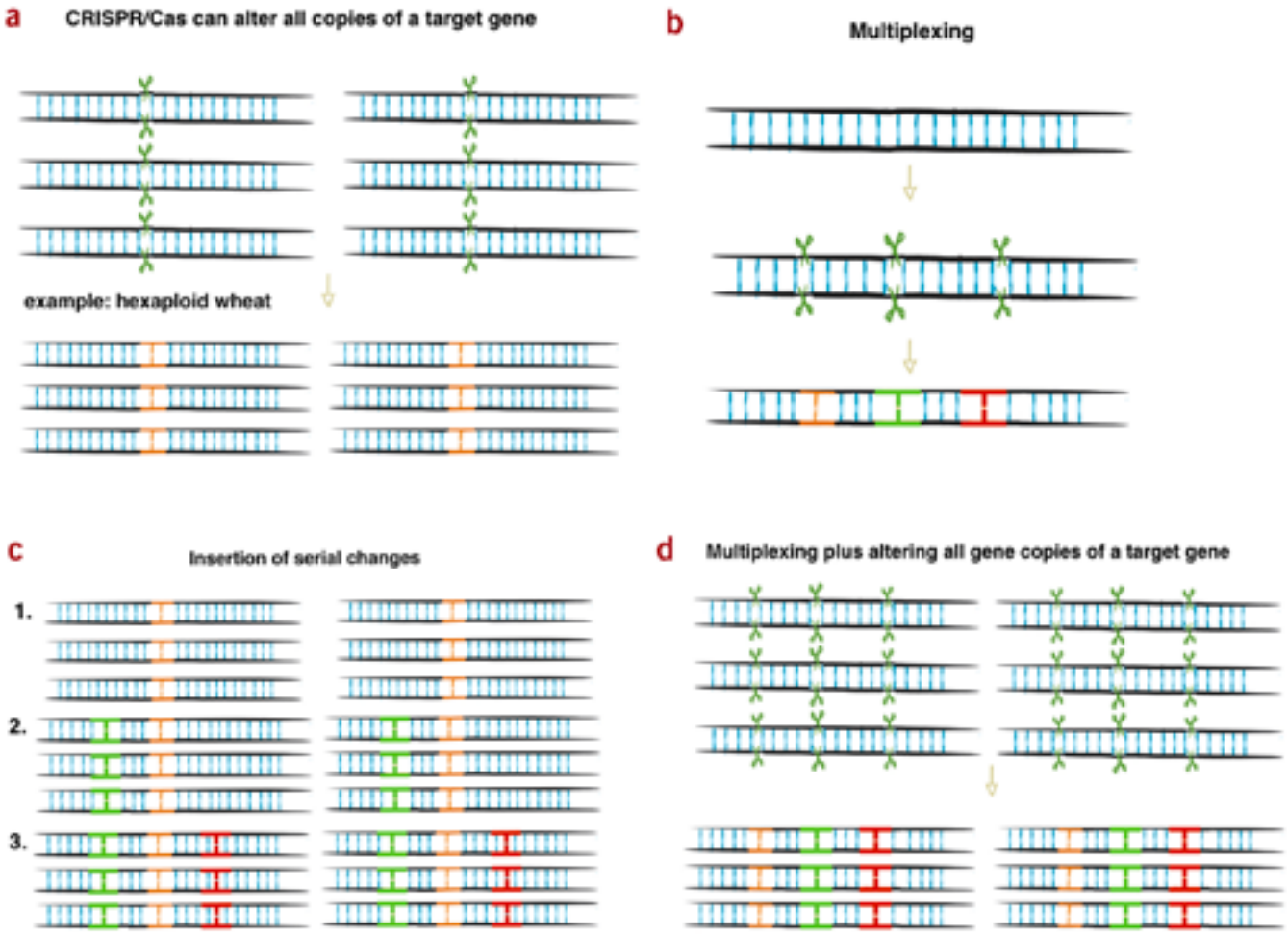


Fig. 4: The patterns of genetic alteration caused by New GE are different to those resulting from conventional breeding: (a) The use of CRISPR/Cas in most cases results in changes at several specific genetic sites in combination. One reason: typically, all copies of one gene will be changed. Especially in plants, such copies occur in large numbers. (See CRISPR Wheat and CRISPR Mushrooms). (b) The gene scissors can also be combined with several guide RNAs, enabling the cutting of several different genes at the same time (multiplexing). (See CRISPR Wheat and CRISPR Camelina). Furthermore, several changes can also be introduced (c) serially or (d) at once to the plant. (See CRISPR Camelina). The pattern of genetic alterations and resulting new gene combinations (genotypes) very often cannot be achieved by conventional breeding. As such, new biological characteristics (phenotypes) can be achieved which need to be thoroughly examined for risks.

4. Combinations of 'Old' and 'New GE'

The applications of nucleases, especially in plants, involves a multistep process. The applied technical processes can be divided into several steps: (1) The delivery of the SDN components into the plant cells; (2) the processes activated by the nucleases (biotechnological mutagens) followed by (3) the regeneration of the plants from single cells and (4) further breeding which is used to segregate transgenes or other unintended genetic alterations (Testbiotech, 2020a).

The first two steps involve technical processes that particularly need to be considered as potential causes of hazards (risks), while the last step is meant to mitigate or eliminate some of the risks. Therefore, all steps of the process need to be considered in the risk assessment and assessed on the basis of sufficiently comprehensive data.

It can be concluded from existing cases of non-regulated New GE plants in the US, that in most cases, the 'old', non-targeted techniques of genetic engineering, such as transformation by *Agrobacterium tumefaciens* and biolistic methods, also called 'gene canon', are used in a first step to insert the components of the nuclease, i.e. the guiding RNA and some additional supporting DNA, into the cells (Testbiotech, 2020a). In this first step, the additional DNA is inserted in a non-targeted manner. This technical step is necessary to first of all make the plant cells produce the nuclease. It is only the next step which then mediates the intended site directed changes in the genome. The result of the first step is a transgenic plant inheriting additional DNA sequences, e.g. from bacteria, that is needed for the production of the gene scissors.

The transformation via *Agrobacterium* and 'gene canon' can result in complex unintended genetic effects in the genome: examples include insertions containing multiple copies (or fragments) of the transgene and/or rearrangements of the DNA, or also epigenetic alterations in the vicinity of the integration site (Kawall et al., 2020). It also has to be taken into account whether the transgene was subsequently removed by segregation; there might be further unintended changes caused by the transformation process which can remain in the plants unnoticed. If the transgenes are inserted in several copies of just fragments (which is very often the case), their removal by further breeding may become especially difficult.

There have been some attempts to apply methods of introduction of the gene scissors that avoid the generation of transgenic plants at the first step of the process, but they are still rarely used. There are some reasons: if the components of the nuclease are directly introduced into the cells, they might be degraded too quickly, before they can fulfill their task. Therefore, permanent expression of the nuclease in the cells can be a technological advantage, even though it requires the establishment/creation of transgenic plants as a first step.

Risk assessment has to take into account the whole process and all technical steps of New GE to identify unintended effects and risks not directly caused by the nuclease and their intended usages.

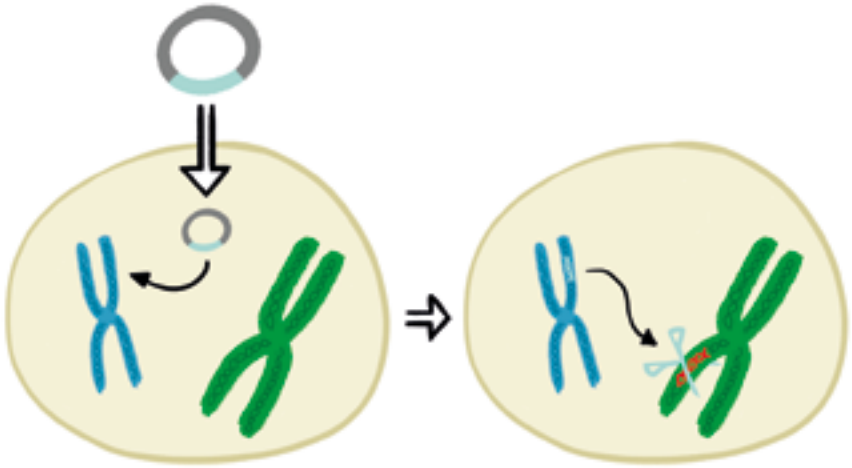


Fig. 5. How to get the nuclease into the cells? In many cases, the nuclease is introduced by using 'Old GE' techniques, i.e. biolistic methods ('gene-canon') or *Agrobacterium tumefaciens*. The DNA needed to produce the nuclease is randomly inserted into the genome of the plants, resulting in a transgenic plant. In a next step, the nuclease is produced by the cell, which can alter the genome at another site in a targeted way. See CRISPR Wheat and CRISPR Camelina.

5. Unintended effects

Gene scissor applications often cause unexpected effects in the targeted genomic region ('on target') as well as unintended effects in other genomic regions ('off target'):

Unintended on-target effects are frequently observed with CRISPR/Cas. There are larger structural changes within the genome as well as unintended insertions of additional DNA-sequences (Kawall et al., 2020). Whereby, the DNA for the production of the gene scissors might be integrated (unintentionally) as well as gene sequences from other origins.

Off-target effects also occur quite frequently. For example, the gene scissors can mistake genome sites in other regions for those they are programmed for due to similarities in the DNA sequence. Off-target effects have already been observed in rice, soybean, maize and barley as well as in animals, such as pigs, mice and rats (Kawall et al., 2020). Unintended proteins can be produced or gene regulation disturbed, causing disruption in the metabolism of plants and animals.

In addition, unintended effects due to the application of 'Old GE' to introduce the gene scissors into the cells also have to be taken into account (see above).

Two examples: in one study, the DNA template encoding CRISPR/Cas9 was unintentionally inserted into the target region. The reason: the gene scissors did not only cut, they also caused their own DNA to be inserted at the target region. In addition, the gene scissor DNA was found in multiple other, possibly random, genomic locations (Li et al., 2015). In rice, there were larger re-arrangements observed in the genome, including deletions and unintended insertions close to the target region. Further off-target effects were identified which are likely to be caused by CRISPR/Cas because of some similarities with the target region. Finally, parts of the gene construct used for the insertion of the nuclease were also identified in the genome of the plants (Biswas et al., 2020).

In order to detect such unintended effects, it is essential to carefully apply available methods to analyse the whole genome, and also the metabolism of the organism: 'Whole genome sequencing' allows the investigation not only of the target sites, but also other regions of the genome. Techniques called 'omics' (transcriptomics, proteomics and metabolomics) are needed to investigate changes in gene expression and metabolism.

Infobox 'flexible safety barriers'

Recent research shows that the emergence of mutations is not completely random but influenced by gene regulation and genome organisation. Relevant factors that impact the likelihood of mutations are, for example, the composition of base pairs (Weng et al., 2019), histone modification, (Lujan et al., 2015; Frigola et al., 2017; Belfield et al., 2018; Huang & Li 2018) and the status of chromatin (Gibcus & Dekker 2013; Luo, 2014; Guo & Fang, 2014; Perez et al., 2019). Greater distances between specific genomic regions causes genes that may be particularly important for the survival of the species to mutate less frequently than others (Fang et al., 2008; Boukas et al., 2020; Halstaed et al., 2020; Monroe et al., 2020). Gene regulation as well as genome organisation also have a substantial impact on the likelihood of repair processes in response to DNA damage (Belfield et al., 2018; Frigola et al., 2017; He et al., 2017; Kawall, 2019; Boukas et al., 2020; Monroe et al., 2020). As a result, it is evident that the occurrence of mutations is not just dependent on random processes followed by selection.

Furthermore, gene duplications play a major role in particular in the genome of plants (Wendel et al., 2016; Gaines et al., 2019). The duplication may have to do with short DNA sequences, larger parts of the genome or whole chromosomes (Otto, 2007): biological characteristics, such as herbicide resistance in weeds, can be fostered through these mechanisms (Gaines et al., 2019) and backup functions established (Jones et al., 2017).

By using these mechanisms, gene regulation and genome organisation can implement and carry out decisive functions: steady changes and (if needed) quick adaption to new environmental conditions on the one hand, and stability in heredity as a precondition for the survival of the species on the other. Evolution is dependent on the balance between chaos and order, change and stability. Gene regulation and genome organisation act as 'flexible safety barriers'. New GE is however made to overcome these safety barriers.

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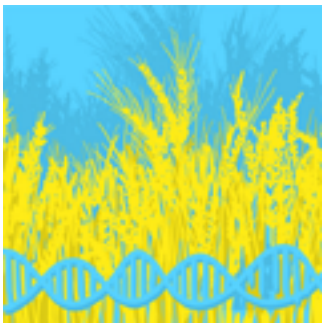
Four crucial questions on the regulation of 'New GE'

First Question: What are the differences between New GE and conventional breeding?



It is very often claimed that the main difference between New GE and conventional breeding is that New GE would be safer. The purported reason: the number of unintended genetic changes is higher in conventional breeding compared to New GE. This is especially emphasised in the cases where conventional mutagenesis is used.

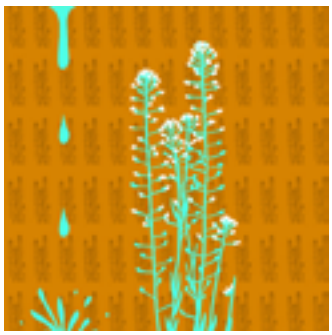
However, these arguments are deceptive. Physico-chemical mutagenesis as currently used and which has been known for nearly 100 years, is meant to enhance genetic diversity in the cells. The desired traits are then achieved through further steps of crossing and selection. Thus, a high degree of genetic diversity is intentional and necessary for the success of conventional breeding. Unlike conventional breeding, New GE aims to introduce genetic change in a targeted way. Therefore, additional changes must be considered to be 'unintended effects'. Due to the technical processes used in New GE, these unintended changes can be very specific and therefore relevant for risk assessment - this is typically not the case with conventional mutagenesis.

In short, the most relevant differences between genetic engineering and conventional breeding are not about the quantity of changes. Quality is much more relevant as are the specific patterns of genetic change:

Tools such as CRISPR/Cas are 'biotechnological mutagens' that, in contrast to physical or chemical mutagens, directly interact with the biological mechanisms in the cells. The use of biotechnological mutagens enables the circumvention of natural mechanisms of gene regulation and genome organisation; they also make the genome available for changes to a much greater extent than ever before (Kawall 2019). Genetic conditions which could be hardly altered by conventional breeding or 'Old GE', can now be genetically engineered. As a result, specific patterns of genetic change are created, such as those explained in 'CRISPR Wheat', where 35 genes were altered at once and in combination (Sanchez-Leon et al., 2018). Even in cases where thousands of the wheat genes might be altered by conventional mutagenesis, no such combination of changes in one plant could be achieved.

Due to the technical processes used in New GE, the resulting patterns of genetic change (genotypes) as well as biological characteristics (phenotypes) and associated risks can be substantially different compared to those derived from conventional plant and animal breeding, or what can be found in nature.

Second Question: Are there specific risks associated with New GE?



In general, the processes as well as the resulting organisms have to be taken into account in order to assess the risks associated with genome edited organisms (Kawall et al., 2020):

1. Old GE techniques are often used in a first step to introduce the nucleases (such as CRISPR/Cas, TALENs) into the cells It is only in a second step that the nuclease (the enzyme) is produced by the cells and then starts 'to cut' at the targeted site. This first step can be linked to many unintended changes of the genome and gene expression.
2. The application of the gene scissors themselves can cause unintended on-target and off-target effects which are specific to the technical processes. These effects largely depend on technical parameters such as (i) the specific nuclease used, (ii) the target organisms, (iii) the targeted genes, (iv) the way in which the nucleases are introduced into the cells, (v) the dosage of the enzyme in the cells and (vi) the duration of the intervention. These technical details need to be taken into account for risk assessment.
3. The intended effects arising from by the application of gene scissors very often results in specific patterns of genetic alteration and new combinations of genetic information. Typically, this outcome is different compared to conventional breeding, even in cases where no additional genes are inserted. The resulting biological characteristics may be linked to substantial risks for humans, the environment and nature.

There are numerous and diverse risks affecting ecosystems, agriculture and food production, e.g. changes in plant composition can impact wild animals such as mammals, birds or insects and their food webs. Changes in the composition of plants can also impact plant interaction and communication with the environment. These risks can affect, e.g. insects (such as pollinators and beneficial species), symbiotic organisms (such as associated micro-organisms) or plant 'enemies' (such as pest insects). There are further specific risks associated with New GE organisms that can spread in the environment. Due to the diversity and complexity of interactions with the environment there can also be next generation effects, such as invasiveness, not observed in organisms developed in the laboratory. Therefore, measures need to be taken against any uncontrolled environmental spread.

The need for a mandatory approval process as foreseen by EU GMO regulation is applicable for all New GE organisms, even if no additional DNA sequences are inserted. The specific technical process used in the New GE application has to be used as the starting point for risk assessment.

Third Question: How should the risks of New GE organisms be assessed within the approval process?

Data on the specific technical process are absolutely necessary to identify unintended effects and risks. EU GMO regulation therefore requests that the process of genetic engineering is the starting point for mandatory risk assessment. For example, in the case of the 'Hornless GE Cattle', a bacterial gene conferring antibiotic resistance was unintentionally transferred into the genome (Norris et al., 2020). This mistake remained unnoticed for several years; the cattle had by this time already produced offspring and had been repeatedly presented as perfect examples of the precision of New GE in the US.

Adequate methods also need to be applied in order to assess the intended complex biological changes arising from New GE techniques. Therefore, the following issues should be taken into account for the risk assessment process:

- In regard to the intended changes, new methods need to be applied to cope with new challenges. In the context of New GE, frequently the aim is to change the biology of plants and animals in great depth. However, in many cases it will be difficult or impossible to find adequate organisms with a history of safe use to compare them with the New GE organisms. At the same time, the European Food Safety Authority (EFSA) sees such comparisons as the most important element in carrying out risk assessment ('comparative approach').
- 'Omics' data are necessary to assess changes in the genome, the transcriptome (RNAs), the proteome (production of proteins) and the metabolome (several levels of metabolic functions) so that the effects of genetic changes in the organism can be assessed;
- The whole pattern of intended and unintended genetic changes and their effects - taking the effects arising from the combination with Old GE into account as well as the effects of the nucleases. Therefore, the whole genome needs to be investigated (Whole Genome Sequencing).
- If it is assumed that the results of 'genome editing' cannot be distinguished from those of conventional breeding, comparative data must be requested as evidence, including whole genome sequencing data.

The genetically engineered organisms should be exposed to a wide range of defined environmental stress conditions, in particular, to test their response to climate change or pathogens.

The application of New GE in plants and animals as well as changes in the composition of their associated microbiomes (such as soil bacteria or fungi). This can have serious implications for the health of soil, plants, animals and humans and therefore has to be investigated in great detail. If New GE plants are released into the environment, the impact on food web has to be taken into account, likewise potential adverse effects on pollinators, beneficial and protected species. Effective measures need to be implemented and prohibitions imposed to prevent the uncontrolled spread of the genetically engineered organisms into the environment.

In addition:

- all relevant genomic data providing information on the exact genetic changes should be made publicly available in data bases;

› labelling should be mandatory, and measures should be taken to protect conventional production in order to protect freedom of choice for breeders, farmers and consumers. These measures are already mandatory under current EU GMO legislation.

Scientifically, it is impossible to assess safety of the New GE organisms without taking the data from the technical process into account. Furthermore, new methods have to be applied to assess the complex biological changes intentionally and unintentionally caused by the New GE techniques.

Fourth question: Can New GE organisms be identified and traced?

Since the application of nucleases, such as CRISPR/Cas or TALENs, will in most cases cause typical patterns of genetic change (see above), these patterns can also be used for identification and traceability.

This was also expressed in a publication jointly prepared by experts from the German regulatory authority (BVL) and DowDuPont (Duensing et al., 2018), confirming significant differences between new techniques of genetic engineering and conventional plant breeding. The publication states: *"(…) 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."*

Consequently, plants changed through 'genome editing' can usually be very clearly distinguished from other plants. The publication states: *"For most products of genome editing, there is a clear signature in the DNA, for instance the exact stretch of nucleotides erased. If that signature is revealed by the developer, the same PCR technology used for detecting GMOs can be applied to the detection and monitoring of genome-edited products in most cases."*

This situation can be exemplified in the case of the so-called non-browning mushroom (Waltz, 2016): several copies of one gene were changed in the mushroom to block the production of a specific enzyme. It is unlikely that any similar mushroom has ever existed on the market.

Even if only point mutations are created in the DNA, these can be used to identify the organisms. As the authors explain, the Cibus plants show a specific alteration in their DNA that makes identification possible (Chhalliyil et al., 2020). However, it is commonly recognised that it is not very often possible, solely on the basis of DNA sequence analysis, to identify the exact new or old GE methods applied to the plants. For this reason, EU regulation requests mandatory approval processes for all GE organisms, and that suitable methods of identifying the plants are made available.

In most cases, the typical patterns of genetic change as well as specific alterations of single DNA sequences will allow the identification and traceability of New GE organisms. Therefore, it has to be ensured that the companies provide the necessary data during the mandatory approval process.

A broader perspective: 'A crack in creation'

CRISPR/Cas was originally found in bacteria as a defence mechanism against viruses. The bacterial system was then adapted to biotechnological applications in the laboratory, i.e. the CRISPR/Cas machinery was taken from its original context in bacteria and adapted to alter the genome of plants, animals and humans. The way in which CRISPR/Cas is used in genetic engineering does not exist in nature.

Applications of New GE are not restricted to agriculture or the laboratory. Instead, an increasing number of applications are targeting wild populations, such as insects, rodents and trees, which are parts of complex ecosystems. As Jennifer Doudna, in her book "A Crack in Creation" (2017) explains, the new techniques of genetic engineering and especially the CRISPR technology can be used to end to 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 era, 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', calls in his book "Regenesis" (2012) for *"the end of the beginning"*. Indeed, apart from genetically engineered organisms, until now, all organisms can be considered to be the 'natural offspring' from the 'first cell' without being technically designed by mankind: natural mechanisms, such as gene regulation, genome organisation and pattern of reproduction, developed during evolution as flexible safety barriers still work for all of them, regardless of whether they are domesticated or not. The rise of genetic engineering is now changing this situation.

If researchers are currently promoting a *'crack in creation'*, the criterion of 'naturalness' will become hugely important to regulatory decision-making. Mankind has already damaged nature substantially: large parts of biodiversity have been extinguished, species and their ecosystems are being lost. Now the human species is interfering with basic principles of heredity, to change what one could call 'the nature of life' (Testbiotech 2020b).

Following generations have the right to experience and benefit from natural diversity of species and biological diversity. For example, in the German law for nature protection it states (Article 1): "Nature and landscape, because of their own value and as basis for live and health of humans also in responsibility for future generations (…) have to be protected in a way that (1) biodiversity (3) diversity, character and beauty as well as the recreational value of nature and landscape is protected in perpetuity (..)."

This right of future generations also implies the protection of biodiversity against the uncontrolled spread of New GE organisms.

Especially problematic in this context are so-called gene drives, which can also be established with New GE (see Figure 6). The purpose of gene drives is to spread artificial genetic constructs much faster throughout natural populations of wild species than would be expected naturally. There are plans to apply gene drives in animals, such as insects (such as flies and mosquitoes), or rodents (rats or mice). The aim is to replace or eradicate natural populations. Once started, the process cannot be controlled effectively or reliably. The damage to humans, the environment and nature could be severe.

The EU allows releases of GE organisms only in cases where they are proven to be safe and if they can removed from the environment if urgently required. These preconditions are however not met for, e.g. genetically engineered gene drive mosquitoes. In general, the environmental release of New GE organisms which cannot be effectively controlled in space and time cannot be allowed.

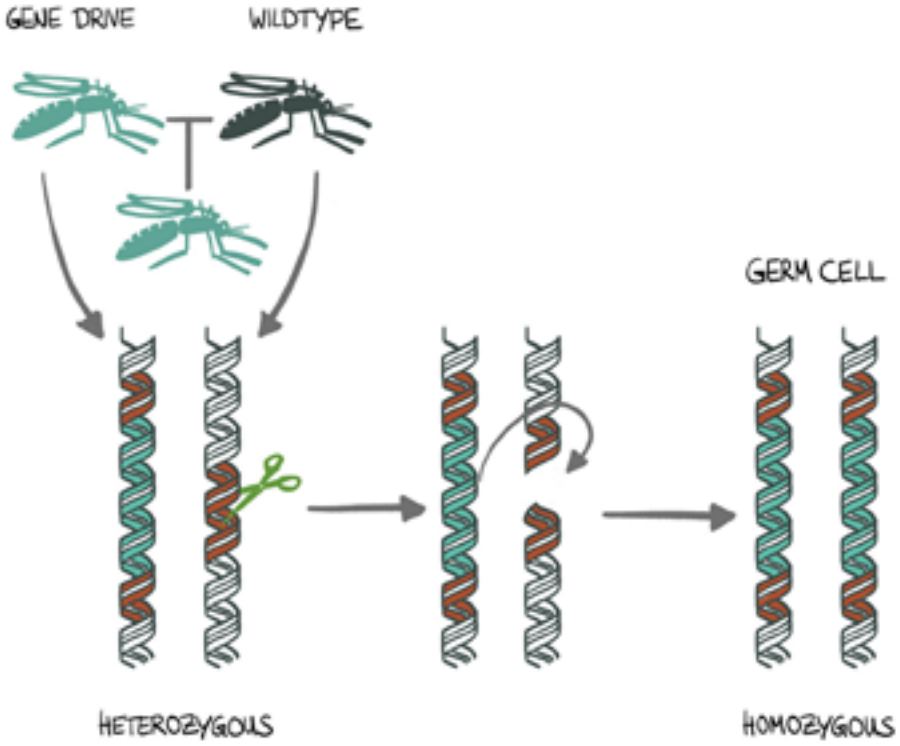


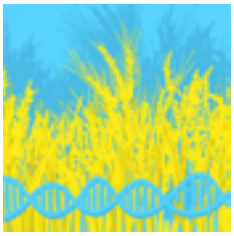
Fig. 6: In cases of gene drive organisms, the DNA to activate the gene scissors CRISPR/Cas is transmitted to the next generations. The nuclease is meant to be activated in each generation and to genetically engineer the target genes so that all offspring become homozygous in regard to the altered genes. Spread of the artificial genes is secured and can be rapidly established in whole populations of wild animals (such as insects and rodents). The aim is to suppress, eradicate or replace the target species.

Annexes

Selected Examples

Testbiotech has selected several examples to explain the potential and risks of New GE. Each example demonstrates that the genetic and biological characteristics of New GE organisms need to be assessed in detail, starting with the technical processes. Small changes in the genome can have major effects. More examples can be found at https://www.testbiotech.org/en/limits-to-biotech

CRISPR Wheat



Scientists working for the US company, Calyxt, targeted a group of gluten proteins (gliadins) in wheat thought to cause inflammatory bowel diseases (celiac disease). These genes occur within a large family of genes that often are present in so-called gene clusters (i.e. in multiple copies) at different locations in the genome. So far conventional breeding has been unable to reduce the large number of genes and gene copies. With the help of the CRISPR/Cas gene scissors, scientists succeeded for the first time in 2018 in switching off a great number of these genes: 35 of 45 genes that produce gliadins were 'switched off'. The result is a unique pattern of genetic modification in wheat. These changes can also trigger unintended biological properties, e.g. changes in other plant components.

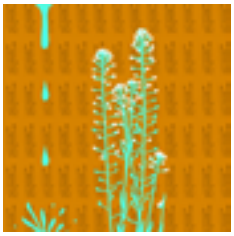
Hornless GE Cattle

Cattle were modified with New GE in 2015/2016 to make them hornless. It was only in 2019 that scientists found genetic material of the bacteria used in the process had also been introduced into the genetic material of the cattle. The process involved using so-called plasmids that were originally found in bacteria. These are synthesized in the laboratory as tools for the transfer of genes (vectors). It was only several years after the cattle were first presented to the public as a perfect example of successful New GE modification, that a complete DNA-sequence conferring resistance to antibiotics in bacteria was detected in their genomes. If the genetically engineered cattle are used for breeding as planned, the unwanted genes can spread rapidly through dairy herds. To detect such risks, the process of genetic engineering always has to be taken into account in the approval process.

CRISPR Mushrooms

These edible mushrooms were created in the US using New GE techniques, i.e. (CRISPR/Cas), to stop cut surfaces from turning brown; the mushrooms were also meant to have a longer storage and shelf-life. This was achieved by destroying the structure of a certain gene present in the fungus in several copies. Using CRISPR meant that the fungus was changed in all copies of the target gene. Such a pattern of genetic change would not appear spontaneously. The responsible US authority, APHIS, approved the mushrooms in April 2016. This was because it was, in their view, sufficient that the developers of the fungus said that no additional DNA had been inserted. As a result, there is no scientific publication on how exactly the properties of these mushrooms were intentionally or unintentionally changed. This example shows that without a legally prescribed authorisation procedure, there is insufficient data to assess the risks associated with consuming genetically engineered organisms.

CRISPR Camelina

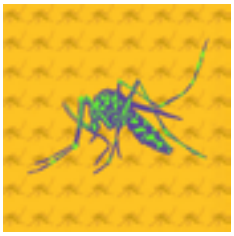


One focus of New GE is on the production of agro-fuel. Some plants in which 18 sites on the genome have been changed using CRISPR/Cas gene-scissors, have already been released for cultivation. These plants show patterns of genetic change and altered oil quality that would not be possible, or at least very difficult, to achieve with conventional breeding, even though no additional genes are inserted. Camelina is one of the oldest cultivated plants in Europe. The plants can survive and multiply in the environment as well as cross into natural populations. This example shows that a legally required mandatory approval process is necessary to obtain precise information on the genetically engineered changes. Only then can the plants be identified if necessary and their uncontrolled spread prevented.

Monarch-Fly

A gene in fruit flies (*Drosophila melanogaster*) was manipulated using the gene scissors CRISPR/Cas to make it similar to a gene found in monarch butterflies (Danaus plexippus). By changing only four base pairs in total, the fruit flies became resistant to toxins produced by certain plants. As a result, the flies can absorb toxins and become poisonous to predators. Mass release of such flies could have serious consequences for the food web and ecosystems. Minor changes to a single gene can have significant impacts on nature, even if no additional genes are inserted into the genome.

Gene-Drive Mosqiotoes



New GE can also be used to alter wild populations. So-called 'gene drives' have been developed to change, decimate or even exterminate natural populations, e.g. pests. The essential feature of a gene drive is that it can bypass the rules of natural inheritance. Furthermore, the inserted genes can spread faster in a population than would naturally be the case. The technology is meant for use in, for example, fruit flies that are regarded as pests in agriculture, mosquitoes that can transmit diseases and rodent pests. The problem: once released, it is hardly possible to stop the experiment. Releases of genetically engineered organisms, whose spread cannot be controlled, must not be permitted in the EU.

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Glossary

Compiled with support of Project Genetic Engineering and the Environment (Fachstelle Gentechnik und Umwelt)

Agrobacterium tumefaciens
Agrobacterium is a soil bacterium that can naturally infect plants and causes tumor-like growths in the plant. The *Agrobacterium* is used by plant scientists as a molecular tool to create genetically engineered plants. The genetic elements of the tumor-like growths are located on a piece of extra DNA that is arranged as a ring (i.e. a plasmid). This plasmid is transmitted into the cell by the bacterium when the plant is infected. This so-called T-DNA is then incorporated at random in the genome of the plant. Scientists use the bacterium as a transport vector to introduce foreign genes, that they incorporated into the T-DNA, into plants. Instead of integrating the genetic material for the tumor-like growths, genes are built in, that the scientist would like to introduce into the plant cells.

Base pairs
Bases are organic molecules that are important components of DNA and RNA. They are often referred to as the letters of DNA. The four nucleobases adenine (A), guanine (G), cytosine (C) and thymine (T) are used in DNA.The opposite bases on the DNA double strand are referred to as base pairs.

Biolistic procedures
Biolistic procedures are among the methods of 'old' genetic engineering. In this process, pieces of DNA are applied to small beads and shot directly into the nucleus of a cell. The synthetic DNA or its fragments can then be incorporated into the genome in a non-targeted way, often inserted in several copies.

Biotechnological mutagens
Biotechnological mutagens can be used to specifically intervene in biological processes within a cell, at the level of the genome or gene regulation. These can in particular be nucleases (gene scissors) that can induce changes in the genome.

Chromosomes
Strongly condensed form of the DNA.

Conventional/classical mutagenesis
In conventional mutagenesis, ionizing radiation or chemical substances (physical and chemical mutagens) are used in plant breeding to generate mutations. This enhances the spontaneous mutation rate in the genome and increases the genetic diversity, which is the starting point for further breeding.

Conventional plant breeding
The aim of conventional plant breeding is to change or improve the properties of plants. Plants with certain properties are selected, crossed with one another and the plants of the next generation are then selected for the desired properties. These plants can then be used for the next breeding cycle. Physical or chemical mutagens are often used to increase genetic diversity at the beginning of the selection process.

CRISPR/Cas
CRISPR/Cas is the abbreviation for Clustered regularly interspaced palindromic repeats/CRISPR associated. CRISPR are small regions of bacterial DNA that aid in the immune defense against invading viruses. Cas is the cutting component of the CRISPR/Cas system (i.e. nuclease) and cuts the double strand at the target region of the DNA. The CRISPR/Cas system was adopted from bacteria as a molecular biological technology and modified with the aim to engineer the genome of other organisms such as plants and animals in a targeted way.

Cytogenic factors
Cytogenic factors are based on mechanisms and factors of epigenetics, gene regulation and genome organisation. As result of recent research it is assumed these factors are impacting the likelihood for spontaneous mutations to occur in a way their emergence can not be regarded as completely random.

DNA
DNA stands for deoxyribonucleic acid. DNA is a macromolecule that carries genetic information within the order of base pairs. It consists of nucleotides. The DNA forms a double strand wound like a helix. In addition to the nucleus, there is also some DNA in the chloroplasts of plants and mitochondria of animals.

DNA strands
The genetic information of humans, animals and plants is made up of two long, filamentous DNA molecules (two DNA strands), which are linked to one another by cross-connections (base pairing through the formation of hydrogen bonds).

Domestication
Plants, but also animals, were bred by humans for many centuries to adapt them especially for human nutrition, which is a process called domestication.

Enzyme
Enzymes are proteins that have a specific function and implement a specific reaction inside of cells. They are often referred to as bio-catalysts.

Epigenetics
Epigenetics describes mechanisms and in some cases also heritable changes in the genetic material that are not based on changes of the DNA sequence (order of base pairs). Epigenetics regulates the activity of genes during development of living beings or in response to the environment.

Epigenetic markers
Epigenetic markers refer to small appendages to the DNA itself and also to proteins to which the DNA is attached. They are regulating the gene activity (gene expression).

Gene
A gene is a section of DNA that carries information for the development of characteristics of an individual and is used for the formation of RNA, which is then translated into a protein.

Gene cluster
Gene clusters describe DNA areas, where genes are present in increased copy numbers, often found in the same region of the DNA.

Gene Construct
A gene construct is a DNA sequence which was synthesized in the laboratory and contains the actual DNA sequences for the development of new biological properties (often proteins, enzymes). In addition, typically it consists of regulatory sequences (start and stop signals), as well as elements that enhance gene activity (promoters). These gene constructs are often synthesized in plasmids (so-called vectors), which can then also be used to transfer the genes.

Gene copies
Several, identical DNA sequences of a gene in the genome are called gene copies.

Gene Drives
Gene drives are techniques that ensure the rapid spreading of genes within a wild population. The genetic traits are inherited more frequently than genes that are inherited according to Mendelian rules. Genetically engineered gene drives can increase heredity to the next generation by up to 100 percent.

Gene expression
The process of using gene information for the synthesis of a functional gene product is called gene expression and is a prerequisite for gene activity.

Gene family
Members of a gene family are genes that are very similar in their DNA sequence and functions.

Gene probe
The guide RNA, which guides the CRISPR/Cas system to the target region on the genome, can also be called gene probe.

Gene regulation
Gene regulation refers to the regulation of switching on and off of genes, i.e. the control of gene expression, especially during the development of an embryo and in response to different environmental conditions. The repair of gene sequences and other mechanisms of genome organization are also influenced by gene regulation.

Gene scissors
Gene scissors are enzymes, more specifically nucleases. The term gene scissors is often used for the targeted nuclease CRISPR/Cas9 to convey how it works in simple words. Other nucleases are TALENs or zinc finger nucleases. The gene scissors cut at certain regions of the genome and often cause a change within a target region.

Genome
The genome describes the entirety of the DNA of an individual that is (to its largest parts) found in the cell nucleus of plants and animals.

Genome Editing
Genome editng is a synonym for New GE. It comprises molecular biological techniques that are intended to change the genome of an organism (humans, animals, plants) in a targeted way. These techniques include among others site-directed nucleases (gene scissors). In contrast to transgenic organisms, genes do not necessarily have to be transferred across species. Most important tool are the gene scissors CRISPR/Cas.

Genetic linkage
If genes are located in close proximity on a chromosome, they are very likely inherited together, so they are genetically linked to one another.

Genome organization
The genome organisation and involved factors and mechanisms impact the order of the genes and the way how DNA is packed and structured in its entirety. The structures, mechanisms and factors influence gene activity and the likelihood of new mutations. For example, genomic areas which are of specific importance for the survival of the species, very often are in larger distances to so-called 'hotspots' where mutations can occur quite frequently. These distances can help to protect these areas from being mutated too often. Additional gene copies protect against the loss of important biological functions. Although the genome is subject to constant changes over generations, these mechanisms help to stabilize biological functions which are essential for the survival of the species.

Genotype
A Genotype means a combination of several genes which in their entirety generate a specific phenotype (trait).

Genetic engineering
Techniques used for genetic engineering are using synthetic molecules produced in the laboratory (biotechnological mutagenes). Typically these molecules consist of nucleid acid (DNA or RNA). In some cases, also proteins (nucleases) get used. These molecules are introduced into the cells with the aim to change the biological characteristics of the resulting organism in a targeted way. To achieve this goal, the DNA sequence of the organisms or the gene expression is altered.

Gliadins
Gliadins are proteins and components of gluten in wheat and rye that are often associated with good baking quality. They are responsible for celiac disease (an inflammatory bowel disease) in humans.

guide RNA
The guide RNA is a short RNA molecule that guides the CRISPR/Cas system to the target region on the genome to generate a change there. It can recognize the DNA target region, bind it and bring the gene scissors into the correct position for cutting the DNA double strand (see gene probe).

Homologous chromosomes
In the cells of higher plants and animals, chromosomes exist in multiple copies (usually two chromosome pairs (diploid), but some plants have more than two paired sets of chromosomes (polyploid)). The homologous, identical chromosomes match each other in terms of shape, structure and gene sequence.

Knock-out
A gene knock-out is a change of the gene sequence in the genome of an organism switching off the respective gene, so it no longer gets expressed. Therefore, the corresponding protein can then no longer be formed.

Metabolism
The metabolism comprises chemical reactions in an organism that are involved in the construction, degradation and remodeling processes inside of cells.

Metabolomics
With the use of metabolomic techniques the composition of all components in the metabolism (metabolic products) of cells and tissues can be examined.

Microbiome
The microbiome of multicellular organisms (humans, animals, plants) comprises the entirety of all microorganisms that naturally colonize such an organism. In humans, the number of microorganisms in the intestine is actually higher than the number of cells in the body. Microbiomes and their "hosts" exchange nutrients and biologically active molecules with one another and can also influence one another's gene regulation.

Multiplexing
By using several guide RNAs it is possible to change multiple (different) target regions of the genome at the same time. But even if only one guide RNA is used, several genomic regions can be changed at once, since genes are often present in several copies in the genome.

Mutagens
Mutagens are substances that can be used to increase the occurrence of mutations in the genome. These are usually physical or chemical, unspecific stimuli (stressors) such as radiation or chemical substances. The results are subjected to the mechanisms of gene regulation and genome organisation, they cannot be predicted. CRISPR/Cas can be defined as biological or biotechnological mutagens, but these tools differ significantly from the physical and chemical mutagens in their specific mode of action.

Mutagenesis
Mutagenesis describes the generation of mutations in the genome of living beings, although it is important to distinguish how these mutations were induced: on the one hand, mutations occur spontaneously; on the other hand, mutations for research or breeding purposes are generated by the treatment of chemical substances or physical radiation. The use of CRISPR/Cas is sometimes also referred to as mutagenesis, but these biotechnological processes differ significantly from conventional mutagenesis in terms of their specific mode of action. Using CRISPR/Cas genes can also be specifically changed that are otherwise protected by their genome organization and specific repair mechanisms.

New genetic engineering
New genetic engineering techniques (for example CRISPR/Cas9, TALENs or zinc finger nucleases) enable the change of genetic information of an organism (humans, animals, plants) in a targeted way.

Nucleases
Nucleases are groups of an enzymes that can cut nucleotides, very often causing DNA double-strand breaks. The nucleases often are referred to as genetic scissors.

Nukleotides
A nucleotide is made up of three components:

- Base - one of the five bases, adenine (A), guanine (G), cytosine (C), thymine (T) (for DNA) or uracil (U) instead of thymine (for RNA).
- Sugar - in this case, carbon atoms arranged in a ring formation, classified as ribose or deoxyribose
- a phosphate group

‚Old' genetic engineering
The wording ‚old' genetic engineering is referring to previous applications of genetic engineering techniques in plants. It encompasses various methods (e.g. biolistic procedures or use of *Agrobacterium tumefaciens*) with which it is possible to introduce DNA into organisms or cells and incorporate it into their genomes. The methods of the 'old' genetic engineering are often used to introduce the tools of the new genetic engineering (i.e. genome editing) into the cells.

Off-target
Regions of the genome, that are not in direct proximity to the target region of the site-directed nuclease, are referred to as off-target.

Omics
Omics examine the entirety of certain components of the cell. In genomics, for example, the entirety of DNA is examined; in proteomics, the entirety of all proteins within a cell.

On-target
On-target refers to the target region of the DNA that is intended to be changed by site-directed nucleases.

PCR technology
PCR (polymerase chain reaction) is an analytical method used in the laboratory with which known DNA sequences can be detected in a DNA sample in a short time frame.

Phenotype
Biological characteristics of an organism.

Physico-chemical mutagens
Physico-chemical mutagens are external influences that can increase the occurrence of mutations in the genome. A distinction is made between chemical (specific chemical substances) and physical (e.g. radiation) mutagens.

Plasmid
Plasmids are used by bacteria to transfer genetic information between them and are small, usually circular, self-replicating double-stranded DNA molecules that exist in bacteria, but are not integrated into the bacterial chromosomes. Plasmids can contain different genes, for example genes that can confer resistance to antibiotics. These resistances can be exchanged between bacteria by the exchange of these plasmids. So-called Ti (tumor inducing) plasmids are found in the species *Agrobacterium tumefaciens* or *A. rhizogenes* and can be transferred into plants. Plasmids are important tools in genetic engineering. They are also known as vectors and are used to reproduce or transfer genes. Ti plasmids, for example, are used to transfer genes in plant cells. The DNA encoding the gene scissors, for example, can be introduced into a cell. The methods of old genetic engineering are used especially in plants.

Polyploidy
Polyploidy means that there are more than two chromosome sets in the nucleus of an organism. Polyploidy is common in plants, such as corn, which has six chromosome sets and is hexaploid.

Proteomics
In proteomics, the composition of all proteins in cells and tissues is examined.

RNA
RNA stands for ribonucleic acid. Similiar to DNA it also consists of nucleotides, but it contains the base uracil instead of thymine, which is a building block of DNA. The sugar component of RNA is different from that of DNA, namely ribose instead of deoxyribose. RNA has important functions as a link in the translation of genes into proteins and also has important regulatory functions in the cell.

SDN
SDN stands for site-directed nucleases and combines techniques such as CRISPR/Cas9, TALENs and zinc finger nucleases that are intended to cause changes within a target region of the genome.

SDN-1
Site-directed nuclease-1 (SDN-1) describes applications of targeted nucleases like CRISPR/Cas, that are introduced into a cell causing a double-strand break at a target region of the DNA. These double-strand breaks activate repair mechanisms in the cell, which introduce changes in the target sequence. No DNA templates are brought into the cell for the repair of the DNA double strand break in SDN-1 applications.

SDN-2
In contrast to SDN-1, site directed nuclease-2 (SDN-2) applications use small pieces of DNA (a few base pairs long) that are produced in the laboratory and additionally introduced into the cell. The DNA pieces serve as repair templates for the DNA double strand break. Scientist are able to introduce specific changes to the target sequence by using DNA donor templates.

SDN-3
Large pieces of DNA (e.g. entire gene regions) can also be introduced into the cell together with the CRISPR/Cas system and specifically incorporated into the target region surrounding the DNA double strand break. This type of application is summarized under site directed nuclease-3 (SDN-3).

Sets of chromosomes
In the nucleus of a cell, the chromosomes can exist in different amounts. For example, humans have a diploid pair of chromosomes with 23 chromosomes coming from the mother and 23 from the father.

TALENs
TALENs are transcription activator-like effector nucleases, which belong to the site-directed nucleases of genome editing techniques.

Target region
The DNA sequence that is to be engineered by site-directed nucleases is known as the target region (or target site). It is recognized, for example, using CRISPR/Cas by the gene probe (guide RNA) and then cut by the gene scissors.

Transgene
Transgenes are genes which are transferred across the order of species by genetic engineering techniques.

Transgenic organism
Transgenic organisms are organisms in which one or more genes, that do not originate from their own species, have been inserted into the genome using genetic engineering techniques.

Transgenic plant
Transgenic plants carry foreign genes in their genomes that were introduced using genetic engineering methods.

Transcriptome
The entirety of all RNA molecules within a cell or a tissue.

Whole genome sequencing
Whole genome sequencing are high-troughput DNA sequencing techniques that decode the entire genome with the exact sequence of the bases.

Zinc finger nucleases
Zinc finger nucleases are members of the site-directed nucleases. The zinc finger domain binds to a specific target region of the DNA, and then the nuclease domain cuts the DNA.