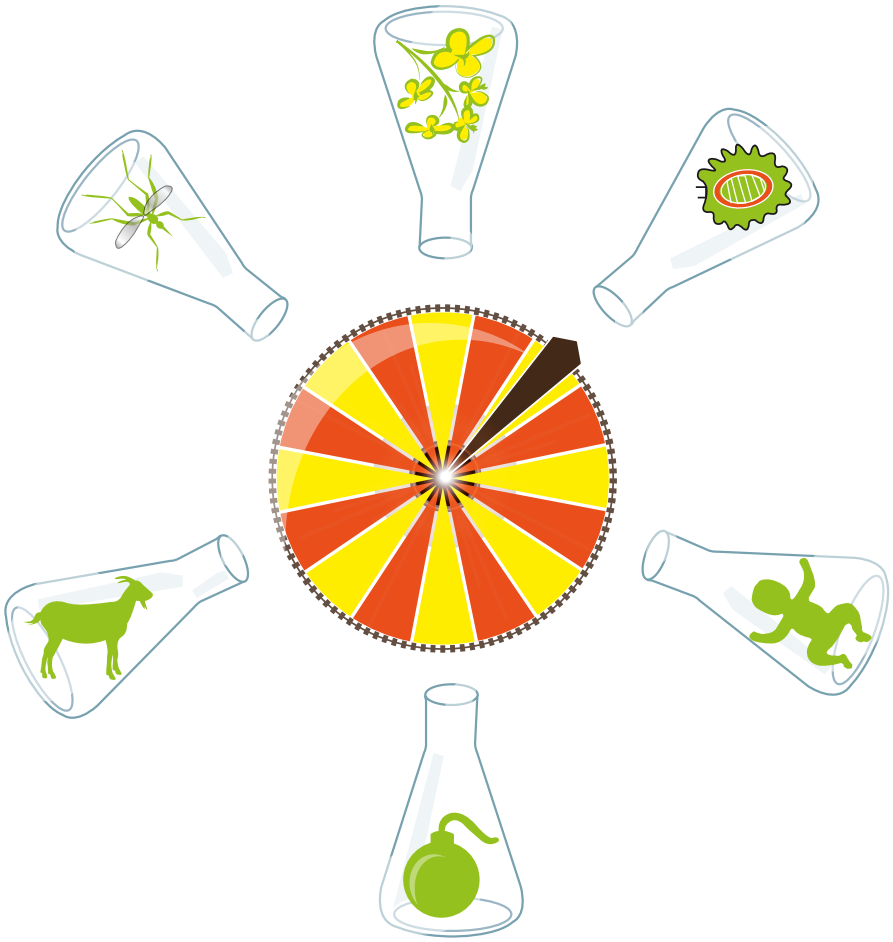

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Playing Russian Roulette with Biodiversity

Uncontrolled applications of gene editing threaten biodiversity, the rights of consumers and farmers, as well as the future of animal and plant breeding

Christoph Then & Andreas Bauer-Panskus September 2017

www.testbiotech.org

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Summary

The use of gene editing tools, such as the "gene-scissors" CRISPR, allow new ways of genome manipulation. They make it possible to edit the genome by removing or changing natural genes, as well as introducing artificial DNA. Gene editing has a huge range of potential applications in agriculture and the environment not only for crop plants and livestock, but also for natural biodiversity, such as insects, wild animals, trees and grasses. These news methods of genetic engineering even make it possible to increase the frequency with which the genetic changes are inherited in the following generations: So-called gene-drives can potentially manipulate the genome of whole natural populations of insects, plants or mammals. In effect, humans are planning to intervene in the "germline" of biodiversity.

As things currently stand, our generation needs to make a decision which could affect the future for years to come. If we get this wrong, it could rob future generations of being able to decide for themselves on the future of breeding, agriculture and food production. Indeed, our hubris around genetic technology could potentially lead us to leave our fingerprint on biodiversity and change it forever.

New methods of genetic engineering are presented as especially safe

The current political, economic and social climates appear to be favourable to biotech companies: The economy, politicians and science have all gathered behind a principle of innovation that puts the technically feasible and financial gain above long-term interests in protecting the environment and future generations. Scientific arguments are frequently made to fit commercial interests and, in many instances, there is a lack of critical independent research. This is creating a climate in which society is in danger of becoming blind to the risks involved.

The companies operating in this sector emphasise that the new methods of genetic engineering are more precise in making changes in the genome, with fewer unintended effects than with previous technology. These arguments are directed at economic interests: The new methods are presented as being so safe that they can be marketed without needing to undergo risk assessment or comply with labelling requirements. In fact, the success rate fluctuates strongly according to method, type of cell and organism – an unmistakeable sign that the methods are still associated with many risks and uncertainties.

Even one accident can have dramatic consequences

The new methods of genetic engineering are associated with a considerable number of risks. Removing strands of natural DNA, blocking gene functions or inserting additional genes can lead to health risks when the resultant plants or animals are used in food production.

It can also impact on soil life, the health of crop plants and livestock, ecosystems and pollinators such as bees, as well as on natural food webs. Both the population of these species and their interactions with ecosystems may be endangered if their changed genetic material is allowed to spread uncontrolled into the environment. These organisms can also become invasive and displace other species.

For closer consideration of the risks, it is important take the whole network of biodiversity, its means of biological communication and ecological interactions into account, in addition to recognising the limits of our current level of knowledge.

Even if in many cases there will supposedly be no evidence of problems, just one accident can have serious consequences for biodiversity, the future of animal and plant breeding, as well as human health. This kind of accident could happen today, tomorrow or even after more than a hundred years. Once this game of Russian roulette has started there will be no reliable way of controlling it.

We must not give up control

Genetic intervention in biodiversity threatens to be irreversible and impact the future of plant and animal breeding, with all the possible consequences. Any possible future decisions and freedom of choice for future generations would be thrown overboard together with the precautionary principle.

The current EU precautionary principle would be seriously undermined if there is no requirement for risk assessment and labelling. It would also be impossible to protect agriculture that does not use genetic engineering technology. Farmers and consumers would lose freedom of choice and decision-making.

Leaving genetically engineered organisms created with new methods of genetic engineering out of GMO regulation would, in fact, mean there would be no data for independent risk assessment. Neither would there be any information available that could be used to identify any such organisms in the case of intended or unintended release. Technical faults, unintended side effects, risks and biological hazards associated with these developments can rapidly become unmanageable and uncontrollable burdens for following generations.

In some ways this development is being forced: Biotech companies are not only talking about blocking genes, but also about "knocking out" GMO regulation and transparency for consumers. The biotech industry is pushing for a situation where there is no alternative to using their patented plants and animals.

Who stands to profit from this situation?

Well-known biotech companies, such as Bayer, Monsanto and DuPont want to expand their businesses; and other animal genetics companies, such as Genus are already positioning themselves in readiness. They promise to ensure global food security, but are driven by short-term financial gain that is secured by taking out patents on plants and animals.

Overall, rapid developments in new methods of genetic engineering are making it much cheaper to make changes to the genome; and the time needed for the process is considerably shorter. It is possible that both the type of changes and the number of genetically engineered organisms will increase rapidly within the next few years. If the biotech industry is able to follow through with their plans, dozens of such organisms will be used in agriculture and released into the environment.

The limits of risk assessment

A differentiation of unintended side effects associated with new methods of genetic engineering must be made between those that are relatively easy to recognise in DNA (off-target and on-target), biological effects in the cells of the organism and those which emerge in interaction with the environment. These are often unpredictable and therefore much more difficult to assess. Accompanying risks for humans and the environment must in each case be investigated by independent experts. Releases must not be allowed if the uncertainties and risks cannot be adequately assessed.

Examples of risks and side effects are:

- Mistakes can be made in regard to the correct genome target and the scissors can cut the genome at wrong place – this can lead to unintended changes at locations in the DNA that were not meant to be cut (off-target).
- If the nuclease cuts the target DNA sequence, additional DNA can often be either accidentally or unintendedly inserted; and at the targeted location there are often further unintended changes in the structure of the DNA (on-target).

Summary

- The removal of natural DNA sequences can lead to shifts in DNA reading frames; affected DNA sequences can be skipped (e.g. Exon skipping) and, amongst other things, proteins that are changed in their structure may form. This can lead to the emergence of unexpected and surprising biological effects in the cells or the organism that are not immediately predictable at the DNA level.
- If intervention in the genome is actually "successful", constituent parts of food plants can be unintendedly changed e.g. there can be an increase in allergenic plant constituents.

Some unintended effects may only emerge with a specific genetic background. These effects are particularly relevant when natural populations of plants and animals are changed and have a greater genetic diversity (heterogeneity) than the plants and animals that have been bred over hundreds of years.

The uncontrolled spread of genetically engineered organisms can have disruptive consequences for ecosystems and food webs:

- Food webs from insects to birds and mammals can be particularly affected by changes in plant populations.
- The exchange of information – e.g. the communication between pollinators and plants can be disrupted.
- The associated microbiomes, microorganisms that are symbiotic with roots of plants or the gut of humans and animals can be altered in a way that soil life is disturbed, or plants, animals and people become more susceptible to disease.

These risks cannot be considered equivalent to those that emerge from conventional breeding or random mutagenesis: Here the cells and the organisms have different means of regulating changes in the genome (random mutations or new gene combinations) so that the phenotype of the plants and animals is very often not changed, or only changed within certain parameters. New biological traits that emerge can adapt over longer periods of time to the environment. These natural mechanisms of genetic regulation are overridden by methods of genetic technology e.g. through simultaneous changes at several genome locations on different chromosomes, and the mass release of organisms with biological traits that have not been tested in the evolutionary process.

1. New methods of genetic engineering – what is the issue?

The terms gene editing or genome editing are used collectively to describe new methods of genetic engineering that are supposedly more precise than previous methods.

- One of the basics of the new methods of genetic engineering is the option to artificially synthesise nucleotides i.e. the basic structural units of the genetic substance DNA (deoxyribonucleic acid) and the messenger substance RNA (ribonucleic acid) in the laboratory. Synthetic DNA and RNA can be altered in its structure to fit a specific aim and purpose. This can lead to the emergence of structures that have not originated from evolutionary processes.
- Short nucleotide chains (oligonucleotides) can be inserted directly into cells so that these cells can serve as a template to modify their own DNA or interfere in gene regulation.
- Currently, the most important tools in this arsenal are nucleases, so-called "gene-scissors". These are enzymes (proteins) that can open up DNA. This newly developed technology targets and cuts the DNA at a specific sequence. DNA can be removed, changed or inserted at this location. This report focusses on these methods and, in particular, on the CRISPR systems.

The methods named above can be combined. The genomes of completely different organisms can be changed in small or large sections and DNA can be tailor-made to fit specific economic purposes. The organisms and their genetic and biological traits created in this process have not been able to adapt to the environment through natural evolution.

The new methods of genetic engineering make it easier to genetically change organisms and shortens development times. Therefore, it can be expected that a large number of these genetically engineered organisms will be released and used e.g. in agriculture.

1.1 Nucleases

In recent years, a number of different nucleases have been developed that enable a restructuring of DNA, which is the basic carrier of molecular genetic information. Currently, the most important of these is CRISPR-Cas9. This is a kind of gene probe made up of ribonucleic acid (RNA) and a protein, the enzyme that can "cut" the DNA (see Figure 1). The RNA is able as it were to reproduce mirror images of structural units of DNA. Via its specific RNA sequences, the CRISPR-Cas9 system can be "programmed" to a specific target. This enables genes to be silenced and/or the insertion of additional DNA into the genome.

Using CRISPR-Cas9, DNA can be changed at several locations simultaneously: The nuclease can alter the genome at all the locations where the target sequences are found. There are often groups of genes that have similar or identical structures – and they can all be changed in one single step. It is also possible to "program" the enzymes to target several target sequences at the same time. This means it is also possible to change different genes in just one step.

The gene-scissors – CRISPR-Cas9

Nucleases are proteins (enzymes) that can cut open DNA – therefore the name gene-scissors. Gene-scissors have been known for some time although it was only possible to "cut" the DNA at relatively few locations. In recent years, various new nucleases have been developed that are more versatile, faster and easier to use. Currently, the most important nuclease is CRISPR-Cas9 that was first described in 2012/2013. A new variation is the CRISPR-Cpfl, which is supposed to cut much more precisely.

The nuclease is intended to cut through both DNA strands. This will trigger repair mechanisms in the cell that will attempt to repair the DNA. As a result, changed DNA structures (mutations) frequently develop at the locations where the nuclease has made the cut, thereby allowing gene functions to be disrupted or blocked. This is called "knock -out" or changing of natural genes. Additional DNA (synthesised in the laboratory) can be inserted into the genome of the cell (called "knock-in") by using the CRISPR-Cas9 system. CRISPR-Cas9 technology can also be used to change the genome at several locations simultaneously. The exact way in which the nucleases act is by no means fully understood.

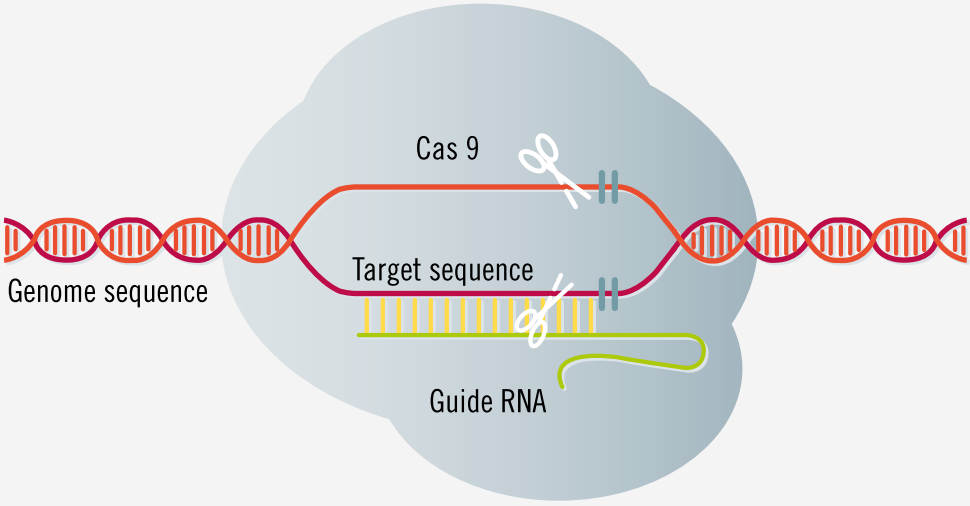

Figure 1: Nuclease (DNA-scissors): CRISPR-Cas9 – Clustered Regularly Interspaced Short Palindromic Repeats

How do the gene-scissors get into the cell?

In a first step, the DNA of the nuclease is inserted randomly into the genome of the cell. In a second step, the DNA scissors are activated to target the location to be changed. At this stage, there are frequently unintended changes in the genome, and in some circumstances, the nuclease can remain active in following generations and lead to unintended changes in the DNA at later stages.

There is also the possibility to combine the nuclease, consisting of protein and RNA, outside the cell. This can then be channelled into the cells as pre-synthesised gene-scissors. In this case, the DNA for the gene-scissor mechanism is not inserted into the genome. The effect is transient. The DNA-scissor is broken down in the cells. If the process is successful only the targeted locations are changed. (see e.g. Weeks, 2017)

This transient method can be relatively successfully used in so-called protoplasts: This is where the cell walls are removed so that it is easier to introduce the proteins into the cells (see for example Gil Humanes et al., 2017). This method can only be used with specific species of plants and, in particular, not with crop plants, such as maize, wheat and rice. It often involves more rigid methods such as particle bombardment: For example, metal particles are coated with nuclease constituents and then using pressure are fired into the cells (see e.g. Weeks, 2017).

A further transient method of introducing the gene-scissor into the cells is by using viruses as a vehicle. These are meant to activate the nuclease without inserting their own DNA into the genome of the plants (see e.g.Weeks, 2017).

There are also methods that introduce plasmids into cells that should base as genetic information to synthesise the nuclease in the cell without introducing the relevant DNA into the genome of the cells (Chilcoat et al., 2017).

By using transient methods, the DNA in cells can be changed or removed, but the insertion of additional genes is technically problematic (see e.g. Liu et al., 2017).

Especially for economic and legal reasons, the biotech companies are pushing these methods to the forefront of their activities: They hope thereby to escape legal regulation for assessment and requirements for labelling if no additional DNA is inserted into the plants and animals.

Variants of the CRISPR systems

The cutting mechanism of the nuclease can be wholly or partially deactivated. In this way, CRISPR variants that will only cut one strand of DNA (nickase) can be obtained, or that will only target single bases (the "letters" of DNA – adenine (A), cytosine (C), guanine (G) and thymine (T)) to be changed.

In addition, with some modifications, CRISPR-Cas nucleases can be used to cause biochemical changes on the chromosomes to change gene activity (epigenetics). In this way, genes can be silenced or activated.

1. New methods of genetic engineering – what is the issue?

Gene-scissors are often put into three groups according to the function of the nucleases – whereby the term SDN or Site-Directed Nucleases technology is often used:

SDN 1 technology – introduces a double strand break (both DNA strands are cut) that is then repaired via cellular repair mechanisms, whereby each strand is changed differently in its structure (non-homologous end joining, NHEJ). As a result, random mutations are created at each site via which the respective genes can be deactivated.

SDN 2 technology – additional DNA is inserted with help of the nuclease. The inserted DNA serves as a repair template (matrix) and enables a homologous repair of both DNA strands (homology-directed repair, HDR). The structure of the DNA is repaired in short sequences, but not randomly changed. Natural gene functions are also thereby often deactivated. In addition, this method is frequently used in combination with SDN 3 technology. The success rate is usually lower than with SDN 1 technology.

SDN 3 technology – additional (longer) DNA sequences are introduced into the cells with the nuclease. The longer DNA sequences can establish new biological functions. The success rate is often very low.

Many of the biotech companies would like to exclude SDN 1 and 2 technology from genetic engineering regulation.

1.2 Gene-drives

In so-called gene-drives, the gene responsible for forming the nuclease is permanently established in the genome. The enzyme is thereby reproduced in following generations and should ensure that changes are repeated on all DNA sequences as targeted. This means that the genes introduced in gene-drives spread much faster: Normally, according to Mendel, genetic information of sexually reproducing organisms splits up in following generations, whereas organisms with a gene-drive pass on changed genetic information homozygously so that the same genetic information is inherited by all offspring.

The aim, thereby, is frequently not "only" the genetic engineering of crop plants and animals. One future goal is the genetic engineering of natural populations. The technology could, for instance, be used to eradicate pests or make weeds more susceptible to herbicides. (National Academies of Sciences, Engineering, and Medicine, 2016).

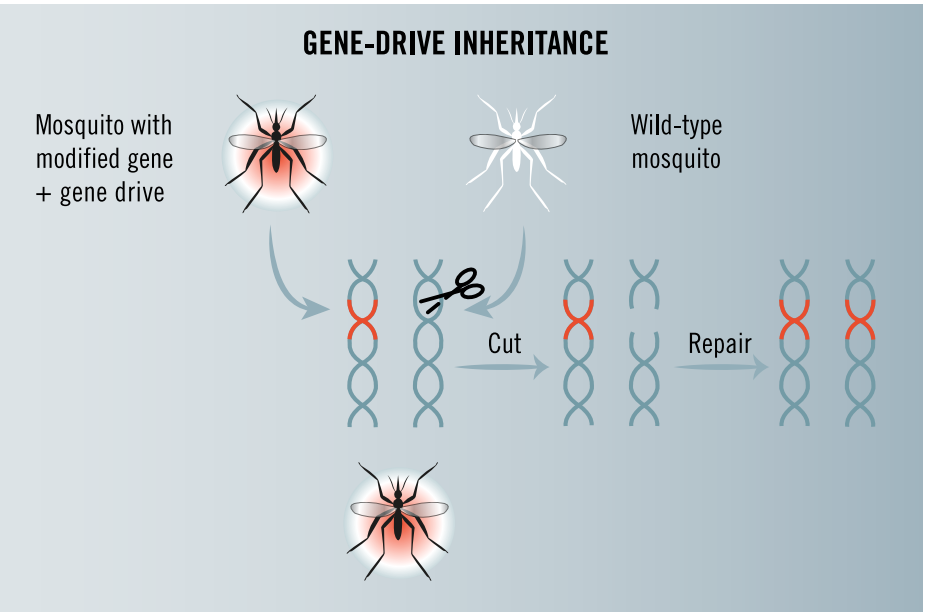

Figure 2: Gene-Drive (Mutagenic Chain Reaction): The genetic changes are passed on to each generation including onto the partner chromosome so that inheritance is homologous. This allows the new DNA to spread much more rapidly through populations (derived from Gantz & Bier, 2015).

1.3 Which plants and animals will be genetically engineered

There is a continual increase in the number of publications that describe which plants and animals have been "successfully" changed using gene-editing methods. Apart from CRISPR technology, other methods used include the TALEN DNA-scissors and so-called meganucleases, although these are more difficult to handle.

In the USA, a maize event produced by DuPont and edible mushrooms have, amongst others, been approved, but are not yet on the market. This particular maize event has a changed starch content and the edible mushrooms are non-browning.

Table 1 – Examples of organisms that are genetically engineered but are not regulated in the USA (original information: Waltz, 2016)

Crop	Trait	Developer	Technique	Year
Green Foxtail	Delayed flowering	Danforth Center	CRISPR-Cas9	2017
Potato	Reduced black spot (PP05 potato)	Simplot	TALEN / Agro-bacterium	2016
Potato	Improved processing characteristics (PPO_KO potato)	Calyxt	TALEN	2016
Waxy corn	Altered starch	Pioneer	CRISPR-Cas	2016
White button mushroom	Anti browning	Penn State University	CRISPR-Cas	2016
Wheat	Resistence to powdery mildew	Calyxt	TALEN	2016
Maize	Increased starch	Agrivida	Meganuclease, method: CBI	2015
Rice	Disease resistance	Iowa State University	TALEN	2015

In many other cases, a "proof of concept" is already available. There are, amongst others, publications on the application of CRISPR on alfalfa, barley, potatoes, maize, poplar trees, petunias, rice, lettuce, soybeans, sorghum, tomatoes, wheat and lemon trees (Tang & Tang, 2017).

Gene-editing and nucleases are being tested on livestock, such as pigs, cattle, sheep, (Tan et al., 2016) poultry (Wang et al., 2017a) and insects such as, honey bees, flies, mosquitos and butterflies (Taning et al., 2017).

In most cases, the natural gene functions have been "knocked out", and in a few cases, new gene functions have been introduced. The chances of success differ depending on the type of plant and the size of the genome (Hilscher et al., 2017; Zhu et al., 2017). Wall cress (Arabidopsis) is frequently used in trials – it has a relatively small genome and is therefore more suitable for this purpose.

The genetic makeup of plants, such as maize, wheat, rapeseed, and sugar beet, is considerably more complex with several paired sets of chromosomes.

There are also limitations with some animals: The success rate of using nucleases on rats and mice, whose embryonal stem cells can be reproduced in the laboratory, is much higher than, for instance, with cattle (Tan et al., 2016). In addition, there are numerous bioethical concerns (Then, 2016a).

1.4 What are the aims?

The arguments in favour of new gene-editing technology often refer to the possible advantages regarding global food security or the reduction in substances that are sprayed onto crops. However, the real aims that are, for instance, formulated in patent applications, are often completely different. In many instances, synthetic gene technology is seen as the continuation of the aims of genetic engineering, such as the reduction in herbicide use, that have already failed. For example:

- The US company DuPont wants to produce plants that are resistant to the herbicide glyphosate by using nucleases in CRISPR technology. Similar genetically engineered plants have been on the market for over 20 years (WO2016007347).
- The Swiss company Syngenta wants to use the new nucleases to further genetically engineer the insecticide-producing maize MIR604 that is already cultivated in the USA (WO2016106121).
- The US company Recombinetics intends to genetically engineer pigs and cattle so that they produce a higher muscle mass (WO2012116274).

Other aims are new but extremely problematic in regard to risks. For example, using gene-drives to combat pest insects, whereby the genetic engineering can lead to the collapse of whole populations (Gantz & Bier, 2015). In another instance, the intention is to genetically engineer the growth of trees to make them more suitable for the paper industry (Fan et al., 2015).

Other applications that directly affect consumers have hardly any benefits e.g. edible mushrooms and potatoes that when cut do not discolour (see Table 1). Such products are more than likely to simply mislead consumers rather than satisfy their desire for fresh food.

There are also projects whose use can be more seriously discussed, such as higher yields or adaptation to climate change. However, it quite frequently seems clear that conventional breeding methods are a better option than using genetic engineering to achieve these goals, this is because conventional breeding methods have been more successful in this respect. There are good reasons for this: Unlike genetic engineering technology, conventional plant breeding takes the whole cell system and natural genetic regulation into account. Therefore, it is able to utilise the activity and combinatorial effects of thousands of genes and can, for instance, produce plants with higher yields and, at the same time, improve adaptation to environmental conditions. Genetic engineering, on the other hand, only uses individual structural units that are frequently not sufficiently adapted to the whole system.

2. Risks and side effects

The advocates of the new methods of genetic engineering often create the impression that they can use DNA-scissors (nuclease) CRISPR-Cas9 technology to make precise interventions in the genome, with no side effects. However, in reality there are very frequently unintended side effects. All of the currently available methods can have side effects that carry a risks for people and the environment. How extensive these are depends on where changes are made in the genome and how the DNA-scissors are inserted into the cell, as well as which types of cell or organisms are targeted for manipulation.

2.1 Varied rates of success

The success rates for gene-editing with CRISPR-Cas9 technology are very varied. Success rates of less than 1% and up to over 50% are achieved depending on which animal or plant species is being targeted, the aim of the genetic engineering, the type of cell used and the technical methods employed (Hilscher et al., 2017; Zhu et al., 2017). Overall, they have considerably higher success rates than with previous genetic engineering technology. Nevertheless, it is obvious that the new methods of genetic engineering have numerous technical problems and uncertainties. The result of the intervention in the genome is still not precisely predictable.

The most successful applications of DNA-scissors are those where there is no insertion of additional DNA into the natural genome. Usually in these cases, natural genes are "knocked out".

The DNA for the gene-scissors does not necessarily need to be inserted in the genome for these applications but can, for instance, with specific plant species, be inserted into the plant cells as a pre-synthesised enzyme (transient method). The gene-scissors enter the cell nucleus, cut the DNA and then leave cell repair mechanisms to repair the damaged DNA sequence. The enzyme is broken down in the cell.

For economic and legal reasons, the transient methods are being pushed into the foreground by biotech companies: They hope thereby to escape regulation for approval as well as labelling requirements because no additional DNA has been inserted into the plants or animals (Wolter& Puchta, 2017). This is a legally controversial strategy (Kraemer, 2015; Spranger, 2015) that does not appear to be plausible from a scientific point of view because the genome of plants and animals is in any case genetically engineered by using these methods. Also destroying natural DNA structures by, for instance, removing DNA sequences is a technical intervention into the genome that carries risks even if no additional DNA is inserted.

Success rates of over 50% can be achieved (Zhu et al., 2017) in optimal conditions. However, they can be much lower than this: According to Chilcoat et al. (2017), DuPont used a transient method to change the starch content of a maize variety, but the success rate was only around 0.5%. Despite the obvious susceptibility to error of this method, the US authorities saw no reason to undertake a comprehensive risk assessment of the maize before it was marketed (see Table 1).

In particular, there are many difficulties associated with the use of nucleases to insert additional DNA. To achieve a sufficient amount of activity, the genome for the synthesis of the DNA scissor is also usually inserted in the genome of the plants. This results, for instance, in lower success rates for soybeans. In model experiments, after hundreds of attempts only one soybean plant was successfully changed. The plant also displayed a large number of unintended gene changes (Li et al., 2016).

"Success" also depends on the structure of the plant genome: Maize, wheat and sugar beet have very large genomes. Rice and the plant often used in laboratories, wall cress, have genomes that are clearer and, therefore, success rates are higher.

2.2 Unintended genetic changes: on-target and off-target

The fact that DNA-scissors have unintended effects at the target location (on-target) is known from numerous publications. In experiments with soybeans, it was found that it was not the desired additional DNA that was at the target location, but often completely different DNA sequences (Li et al., 2016), in particular, often parts of the DNA for gene-scissor synthesis. This means that the instructions for making the tool are inserted into the genome and not the desired DNA sequence that was supposed to be inserted with the help of the CRISPR-Cas9 technology.

This unintended effect i.e. that the DNA for the gene-scissors is inserted into the genome of the plant cells is recognised by experts as a general problem (see e.g. Liang et al., 2017). Above-mentioned transient methods for applications of CRISPR technology have been developed to avoid this problem. In these applications, the protein (with related RNA) is, for instance, processed outside the cell and directly inserted into the cell as a pre-synthesised enzyme. The success rate of this method is dependent on, amongst other things, the respective plant species (siehe z.B. Weeks, 2017).

If the changes do not occur at the intended target but at other locations in the genome they are known as off-target effects. One possible reason for this is confusion about the gene location where the gene-scissors are to be activated. This is a risk, in particular, with maize, wheat and sugar beet whose genomes are present in multiple versions in the cells (four, six, or even eight paired sets of chromosomes – known as called polyploidy). The genome is thereby organised in similar and repeated gene families so that it is more likely that they are confused with the targeted DNA sequence (Zhu et al., 2017). Sometimes the effect that the gene-scissors automatically cut all identical DNA sequences is used. Thereby, all the gene functions in the plants or animals can be blocked or changed in one go. This is a mechanism that does not occur in conventional breeding (see below).

Further inaccuracies are another reason for the variable success rates of CRISPR-Cas technology: So, for instance, the DNA scissors might only cut one of the complementary strands of the DNA (Li et al., 2016). Furthermore, the damage caused when the DNA is cut (deletions) might be very different in its extent and the resulting plants might only have specific changes in a few cells, but not in others (chimera) (Peng et al., 2017; Mao et al., 2017).

Overall, the tendency is to more off-target effects with CRISPR-Cas9 technology in comparison to other gene-scissors, such as zinc finger and TALEN (see e.g. Zhu et al., 2017). Therefore, attempts are being made to make the CRISPR system more reliable. For this purpose, a new enzyme CRISPR-CPf1 has been developed that is apparently less prone to error (Begemann et al., 2017, Mahfouz et al., 2017; Wang et al., 2017b). As yet, there are relatively few publications but, nevertheless, the deletions at the target location of the gene scissors (removal of DNA sequences) are more extensive than with CRISPR-Cas9 (Kim et al., 2017), which can lead to further complications.

A further approach is a change in the CRISPR-Cas9 system so that the DNA is no longer opened (cut) and only the individual letters of the DNA (the bases ATCG) are changed. However, even here the result is by no means what was planned: In tests with rice it was found that the change of the bases was in many cases flawed (Li et al., 2017; Ren et al., 2017).

Similar problems to those with plants have emerged with animals, whereby these are further compounded by bioethical problems.

Overall, tools such as CRISPR-Cas9 are relatively prone to error and this is reflected in the varying success rates. Clearly, precaution and carefulness must have priority in the use of these and similar methods of genetic engineering.

2.3 Unexpected biological effects

In order to understand the biological effects that are relevant for risk assessment, we have to look beyond the level of DNA and consider cells and whole organisms, including their interactions with the environment. Finding these unexpected biological effects is much more complex undertaking than looking at unintended changes at the level of DNA. Moreover, it can often not necessarily be deduced which biological changes will emerge from changes in DNA structure.

However, the DNA analysis can in many cases provide important indications. For example: When genetic changes are made using CRISPR-Cas9, very often the gene information for synthesis of the enzyme is also inserted at the location where the gene-scissors cut (see e.g. Li et al., 2016). If this genetic information is complete, then the mechanism of genetic manipulation can be unintendedly inherited and thereby lead to unintended changes in following generations.

Other biological effects are much more difficult to discover e.g. it was found that unexpected changes can occur when DNA sequences are deleted. For example, CRISPR-Cas9 was used in human cells to remove various gene sequences that are responsible for forming specific proteins. Such gene sequences are known as "exons". The affected cells reacted in an unexpected way: Some of the changed sections of the genes were simply read in a different way; and some specific DNA sequences were skipped (exon skipping). Thus, the cells were still able to form the proteins – in part, in a modified form (Kapahnke et al., 2016; Lalonde et al., 2017; Mou et al. 2017). These effects have so far been overlooked. The reason for this is that for them to be discovered, it is necessary to investigate not only the gene structure but also the formation of messenger substances, such as RNA, and the formation of proteins.

So-called omics technology can be used for such investigations (e.g. for investigation of, amongst other things, the transcription i.e. gene activity; proteome i.e. the formed proteins and the metabolome i.e. the metabolic products in the cell). As yet, these kinds of investigations are not required for the risk assessment of genetically engineered organisms. Mechanisms like exon skipping can have a significant impact on the function of the cell and the whole organism. Sometimes these effects might only emerge with specific environmental conditions e.g. with stress factors.

It also may be the case that only one strand of DNA is affected – this would complicate an exact investigation. In general, the effects differ according to the type of cell; they are also not random and cannot be predicted (Mou et al., 2017). Various causes have been discussed (Sharpe & Cooper, 2017). In future, it can be assumed that many other mechanisms in the cells will be discovered that lead to unexpected biological effects.

One of the many other possibilities that can trigger such unintended effects is when DNA sequences with several biological functions are changed. One example is the gene manipulation of moths (*Cydia pomonella (L.)*) where the genes not only as assumed concerned the sense of smell, but also fertility. This was only noticed when attempts were made to breed the insects that had been "successfully" genetically manipulated in regard to their sense of smell. In fact, the attempts to breed the moths for further investigations failed because they were unable to reproduce (Garczynski et al., 2017).

In reality, the biological functions of many DNA sequences are not limited to a specific function. It is for this reason that scientists using CRISPR-Cas9 believe they may find particularly interesting applications, in particular, when they manipulate sections of genes that have key functions in the genome and which influence the functions of many other genes (Jez et al., 2016).

The more experiments are carried out with CRISPR-Cas9, the longer this list of biological effects becomes. Still, the question remains about whether all the risks will be discovered before organisms are released.

2.4 Comparison with random mutations

The unintended effects and associated risks are often compared with the effects of random mutations and other changes in the genome. In particular, such statements are often found in reports that address regulatory requirements (see e.g. High Level Group of Scientific Advisors, 2017).

From the point of view of the users, these comparisons have a central significance: Due to random mutations - in particular accelerated mutagenesis through radiation or chemical additives in the laboratory, is often associated with many more random changes that occur through genetic intervention in the genome (Songstadt et al., 2017). The same argument is used in regard to breeding through crossing and selection. Emanuelle Charpentier, one of the inventors of the CRISPR technology, is quoted as saying "One should not forget that in conventional breeding much less can be predicted about what will happen to the genes. Plants are crossed and the genes are thrown into confusion. With CRISPR, breeders and farmers can decide for themselves what they need." (translation: Testbiotech)[1]

In essence, these arguments are not scientific but aimed at influencing political decision-makers. The specific risks associated with the genetic manipulation of the genome are thereby relativized and, as far as possible, brought down to the level of the alleged chaos generated in conventional breeding.

At first sight, these arguments appear to be plausible but, in fact, they are hardly appropriate to evaluate the risks and consequences of genetic manipulation of the genome. There are, indeed, continuous mutations in the genomes of plants and animals; plants are often exposed to strong UV light that is known to trigger mutations. It is also not remarkable that continual changes occur in the DNA of plants and animals. What is more remarkable is that very often, only a tiny percentage of these lead to a biological effect. Two essential basic conditions must be fulfilled for species preservation: continuous change and (in certain circumstances very rapid) adaptation to changing environmental conditions. Furthermore, stability in the heritability of basic genetic structures to ensure preservation of the species over longer periods of time is also important. It is interesting that for example the processes influencing the architecture of the genome are not organised at random (see e.g. Al-Shahrour, 2010).

In summary, evolution and genomic organisation require a balancing act between chaos and order, in changes adaptation and stability. Evolution also means consistency and continuity despite change. The creation and survival of species and thereby "higher" complex living beings would otherwise be impossible. There are, for instance, basic genomic structures and mechanisms in the cells of many of our food plants that can trigger polyploidy or can control mobile elements in the genome (Wendel et al., 2016).

Thus, many spontaneous or induced changes in the genome (mutations, "jumping genes", changed

1 Interview with Emanuelle Charpentier (German): http://sz.de/1.3502623

gene activity) are not really random but are subject to regulation mechanisms in the cells/organisms. In fact, the cells can to a certain extent influence which mutations or other changes in the genome are established. Plants and animals are in the same way as microorganisms simultaneously the subject and the object of evolution.

The already mentioned example of exon skipping, is an example of these natural mechanisms of control and correction of gene activity. The cells can compensate for some flaws in the structure of the genome and/or jump over damaged DNA sequences to ultimately still make the required protein (Mao et al., 2017). Plants, in particular, have numerous reactive mechanisms to change or control regulation of their genomes (see eg. Wendel et al., 2017). Methods of genetic engineering try to bypass these mechanisms to achieve the desired result.

How the new methods of genetic engineering actually manage to bypass these mechanisms of genetic stability is illustrated in the example of multiple genetic changes: The genomes of plants and animals mostly contain two paired sets of chromosomes, but the genomes of many important food plants often have more. This is known as polyploidy. One example of a plant with many paired sets of chromosomes is sugar beet, it has eight paired sets of chromosomes. Bread wheat contains six, whereas potatoes, cotton, apples, peanuts and durum wheat each have four. Many other plants, such as peanuts, rapeseed and alfalfa are also polyploid (see e.g. Wendel et al., 2016).

From the perspective of evolution, complex life forms such as plants and animals that have three or more paired sets of chromosomes are a sensible option (Wendel et al., 2016); it means there are always back-up copies if the genetic information on a chromosome is damaged. However, from the perspective of the genetic engineer, it is problematic because in order to establish new biological traits in plants or animals it is preferable to change all copies of a gene, not just one.

Songstadt et al. (2017) write that,

> *"One challenge for targeted mutagenesis is that many plants are polyploid or have undergone past episodes of polyploidy. Consequently, plant genes typically have multiple, redundant genes and extensive gene family networks."*

This is where scientists see a decisive advantage in the application of CRISPR-Cas9 genetic editing technology (Braatz et al., 2017):

> *"In conclusion, the CRISPR-Cas9 system is clearly superior to classical mutagenesis. We propose that in future, all members of a given gene family can be knocked out by a single CRISPR-Cas9 experiment and without off-target effects. Thus, targeted mutation induction will accelerate the introduction of mutants into the breeding programs."*

In comparison to genetic engineering technology, random mutations do cause e.g. in wheat all the relevant locations on the genome to be changed. However, the gene-scissors CRISPR and TALEN have already proven that this is exactly where they are different. So, for instance, Wang et al. (2014) reported a successful change in bread wheat on all six chromosomes at the same time. Further, Clasen et al. (2016) have described the simultaneous change of a gene using the TALEN nuclease on the four chromosomes of potatoes.

This difference between methods of conventional breeding and methods of genetic engineering (that is also relevant for the application of oglionucleotides) shows that it is necessary for genetically engineered plants

and animals to undergo comprehensive risk assessment before any declarations are made about their safety. From a scientific point of view, it is not understandable why it matters how extensive the changed section is and whether DNA is inserted, changed or removed. All such changes have to undergo a detailed risk assessment.

The USA has chosen a different path: Scientists there have, for instance, manipulated edible mushrooms using CRISPR-Cas9 technology so that cut surfaces do not turn brown as quickly, and the mushrooms can be stored for longer. To do this, the function of natural genes was simultaneously blocked at several locations on the genome. The regulatory authority declared the mushrooms safe – without requiring risk assessment for marketing approval. However, these genetic changes can lead to substantial undesirable changes in metabolism and content of the mushrooms. Nevertheless, there have so far – the mushrooms were approved in the USA in 2016 – been no scientific publications on how exactly the traits of the mushrooms were modified, whether intended or unintended.

2.5 Limits to the assessment of health risks

As has been shown, there are unintended effects and side effects that occur with DNA-scissor applications (on-target and off-target), that are also reflected in the varying success rates. These effects can also be expected when genes are blocked or base pairs are replaced.

Undesirable outcomes can emerge even when the intervention in the genome is apparently successful and no unintended structural changes can be found in the DNA at other locations on the genome (see exon skipping). Even if no additional DNA was inserted, the outcome may possibly be very different from effects that occur with random mutations.

Each outcome may also be dependent on stress factors or environmental conditions, specific interactions or the genetic background of specific varieties of plants and animal species. The underlying mechanisms can be genetic, epigenetic (concerning gene regulation) and be a result of interactions with the environment. It has to be taken into account that there are biological mechanisms that play a substantial role in this respect, but which are either unknown or not completely understood.

There are numerous risks that must be taken into consideration if these plants and animals are used in food production: There can, for instance, be an increase in the effect of allergens or phytoestrogens in the plants and animals that have had specific genes removed from their genome.

Many of these effects are only observed after a longer period of time and in interaction with other substances or eating habits. It has to be assumed that current methods of risk assessment are inadequate for determining these risks. Even any meaningful observation of consumers (monitoring) has so far proven impracticable even when the effects on health are drastic (see European Communities, 2005).

There are many other uncertainties that are not taken into account in the risk assessment of genetically engineered organisms. It is, for instance, becoming much clearer how closely ecosystems interact via a network of microorganisms. Plants, animals and humans are inextricably linked to their microbiome (amongst other things, microorganisms that live in symbiosis in the gut of humans and animals and also on the roots of plants) (see e.g. Bakker et al., 2013). The microbiomes of humans, animals and plants are themselves in constant interaction. This is related not only to provision of nutrients, but also to numerous forms of biological communication and interactions that are so far only partially known.

There are, for instance, ongoing discussions regarding to what extent biologically active messenger substances (miRNA) originating in plants or microorganisms can intervene in the regulation of specific genes in humans (Zhang et al., 2012). As yet, this issue has not been included in the risk assessment of genetically engineered organisms – not because it is irrelevant, but because it is a hugely complex issue, as would be investigations.

2.6 Limits to the assessment of environmental risks

A publication on breeding experiments with so-called "Golden Rice" clearly shows the limitations in the assessment of environmental risks. "Golden Rice" produces carotenoids, a precursor of vitamin A. This particular rice was developed with previous methods of genetic engineering and not with new genetic editing technology, although this makes no difference in the observed effects: Crossing "Golden Rice" with the Indian rice variety "Swarna" led to significantly impaired growth in the resulting plants (Bollinedi et al., 2017). There are several causes for this: One is that the inserted gene construct impairs the function of the natural gene that promotes plant growth. Another is that the added gene is active in the leaves of the plants and not in the grains as planned. This means there is a reduction in the content of chlorophyll that is vital for the plant.

These side effects were not discovered in previous investigations. On the contrary, it was thought that the genetically engineered rice plants used here were genetically stable. The significant side effects were only discovered when the transgenic plants were crossed with the rice variety "Swarna" that is grown extensively grown in India. The unexpected biological effects were, thereby, dependent on a specific genetic constellation that was brought about by crossing the plants with "Swarna".

These risks might possibly harm plant breeding and agriculture over long periods of time. Once genetically engineered rice is released, its genome can spread into the genome of wild rice and other varieties of rice, and from wild rice return again and again to the fields (Lu & Yang, 2009) even when the genetically engineered rice is no longer cultivated. Instead of helping to fight vitamin A deficiency, genetically engineered plants could endanger the rice harvest in these regions.

This is a risk for farmers, breeders and consumers alike: Once the genetically engineered organisms have spread into regional varieties or related wild plants, it may be impossible to reliably recall and withdraw them from populations. As yet, genetic effects that emerge from crossing genetically engineered plants with specific varieties are not investigated in risk assessment.

If genetically engineered plants escape into natural populations, it is conceivable that the environment could be endangered through different networks and interactions:

- Natural populations within which genetically engineered genetic information is allowed to spread can be weakened and, for instance, become more susceptible to disease or other stress factors.
- Conversely, the genetically engineered organisms can have a higher fitness than their natural relations and therefore be able to impinge on these and other species.

The uncontrolled spread of genetically engineered non-adapted organisms can possibly have disruptive consequences for ecosystems and food webs:

- The food webs from insects up to birds and mammals can, in particular, be impacted by changes in plant populations.

- The exchange of information e.g. communication between pollinators and plants – can be disturbed.
- The associated microbiomes on roots, leaves in the gut of wild animals or humans can be changed and impact on soil life; or plants, animals and humans can be weakened and become more susceptible to diseases.

Whether, and if, and how much damage genetically engineered organisms that breed and persist in the environment over periods of time e.g. for five, ten or over a hundred years actually cause cannot be predicted. In general, it can be said that the less these organisms can be limited in their spread spatially and temporally, the higher the probability that the risks will lead to actual damage.

The actual probability of damage being caused cannot be predicted, therefore, suitable "pre-factual" preventative measures must be implemented in order to, in particular, avoid uncontrolled spread. Therefore, risk assessment prior to approval, labelling and traceability are absolutely essential. If genetically engineered organisms are not regulated, there will be no data in order to identify an intended or unintended release.

2.7 Extending the risk zone

In recent years, so-called gene-drives have been developed with which whole natural populations can be genetically changed. Until now, plants and animals were mostly bred or genetically engineered for agricultural purposes. Now, however, gene-drives can be used to manipulate the genomes of wild species (National Academy Press, 2016). Human beings are planning, as it were, to intervene in the germline of biodiversity.

Gene drives are planned for use in decimating specific species. One of the possibilities in this respect is the insertion of gene-scissors in vital genes in order to damage certain genes, so that, for instance, only the males of a specific species survive in following generations. These applications are being explored and discussed for use in insects or unwanted wild animals, such as mice (National Academy Press, 2016).

Further applications for gene-drives include the manipulation of some species: For instance, to manipulate mosquitoes so that they are no longer able to transmit malaria (see e.g. Tanning et al, 2017) or to make weeds more susceptible to herbicides(National Academy Press, 2016). This could mean that whole plant and animal populations could be genetically engineered and affected in their entirety; and also spread uncontrolled into the environment.

Various experiments have shown that gene-drives in insects are basically feasible, but have led to intense controversy amongst scientists. Many experts are warning that these organisms should not be released into the environment. With our current level of knowledge, no credible statement can be made on how organisms with gene-drive might behave in the environment. Once they are released, the organisms could cause irreparable damage in ecosystems. Any releases could not be recalled or withdrawn, and effective controls are so far not possible.

3. Economic interests

From the point of view of industry, two strategic questions are relevant for marketing new genetically engineered organisms: Avoiding regulation and expansion of patent acquisitions.

3.1 Avoidance of regulation

In the USA, companies, such as Pioneer/ DuPont and Cellectis/Calyxt have been given approval for releases of some of their plants that were manipulated with new methods of genetic engineering using gene-scissors, such as CRISPR and TALEN, with no detailed risk assessment (see Table 1).

The reasoning behind this is simple: There are less off-target effects with applications of nucleases and oglionucleotides than with conventional breeding and, therefore, plants and animals that have been genetically manipulated with these methods do not need to undergo risk assessment (see e.g. Wolter and Puchta, 2017).

This argument is not convincing from a scientific point of view. Even if there are fewer off-target effects at the level of DNA, the methods of manipulating the genome and their outcomes are very different to random mutations in genetic makeup – as, for instance, the simultaneous change of all similar genome locations on different chromosomes (see above). In addition, biological effects can emerge at the metabolic level of plants that are measurable at DNA level e.g. exon skipping.

3.2 Expansion of patent applications

Some experts are saying that these new technologies are cheaper than previous genetic engineering methods and are, therefore, more affordable for smaller companies and not just the biotech giants.

However, this overlooks the fact that the new methods using nucleases, such as CRISPR-Cas9, are patented just like the manipulated plants and animals. Companies, such as Bayer, Monsanto and DuPont, long since have contracts with the DNA- scissor inventors from the Broad Institute (USA) and the University of California to use their patents.

Moreover, companies file patents on special applications e.g. DowAgroSciences are systematically filing patents on naturally occurring DNA sequences in plant genomes that are supposedly particularly suitable for nuclease applications. Other patent applications are for applications such as those generating herbicide resistances, changed growth, changed contents or for specific technical variations in the application of nucleases (Then, 2016b).

Bayer and Monsanto have filed their own patents on nucleases, their uses and the resulting manipulated plants. Bayer is, hereby, cooperating with other companies, such as Cellectis (which is closely connected with Calyxt), as well as CRISPR Therapeutics. Bayer has a particular interest here - CRISPR Therapeutics, in which one of the inventors of CRISPR-Cas9, Emmanuelle Charpentier, is a shareholder, handed over all applications for use on plants and animals in the agricultural sector exclusively to the company for further use. Monsanto is also securing its interests in the new technology, and in September 2016 it agreed on a licence contract with the Broad Institute (MIT) and Harvard University. This is in regard to the further development of the CRISPR technologies and CRISPR-Cpf1 nucleases.

The influence of the large seed companies will expand further with the patents and also promote the process of concentration in the business. Currently, just three companies, Monsanto, DuPont (meanwhile fused with Dow AgroSciences) and Syngenta, control 50% of the international seed market.

If Monsanto and Bayer merge, this may possibly create a new market dominance for new methods of genetic engineering. The new, merged company would have cooperation agreements with both parties who are arguing about the basic patent i.e. Emmanuelle Charpentier and the Broad Institute (MIT).

Table 2: Overview of patent cooperation between seed giants and the developers of CRISPR technology

Company	Cooperation with
Bayer	CRISPR Therapeutics/Emmanuelle Charpentier
DuPont	University of California / Caribou
Monsanto	Broad Institute (CRISPR-Cpfi)

This development includes animal breeding. Genus, one of the largest companies in the livestock breeding sector, has already announced that it intends to use animals produced with gene-editing technology (Bruce, 2017) and is in cooperation with Recombinetics, a company that is systematically filing patents on pigs and cattle.

Table 3: Examples of patents filed by Recombinetics (USA) for livestock genetically engineered with nucleases

Registration Number	Claims
WO 2012116274 / EP2678434	Methods using nucleases to increase muscle growth in cattle and pigs.
WO 2013192316 / EP2863736	Methods using nucleases to increase muscle mass in certain cattle; and produce hornless cattle.
WO 2014070887 / EP2914714	Livestock that do not reach sexually maturity and can be fattened for longer. Farmers cannot use these animals for breeding.
WO 2014110552 / EP2943060	Hornless cattle for natural and synthetic genetic applications.
WO 2015168125 / EP3136850	Animals with multiple genetic changes.

Conclusions and recommendations

The new generation of genetically engineered organisms pose new challenges for society. The euphoria surrounding technical feasibility threatens to overcome awareness of the risks.

If purely economic interests gain the upper hand, society will no longer be able to control or regulate the process because there will no possibilities for independent experts to assess the risks, identify the organisms and, if necessary, remove them from the environment. Both farmers and consumers will lose their freedom of choice.

Transparency, traceability and the precautionary principle in dealing with new methods of genetic engineering are essential to having alternatives and remaining able to act in future. Therefore, none of the new methods should be excluded from regulation in the interests of short-term profit.

If there is no approval process, then there will be no data on the exact type of genetic manipulation. Furthermore, the consequences of genetically engineered organisms being allowed to spread uncontrolled into the environment can remain undiscovered for many years.

Once this game of Russian roulette begins there will no longer be any reliable control. Even just one "accident" could have considerable effects on biodiversity, the future of plant and animal breeding, as well as human health. This "accident" could happen today, tomorrow or even after more than a hundred years.

Therefore, we need to adopt suitable preventative measures without delay to safeguard the interests of future generations and to protect biodiversity. In this context, Testbiotech has formulated five central questions in order to set limits to genetic engineering:[2]

Protect biodiversity!

If we allow genetically engineered organisms to spread their DNA into native populations, it can be regarded as a 'germ-line manipulation' of biodiversity. This human intervention will impact all future generations of the species concerned as well as their ecosystems. We demand that action is taken against the uncontrolled spread of genetically engineered organisms.

Protect health and environment!

The EU has already authorised more than 60 genetically engineered plant events for use in food and feed. There are too many risks and uncertainties associated with the introduction of these plants into the food chain. We demand that the protection of health and the environment is given priority over and above the interests of the biotech industry.

2 www.testbiotech.org/en/limits-to-biotech

Enable freedom of choice!

EU regulations mean that we can avoid genetically engineered organisms being used in food production; that seeds are protected from contamination and we have mandatory labelling of products derived from genetically engineered organisms. Free trade agreements such as CETA are putting these standards at risk. We demand that freedom of choice is safeguarded over and above the interests of free trade.

Push back against the influence of the biotech industry!

Biotech companies not only use their patents to sell their genetically engineered seeds - they also use them to control biosafety research. Experts with close ties to industry have a strong influence on the work of authorities carrying out risk assessment. We demand the strengthening of independent risk assessment. We need to reduce the influence of the biotech industry on breeding, risk assessment and research.

Give ethical principles a higher priority!

From 2004 to 2015, the number of genetically engineered animals used in laboratory experiments in Germany increased more than threefold each year. And in 2015, the number of genetically engineered animals used in experiments exceeded more than one million for the first time. This development is largely driven by economic interests. Furthermore, genetic engineering in human embryos is being discussed as a new option. We demand that patents on genetically engineered animals and intervention in the human germline are prohibited.

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