



## Testbiotech Factsheet 22-1-2015

# Synthetic gene technologies and their application regarding plants and animals in agriculture

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## Summary

In recent years, there has been much discussion about new technologies and techniques used in plant and animal breeding. These include, amongst others, marker assisted selection (MAS), tilling, protoplast fusion, cisgenesis, oligonucleotide techniques, nucleases (DNA scissors) and direct interventions in gene regulation (epigenetics).

At least for some of the new techniques there is some controversy as to whether they are covered by EU regulation for genetically engineered organisms.

- There are some good reasons for partly or wholly excluding marker-assisted selection (MAS), tilling and protoplast fusion from these regulations because these techniques do not involve the preparation or synthesis of material outside the organism and its insertion into cells, natural gene regulatory systems are not bypassed.
- On the other hand, cisgenesis, which uses isolated genetic material from the same or closely related species has been classified as genetic engineering by the European Food Safety Authority (EFSA).
- The regulatory status of oligonucleotide techniques, the use of nucleases (DNA scissors) and of cell-invasive technologies that directly interfere with epigenetics is controversial. Industry and various experts are calling for these techniques to be excluded from the regulatory framework for methods of genetic engineering.

However, there does not appear to be much room for discussion of existing EU regulations. The EU

takes a coherent, process-oriented approach by regulating all organisms produced by the transfer and insertion or introduction of externally prepared genetic or heritable material (such as DNA and RNA). This is, in particular the case when the insertion or introduction of genetic material is done in a way that does not occur in nature. Thus, oligonucleotide techniques, the use of nucleases (DNA scissors) and of cell-invasive technologies that interfere with epigenetics are covered by EU regulation.

The need for strict regulation of these technologies is also supported by the fact that the technical potential to change and interfere with the genome goes beyond that of the technologies that currently are in use. Risk assessment will need more specific requirements, and in many cases even higher standards will be necessary than those currently applied by the European Food Safety Authority, EFSA.

For several reasons it has been suggested that these techniques and applications (oligonucleotide techniques, the use of nucleases and of cell-invasive technologies that interfere with epigenetics) should collectively come under the heading *synthetic gene technologies*:

1. This shows that developments in genetic engineering are currently moving closer to the methods and approaches developed within synthetic biology.
2. It emphasises the difference to methods such as mutagenesis, smart breeding or protoplast fusion.
3. The technical basis for the applications mentioned above, are substances such as DNA and RNA that are synthesised externally from organisms and then inserted in the cells.

The use of plants or animals produced by synthetic genetic engineering for food production and agriculture should not be permitted at this stage. Many of these techniques and technologies are so new that there are no comprehensive data to complete any reasonable risk assessment. Even for those applications that have been under development for some years, there has been no systematic research into risks.

## 1. A definition of genetic engineering

In the last few years, a range of new technologies have been developed for modifying plants and animals. Several of these new technologies have been available for some time; others are in the very early stages of development. The technologies have been grouped under various names such as genome editing, precision breeding, SMART breeding or molecular breeding. Since these terms cover identical technical areas, current terminology is somewhat confusing. Several of the technologies have to be defined as genetic engineering according to EU regulation. Table 1 briefly describes some of the new technologies and their stage of development.

**Table 1: Overview of selected new plant breeding technologies**

Name of the technology	Method	Covered by EU regulation	Stage of development
Marker-assisted selection	Genetic screening allows plants and animals with desirable traits to be selected	No	Products are on the market
Tilling	Random mutations are caused, followed by selection of those plants which carry a specific, desired mutation	No	Products likely to be on the market
Protoplast fusion	Cells of certain plant species can be fused in the laboratory to combine their genomes	Mostly not	Products are on the market
Cisgenesis	Isolated DNA sequences are transferred that are derived from the same species	Yes	Field trials for example with fruit trees are underway. There are no products on the market in the EU.
Oligonucleotide technology (Oligonucleotide-directed mutagenesis)	Short, synthetic DNA fragments/components are introduced to cells to change the structure of the DNA.	Yes (but contested)	Unlikely that products are on the EU market
Nucleases or DNA Scissors (CRISPR-Cas, TALEN, zinc finger nucleases, meganucleases)	The DNA is cut at specific sites with the help of enzymes that are coupled with tracers to identify targeted sequences. Mutations often occur at that site after DNA repair. Additional DNA segments can also be introduced at those sites.	Yes (but contested)	Unlikely that products are on the market in the EU
Epigenetic/ manipulation of gene regulation (e.g., RNA interference (RNAi) or changes in chromosome structure/methylation)	MikroRNA, amongst others, is used to change the activity of specific DNA regions. The effects can be transient (without changing the DNA structure); however very often the effects are imposed by changes of the DNA.	Mostly	Genetically engineered soy with altered oil content is one of the products on the market

Industry and various experts are demanding that techniques such as cisgenesis, oligonucleotide and nuclease-based technologies and engineering of epigenetics regulation be exempt from the EU regulations.

However, it is clear that the EU regulatory framework for genetically engineered organisms covers all processes involving the transfer and insertion of externally prepared genetic or heritable material (such as DNA and RNA) into cells, meant to change its genetic conditions. Such methods are the technical basis for cisgenesis, oligonucleotide techniques, nucleases (DNA scissors) as well as for cell-invasive methods to change gene regulation. Thus, these technologies have to be regulated in accordance with EU Directive 2001/18. In fact, the European Food Safety Authority (EFSA) has already classed cisgenesis as a method of genetic engineering.<sup>1</sup>

### **Criteria used in EU regulation for genetically engineered organisms**

The EU Directive 2001/18 defines methods for genetic engineering that needs to be regulated as follows;

*“Any organism [...] in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination”* (Article 2)

Relevant methods are:

*“recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, [...] and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation”*

as well as

*“techniques involving the direct introduction into an organism of heritable material prepared outside the organism [...]”*

and

*“cell fusion (including protoplast fusion) or hybridisation techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally”. (Annex I A)*

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1 <http://www.efsa.europa.eu/de/search/doc/2561.pdf>

In contrast, techniques such as random mutagenesis as well as cell fusion (including protoplast fusion) of organisms, which can exchange genetic material through traditional breeding methods do not have to be regulated according to Directive 2001/18 (Annex IB). These exclusions to the regulatory regime can be supported by scientific arguments as these techniques bring about random changes in the genome that are subject to natural gene regulation. No isolated, biologically active materials such as DNA or RNA are introduced to plant cells with the objective of overriding natural pathways of cell regulation; instead, such techniques make use of the plants' biological potential of genetic diversity, plasticity and variability. Nevertheless, risk assessment for individual products of these excluded techniques may be justified on a case-by-case basis.

## **2. A new era of super genetic engineering?**

With the emergence of techniques such as oligonucleotide technology, use of nucleases (DNA scissors) and epigenetic modification many experts are proclaiming a new era of super genetics. After three decades of genetic engineering in plant breeding, most applications failed to control the site of insertion of new DNA and to achieve complex genetic changes. But now these new technologies are considered to enable manipulation the genome and gene regulation in a targeted way, with only minor technical limitations and without triggering unintentional side effects. Terminology such as genome editing, precision breeding or molecular breeding are meant to convey the message that we have left the Stone Age of genetic engineering behind.

It is true that especially so-called DNA scissors do offer new possibilities for changes of the genome. Preliminary studies detailing the application of these techniques to plants (such as Arabidopsis, sorghum, rice and wheat), fish, flies, worms, rats, rabbits, frogs, non-human primates and human cells (see Sander & Joung, 2014) and also to animals used in food production such as cattle (Tan et al., 2013), sheep (Han et al., 2014) and pigs (Hai et al., 2014) suggest that technologies such as CRISPR and TALEN are universally applicable across the biological kingdoms. They enable targeted DNA manipulation, even at multiple sites simultaneously (Bortesi & Fischer, 2014, Segal & Meckel, 2013, Baker 2014).

These approaches are synergistic to methods of DNA synthesis that have been developed in recent years: It is no longer necessary to isolate DNA from an organism in order to transfer genes. Knowledge of the specific DNA structure is sufficient to synthesise these genes in the laboratory. Gene synthesis is not confined to DNA sequences with a native template, but can also provide

artificial sequences. The new technologies such as DNA scissors can insert synthetic DNA anywhere in the genome and even enable radical changes.

## **2.1 CRISPR-Cas**

Nucleases are proteins (enzymes) which can be used to splice DNA, hence the term “DNA scissors” or “gene scissors”. These tools were discovered some time ago, but were only able to be used to “cut” DNA in relatively few places. In recent years, several new nucleases have been developed that in principle allow for targeted DNA introduction or modification at any chosen site in the genome. The current star of the nuclease family is known as CRISPR-Cas. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and consists of a guide-RNA region, which can match with a targeted DNA sequence. RNA is capable of mirroring and 'recognising' DNA structure, so that the CRISPR-Cas system can be directed to specific sequences in the genome. The Cas enzyme, which is coupled with the tracer-RNA, operates as the ‘DNA scissor’ and can ‘cut’ a single DNA strand or both simultaneously. Mutations often occur as the cell’s own mechanisms seek to repair the breaks, causing, for example, genes to be silenced. CRISPR-Cas also allows synthesised DNA to be introduced to the site. The Cas enzyme can also be applied to silence genes without cutting the DNA.

The system is surprisingly simple and efficient to operate. Since the possibilities of the CRISPR-Cas system were first discovered some two or three years ago, publications have grown rapidly and there are already commercial applications for its use in laboratory animals. Other gene scissor systems such as TALEN (Transcription Activator-Like Effector Nucleases) and Zinc Finger Nucleases function along similar lines but have proved more difficult to operate. Despite the fact that multiple uses for DNA scissors have been identified, there is as yet no understanding of how they actually function in detail.

## **2.2 Oligonucleotide technology**

A technique that has been available for much longer than CRISPR is oligonucleotide technology. It is not clear whether products developed using this technique are already on the EU market. Without registration and labelling, plants modified using oligonucleotides may be difficult to detect. It is possible, for example, that BASF’s herbicide resistant Clearfield-oilseed Rape was generated using

this technique. According to BASF, however, this is not the case<sup>2</sup>.

Oligonucleotide techniques use very short DNA fragments that have been made to mimic naturally occurring sequences. In addition, the DNA is modified at a specific site in order, for example, to achieve herbicide resistance. These short, synthetic DNA components (oligonucleotides) are introduced into cells to trigger the cell to adjust its own DNA to the foreign synthetic nucleotide, thus resulting in changes to the DNA at desired locations in the plant's genome. In the process, the artificial DNA is allegedly not directly incorporated into the cell's genome/genetic makeup. The exact mechanisms for these genome modifications are not yet understood (see for example Lusser et al., 2011). It is important to recognise that the technique for introducing oligonucleotides can also be used to change longer DNA sequences, such as in the case of Multiplex Automated Genome Engineering (MAGE). In this case, multiple changes of the cell's genome are effected either sequentially or simultaneously (Carr et al., 2012). According to a well-known proponent of synthetic biology, George Church, these or similar techniques can even be used to rewrite the genome of one life form to match that of another (Church & Regis, 2012).

## 2.3 RNAi applications

RNAi-techniques, which are applied to manipulate gene regulation, are not new as such, but new applications are constantly under development. The first genetically modified plants approved in the USA in 1994 – the so-called Flavr-Savr tomatoes were products of RNAi and were genetically modified to block the plant enzyme involved in degradation of cell walls so that the tomato retains its 'form' for longer. The gene for the enzyme was inserted into the genome in its reoriented form ('antisense') so that it had to be read backwards. The RNA produced by this DNA caused the native gene function to be silenced. Other genetically engineered organisms currently approved on the market include soybeans produced by Pioneer with altered oil quality (Soybean 305423)<sup>3</sup>.

As has become evident within recent years, RNA interference (RNAi) mechanisms are highly complex instruments of gene regulation that are present in vertebrates, insects, plants and other life forms. Possible applications of RNAi have increased steadily in recent years. For example, Monsanto wants to introduce genetically engineered maize that produces RNAi as some kind of insecticide: When pest insects feed on the plant, they ingest the additional RNA which then

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2 Personal communication

3 [www.testbiotech.org/node/1013](http://www.testbiotech.org/node/1013)

switches off genes that are vital to their survival – a mechanism that is not always successful (Chu et al., 2014).

### **3. Less control?**

Effectively these methods enable radical modification of the genome and of the gene regulation of all life forms. Thus, the limits of technical manipulation of life forms have been expanded dramatically. For several reasons it has been suggested that the applications of nucleases, oligonucleotides and RNAi-mechanisms come under the collective term “synthetic gene technologies”. This shows that current developments in genetic engineering are moving closer to the methods and approaches of synthetic biology developments (but synthetic biology actually goes beyond the technologies mentioned). On the other hand, this wording emphasises the difference to methods such as random mutagenesis, smart breeding or protoplast fusion. Further, the common technical prerequisite for these applications are substances that interfere with genetic conditions (such as DNA and RNA) prepared or synthesised externally from organisms to be inserted in the cells.

#### **3.1 Risk assessment of oligonucleotide applications**

Various experts believe that techniques such as oligonucleotide-based approaches should not be classed as genetic engineering but as mutagenesis and therefore exempted from the EU regulatory regime. For example, this is the view of the German Federal Commission for Biosafety (ZKBS). Yet, as discussed, oligonucleotide techniques are fundamentally different to mutagenesis: they are invasive methods that intervene in the genome to achieve very specific modifications. Consequently, potential unintended effects (so-called off-target effects) have to be investigated. It is known that this intervention can indeed unintentionally cause changes to the genetic makeup in other parts of the cell or disrupt gene regulation. (see, for example, Lusser et al., 2012, Vogel, 2012). But so far there has been no systematic investigation into specific risks arising from these changes.

### 3.2 Risk assessment of nucleases (CRISPR-Cas)

Numerous side effects arising from CRISPR-Cas interventions have already been observed: Wrongly identified DNA target regions have led to nucleases unintentionally to cut the genome in the wrong places (see for example Fu, et al., 2013). The effectiveness and specificity of the application in plants is dependent on size and complexity of the genome. Thus, no conclusions can be derived from investigations performed on plant species with a smaller genome (such as *Arabidopsis*) on effects in species such as maize (Bortesi & Fischer, 2014). Further, the results also can vary within the same species if different types of cells are used, and they depend on the targeted DNA within the genome (Bortesi & Fischer, 2014).

Important details about the mechanisms on how DNA scissors operate are still unknown and some effects observed seem to be puzzling. Experiments with plants show the desired changes in the genome manifest a generation later than expected (Feng et al., 2013). For this reason, Gao & Zhao (2014) suggest that modification by CRISPR-Cas is a progressive process. They also state that the conditions under which CRISPR-Cas-mediated changes take place in plants remain unclear. Thus, there appears to be a lot of uncertainty whether the potential for a specific change of the genome is being transmitted to next generation.

For risk assessment, it also should be taken into account that the CRISPR system was originally detected in bacteria, its purpose seems to be a defense mechanism against viruses (see for example Baker, 2014). The application of the system in plants and animals means a transferral of biological mechanisms above biological kingdoms. Cells of plants and animals have a different structure to bacteria or (genetic material in viruses) and the mechanisms of cell regulation are different. Thus, it is not unlikely that its application will cause unintended interactions and effects, because it is not adapted in plants and animals by evolutionary processes.

Many unintended effects might be difficult to discover. Most investigations aim to assess target specificity. It is regarded as 'success' if the intended part of the DNA is changed with a high success rate while off-target effects in other regions of the genome are limited. However, beyond the criteria of an effective targeting there are several other questions of relevance. There are several effects that might be triggered by the inserting into cells of nucleases consisting of enzymes and RNA. For example, CRISPR-Cas can cause gene silencing without any change of the DNA being recognised (Bortesi & Fischer, 2014). Processes that involve defense mechanisms in the cells and degradation

of the inserted material should also be considered. For example, the production of new miRNA might be triggered in the targeted cells. This may not only change the metabolism in the plant, but the miRNA might also be transmitted at the stage of consumption and interact with mammalian cells (see for example Zhang et al., 2011).

In general, risk assessment in this context seems to be quite complicated and has to be performed case by case since there are many impact factors. For example, as mentioned, CRISPR-Cas can show different results within a species if applied to different cells. Thus, specificity not only depends on species, but also on the type of cell and the position of the DNA that is targeted. This means a wide range of potential, case specific side effects has to be expected. And some of these effects might only show up under specific environmental conditions or after a number of generations.

Further, many technical modifications can be applied to the design of the CRISPR-Cas enzymes. The nucleases are adopted from case to case to make them efficient and easy to handle. A specific risk assessment will be needed for each of these technical variations.

### **3.3 Risk assessment of RNAi applications**

RNAi techniques pose multiple risks. For some time, the risks were considered to be minimal because no new proteins are produced. Yet recent reports from China have caused a great stir: Chinese researchers claim to have demonstrated that microRNA (miRNA) produced in plants is directly taken up by humans and animals during consumption (Zhang et al. 2011). These miRNA are biologically active substances that might intervene in human and animal metabolism. Lukaski & Zielenkiewicz (2014) had similar results. They found, for example, miRNA of plant origin in the milk of animals after feeding.

The discussion showed that the biological mechanisms underpinning miRNA are far from sufficiently well researched. This has been confirmed in conferences held by USA and EU authorities in 2013 and 2014 that were the first to systematically address the risks of RNAi. At present, we are not in a position to conclude on how much miRNA is taken up from the gut, or to predict what effect these new genetically engineered plants will have on humans or the environment (see EFSA, 2014).

#### 4. Conclusions and recommendations

In general, it has to be acknowledged that plants and animals used in agriculture have to be safe for all consumers and the environment, and safety has to be guaranteed for all stages of vegetation and all environmental conditions. This means risk assessment is much more complex than for many other products that are of stable chemical structure, produced and applied under defined or very specific conditions.

Overall, we know far too little about these new techniques to be able to make well-informed decisions about their use. As yet there is no adequate data about the nature or the consequences of unintended effects these techniques might trigger in plants and animals; nor is there any data on how modified plants might react under conditions of stress or interact with ecosystems/their environments. Nevertheless, publications to date show that the effects may differ from case to case. Calls to have these technologies exempted from regulations have no scientific basis.

From this perspective, the following points are particularly important:

1. Introduction of these controversial new techniques into agriculture should not be permitted on the basis of current knowledge. Some techniques are so new that there are not sufficient data to properly assess their risk. Other techniques are older, but risks arising from them have never been systematically investigated.
2. Industry demands to exclude synthetic gene technologies from the EU regulations must be resisted. Allowing the release of relevant plants, insects or animals without safety assessment and systematic monitoring and labelling of the resulting products would be irresponsible. On the contrary, risk assessment has to be strengthened to comply case by case with the complexity of the risks posed by of the new techniques.
3. Since the methods of synthetic gene technologies enable radical modification of the genome and of the gene regulation of all life forms, new ethical questions are emerging. For example, regulatory initiatives to protect the integrity of the genome of species have to be considered.
4. No release of organisms derived from the new technologies should be allowed, especially if spatio-temporal control within the environment is not guaranteed.
5. Trade agreements such as CETA and TTIP must not be used to introduce provisions that allow exemption of these new technologies from EU regulation.

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