CRISPR-Cas – Revolution from the lab

COGEM Report and advice CGM/141030-01

1. Introduction

In recent years great strides forward have been made with techniques for introducing targeted changes in the hereditary material, collectively called ‘gene editing’ or ‘genome editing’. These new techniques raise questions about the limitations and scope of the legislation governing genetically modified organisms (GMOs). COGEM has drawn attention to these new developments in previous topic and advisory reports.

A new technology that makes use of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and their associated protein (Cas), abbreviated to CRISPR-Cas or CRISPR, promises to considerably speed up the advances being made in genome editing. In a very short time CRISPR has been taken up in numerous fields of research.[1] The CRISPR-Cas system is based on the recognition of specific sequences in the genome and the use of a nuclease to introduce breaks in the DNA.

1.1 History of genome editing

The possibility of making targeted modifications of the DNA in a living cell using sequence-specific nucleases was first described in 2002. Since then several of these nucleases have been discovered, such as zinc finger nucleases, Transcription Activator-Like Effector Nucleases (TALENs) and the CRISPR-Cas system in which the Cas9 protein makes a break in the DNA.[2]

In 2002 the zinc finger nucleases were used for the first time to make specific changes in the genome of the fruit fly (*Drosophila melanogaster*).[3] In 2005 these zinc finger nucleases were used to repair a mutation in human cells[4] and since 2008 the technique has been used in a growing number of applications. In 2009 COGEM published a topic report on the use of zinc fingers.[5]

The use of TALENs in yeast was described for the first time in 2010.[6] Since 2011 this technique has been used for various applications, including making changes in the genomes of *Arabidopsis thaliana*, *Caenorhabditis elegans* and human cells.[7,8,9,10]

The use of the CRISPR-Cas system was first described in 2012. Since then many scientific articles have been published on applications in which this system is used (see Table 1). For example, it is possible to make human stem cells less susceptible to infection with HIV-1, repair genetic defects and turn genes on and off in plants and animals.[11,12,13,14] The CRISPR-Cas system is highly promising and may be hugely significant for both the medical and plant biotechnology sectors.

The technology has advantages over previous techniques such as zinc fingers and TALEN because it requires no knowledge of protein technology, the protocols for making a CRISPR-Cas molecule are relatively simple and the molecules can be synthesised quickly. Numerous manufacturers supply gene-editing kits that use CRISPR-Cas, making genome editing available as a standard technique for every research laboratory.

Table 1: Number of scientific publications on the CRISPR-Cas system[1]

2010	0
2011	1
2012	3
2013	100
2014[2]	235

1) search terms in PubMed: CRISPR Cas9
2) to 30/09/2014

The potential of this technology and its rapid uptake throughout the research world is reflected in the exponential increase in the number of publications on CRISPR-Cas. The technology is also used in the Netherlands.

This report examines how the CRISPR-Cas system works, the possible applications of the system, the potential environmental risks, the applicability of the GMO regulations to this new technology, and aspects of the GMO regulations that need to be re-examined in response to the uptake of this technology.

2. The CRISPR-Cas system

The CRISPR-Cas system recognises specific DNA sequences and can therefore be used to make targeted changes in the hereditary material. The system makes it possible to regulate gene expression, remove genes or parts of genes, introduce new genes or DNA fragments and reveal the sites of certain sequences within the genome. The CRISPR-Cas system is specific, efficient and relatively easy to use.

2.1 The CRISPR-Cas system is found in bacteria

The CRISPR-Cas system is part of the immune system of the bacterium *Streptococcus pyogenes*. Similar systems have been discovered in 90% of the *Archaea* (ancient bacteria which have no cell nucleus like other bacteria, but in which the transcription of genes appears to proceed in the same way as in eukaryotes, which do have a nucleus) and 40% of bacteria.[15,16,17]

The CRISPR-Cas system consists of two RNA molecules and a protein. The first RNA molecule can bind to a specific DNA sequence of 20 nucleotides, the second RNA molecule binds to the first, and then the Cas protein can bind. When a protospacer adjacent motif (PAM) is present next to the sequence to which the first RNA molecule binds, the Cas9 protein cuts each strand of the double helix DNA molecule, thus causing a double strand break in the DNA. To make these cuts the Cas9 protein has two different nucleases, one for each strand of the DNA. The PAM sequence consists of the base sequence NGG, in which N can be any of the bases.

2.2 Use of the CRISPR-Cas system as a genome editing tool

In 2012 it was reported that in bacteria both RNA molecules can be combined to act as a 'single guide RNA' (sgRNA) (Figure 1).[18] In 2013 it was discovered that this can also be implemented in eukaryotic cells,[19] a discovery that prompted a rapid increase in the amount of research into the CRISPR-Cas system. The application of CRISPR-Cas as a gene or genome editing system is based on the possibility of introducing breaks into genome sequences at specific places and on the use of cellular DNA repair mechanisms to introduce mutations and deletions or insert new sequences.

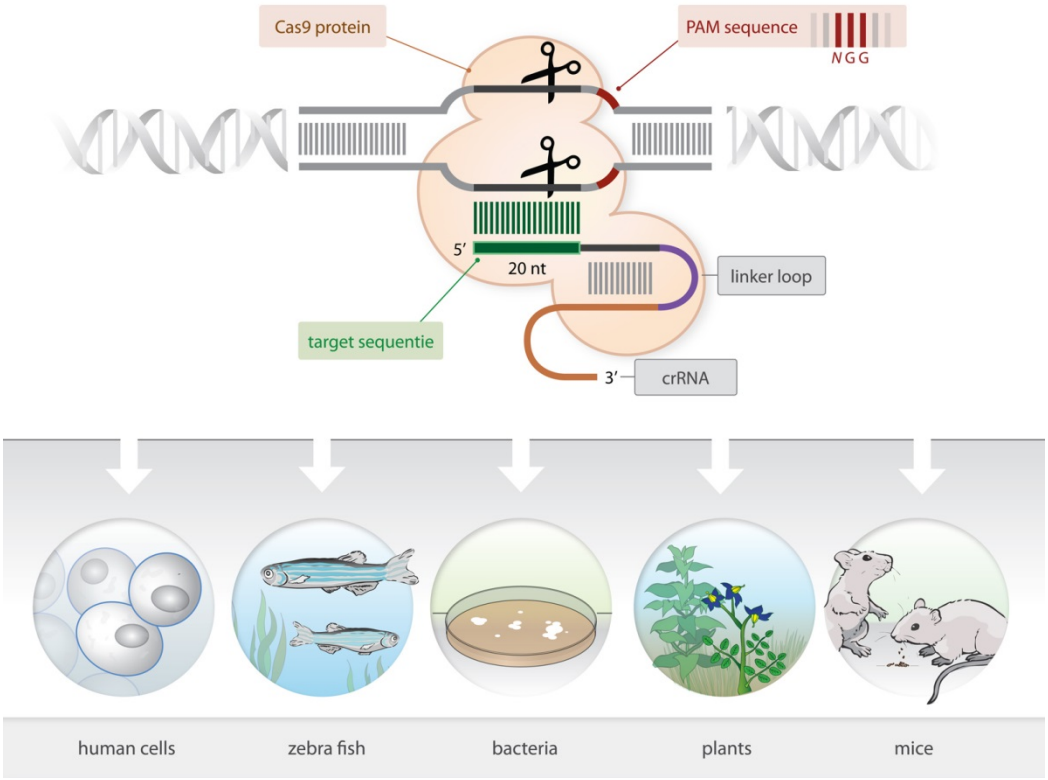

Figure 1: The CRISPR-Cas system

2.2.1 DNA repair mechanism repairs a double strand break

As a break in the DNA can be lethal for a cell, its natural DNA repair mechanism is triggered to repair the break.[20] Cells have two mechanisms at their disposal to do this: homologous directed repair (HDR) and non-homologous end-joining (NHEJ) (see box).

Homologous directed repair (HDR)

Homologous recombination is a very precise mechanism for repairing breaks in DNA.[20] It is a sort of copy/paste process in which an undamaged homologous DNA sequence serves as an information source for the repair.[20] A comparable sequence in the genome, for example the sequence on the sister chromatid, can serve as homologous DNA.[20]

Non-homologous end-joining (NHEJ)

Another way of repairing a double strand break involves joining together the broken strands of DNA, which is called non-homologous end-joining. This requires little or no sequence homology and often results in deletions or insertions at the site of the break.[20,21,22]

The CRISPR-Cas system makes it possible to introduce specific mutations or genes into a genome by means of homologous recombination (HDR). During the modification a DNA molecule must be introduced at the same time as the sgRNA and the Cas9. This DNA must have sequences on either side of the new sequence that are very similar to the sequences on either side of the double strand break.[23] In addition, CRISPR-Cas can be used to introduce small insertions and deletions (indels) in the genome, because a break in the DNA will be repaired by the NHEJ mechanism.

2.2.2 The CRISPR-Cas system in eukaryotic cells

To make targeted changes in the genome of eukaryotic cells there must be a method or technique for introducing the CRISPR-Cas system into the cell. Unlike prokaryotic cells (such as bacteria and archaea), eukaryotic cells have a cell nucleus, which means the Cas9 must be given a localisation signal to ensure it is transported into the cell nucleus.

The best method for introducing the elements of the CRISPR-Cas system into eukaryotic cells depends on various factors, including the type of cell and the organism concerned. Different methods are used for mammal cells than for plant cells. For plants use is mainly made of the bacterium *Rhizobium radiobacter* (previously *Agrobacterium tumefaciens*).[24] A plant can then be cultured from the resulting transformed cell. The CRISPR-Cas system can also be expressed constitutively in the cell with the help of a transformation vector, which is not inserted into the genome. For some plant species it is possible to introduce the CRISPR-Cas complex into protoplasts (without the intervention of a vector or *R. radiobacter*) and regenerate a whole plant from the protoplasts. Vector systems based on plant viruses are also being developed to introduce the CRISPR-Cas system into plant cells.[25]

Various methods are used to introduce the CRISPR-Cas system into mammalian cells. A much used method is to insert a plasmid or other DNA vector containing the gene that codes for the Cas9 protein, which then produces sgRNA.[23,26] Another method is to introduce the sgRNA (or both RNAs separately) into the cell together with the mRNA that codes for the Cas9 protein.[27] A third method is to inject the complex containing both the Cas9 protein and the sgRNA.[21]

2.2.3 CRISPR-Cas sequences not present in the final organism

Besides the changes introduced into the genome of the cell, in a number of cases the elements of the CRISPR-Cas system themselves (Cas9 gene and sgRNA coding sequence) will also be inserted into the genome. This is the case, for example, when a plant cell is genetically modified and a plant is regenerated from the cell. However, in most cases none of the elements of the CRISPR-Cas system will still be present in the final organism. In the example of the genetically modified plant created by means of *R. radiobacter* transformation mentioned above, the inserted CRISPR-Cas sequences can be removed after outcrossing by continuing the breeding and multiplication process with the progeny that do not contain the construct but do possess the desired change in the genome. Recognition or detection of these organisms is then only possible if the exact changes made to the genome are known.

3. Applications of the CRISPR-Cas system

Genetic modification has gone through a phase of rapid development in recent years and scientists are continually looking for more targeted and more efficient modification techniques. The CRISPR-Cas system can be used to engineer the correct change at any desired site in the genome.

Using CRISPR-Cas it is possible to make the following changes at specific sites in the genome (Figure 2):

- make small and large deletions,
- introduce point mutations,
- insert sequences or genes.

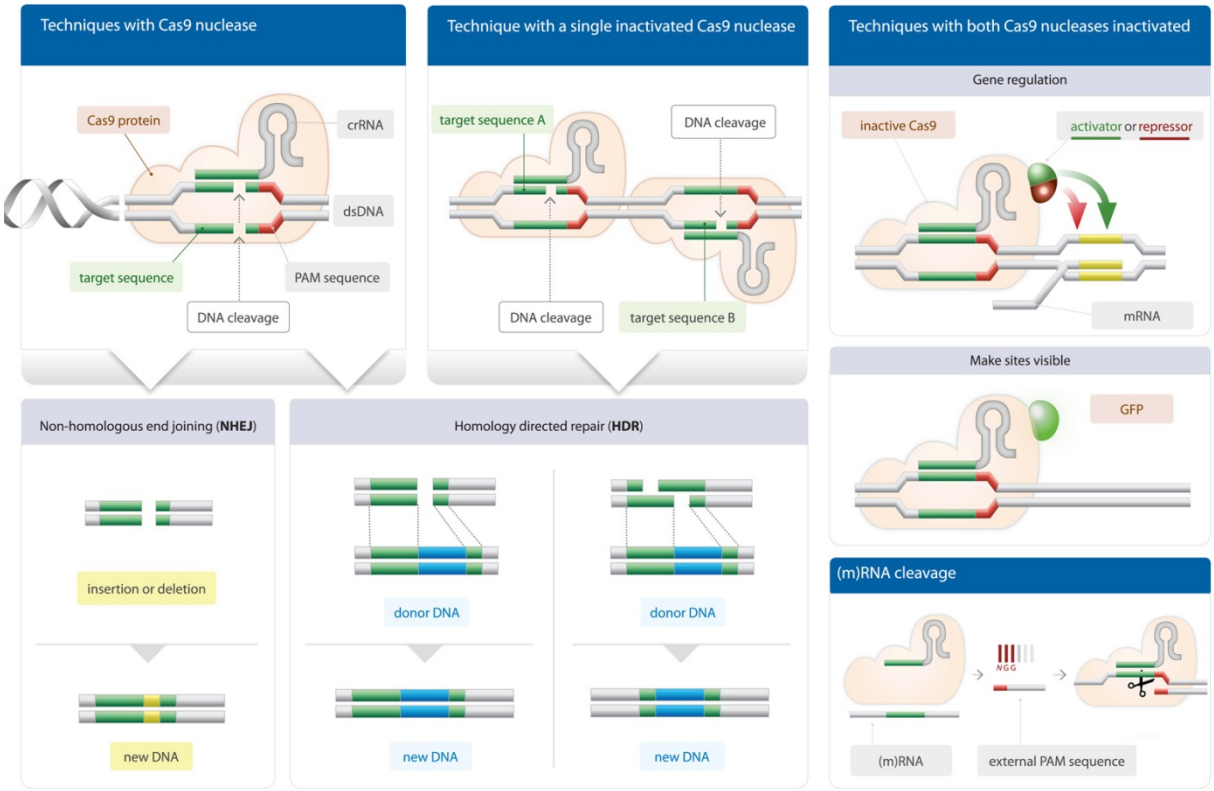

Figure 2: *Applications of CRISPR-Cas*

Variations on these applications can be made by inactivating one or both Cas9 nucleases:

- If one of the Cas9 nucleases is inactivated, a single strand break can be introduced into the DNA molecule. If two different target sequences are used, specific parts of the DNA molecule can be removed.[28,29,30]

It is also possible to influence the expression of genes without introducing changes into the genome sequences:

- If both nucleases of a Cas9 protein are inactivated, it can bind to the DNA but cannot cleave it. If specific domains (activators or repressors) are linked to the Cas9, genes can be regulated.[31,32,33,34,35,36] This technique can also be used to make certain sites on the DNA molecule visible for research purposes.

A surprising development is that CRISPR-Cas can be used not only as a genome editing system but can also bind to (m)RNA and cleave it:[37]

- This can be used to investigate (m)RNA functions, influence gene expression without changing any DNA sequences, and also to combat RNA viruses.

The possibility of introducing targeted changes into the genome paves the way towards numerous research applications: gene functions can be investigated by ‘knocking out’ genes, by changing

gene sequences or inserting genes; gene regulation and associated elements can be studied better; 'reverse genetics' is made easier, etc.[38]

Many experiments have already been carried out on cell lines,[19,39,40,41] and also on fruit flies,[42] zebra fish,[21] mammals,[22,41,43,44,45,46,47] non-human primates[27] and plants.[48,49,50,51] COGEM expects that many studies will be carried out using this technique in the coming years and that commercial applications will soon become available. It should be noted that this technology is still in its infancy and that new and unforeseen applications will appear over the years. Some applications of the CRISPR-Cas system are described below.

3.1 Applications of the CRISPR-Cas system in the medical sector

Much research is being done on the use of CRISPR-Cas in the medical field. Although many of these studies are in the developmental phase, several studies have been reported in which CRISPR-Cas is used for the genetic modification of mammals, such as mice,[43,52] rats,[43,44,45] cattle[53] and non-human primates.[46] CRISPR-Cas has advantages over the current systems for producing genetically modified (GM) animals. A GM animal can be obtained by injecting a CRISPR construct into single cell embryos, which reduces the 'development time' from more than a year to just a few weeks.

In one study cataracts in mice were cured.[22] Cataracts are a dominant trait, which means that the progeny of a mouse with and a mouse without cataracts will inherit the disease. The genome of affected mice lacks one base pair, causing a stop codon to be prematurely introduced and consequently a shorter protein is made, which in turn causes cataracts. To solve this problem, the researchers injected fertilised mouse egg cells with the components of the CRISPR-Cas system. The chosen sgRNA ensured that a double strand break was introduced next to the missing base pair and the researchers demonstrated that the double strand break is repaired by both HDR and NHEJ. Genetic modification occurred in about half (36 of 78 mice) of the live-born mice pups, and in 67% of these (24 of 36 mice) the effect of the mutation was nullified, resulting in good vision.

The CRISPR-Cas system can also be used in adult animals to develop a disease model system. It has recently been shown that it is possible to turn oncogenes and tumour suppressor genes in liver cells on and off, thus creating a model system for liver cancer.[54] In addition, the CRISPR-Cas system has recently been used to cure adult mice of a hereditary liver condition.[55]

Applications that can cure human diseases are coming within reach. Various studies have been published in which a genetic abnormality caused by an error at one place in the genome has been corrected.[14,56] In addition to therapeutic applications in which gene defects are corrected, researchers are working on using CRISPR-Cas to influence gene regulation. Other research is looking into whether the system can be used as an antiviral agent[57] or to suppress bacteria. A bacteriophage (a virus that only infects bacteria) into which CRISPR-Cas has been introduced can track down specific bacteria or specific sequences, such as antibiotic-resistant genes or virulence factors.[58]

Therapeutic applications of CRISPR-based systems still have to surmount a number of obstacles, such as reducing non-specific activity caused by off-target binding and developing efficient vector systems for introducing the systems into the target cells. This last obstacle can be surmounted by combining CRISPR-Cas with viral expression vectors, as already in use and in development for gene therapy.[59]

3.2 Applications of the CRISPR-Cas system in plant biotechnology

The first applications of CRISPR-Cas in plants were reported in 2013. Some of these publications demonstrated that the system is effective in plant cells and that mutations or gene sequences can be introduced into model systems such as *Arabidopsis* and tobacco.

For *Arabidopsis* it has been reported, for example, that it is possible to modify an inactive gene in such a way that it becomes active and its trait is passed on to the next generation. It is possible to introduce these changes without making any other modifications in the genomic DNA.[60]

Besides model systems, work is in progress on important agricultural crops, such as tomato[61] and bread wheat (*Triticum aestivum*). Bread wheat is hexaploid (42 chromosomes: 2n = 6x = 42), which makes genetic modification a challenge because changes have to be made at several different places in the genome. Researchers have shown that it is possible to make bread wheat resistant to powdery mildew by modifying the same gene on several chromosomes. They made use of two techniques: TALENs and the CRISPR-Cas system.[62] As several important agricultural crops are polyploid, an application that makes it possible to introduce the same modifications to several chromosomes at the same time is of great interest to plant biotechnologists and breeders.

The same genes sometimes occur at several places in the genome. These paralogous genes are created by duplications and consist for the most part of the same sequence. Introducing mutations into all the relevant versions of these paralogous genes at the same time offers breeders the possibility of altering the characteristics associated with these genes.

Besides altering or introducing gene sequences, another possibility being considered is introducing mutations in promoters or other regulatory sequences to increase or reduce the expression of a gene.

The great potential of the technique and the relative simplicity and user friendliness of the CRISPR-Cas system has in a very short time led to its widespread use in the research community and plant breeding industry. This suggests that commercial applications in the agricultural sector will follow.

4. Risks of CRISPR-Cas

Expectations of the CRISPR-Cas system in the scientific literature are high. Little has yet been published about the potential risks of CRISPR and its applications, partly because of the limited practical experience gained so far.

4.1 Off-target effects

The most important disadvantage or risks of the CRISPR-Cas system stated in the literature are possible off-target effects.[63] These are caused when the sgRNA binds to the wrong place in the hereditary material. When the DNA is then cleaved and repaired, mutations may occur in places where they are not intended[22] and may have unwanted effects, such as changes to a protein molecule.

The main cause of possible off-target effects is a lack of specificity of the sgRNA, which must be high enough for the hereditary material to be recognised at just one site. The sgRNA is 20 nucleotides long, which, given the size of the genome of eukaryotic organisms, means that these binding sequences may occur at several places within the genome. In addition, the sgRNA can sometimes bind to DNA sequences that are near-identical to it. The chance of this occurring is greater when the nucleotides of the sgRNA are situated further away from the PAM. In some cases it is sufficient for 15 of the 20 nucleotides to be an exact match.[64,65,66] In these sites double strand breaks can occur which can then cause unwanted mutations.

One of the best ways of preventing off-target effects is to ensure that the selected sgRNA cannot bind to another position within the genome. A suitable sgRNA can be chosen on the basis of a bioinformatic analysis of the genome sequence. There are also computer programmes and web tools that can predict off-target binding.[67,68,69]

The number of off-target effects is also highly dependent on the experimental conditions, especially the amount (concentration) of Cas9 proteins and sgRNAs. High concentrations lead to more off-target effects.[63]

Another method of preventing off-target effects is to inactivate one of the nucleases of the Cas9 protein so that only one of the two DNA strands is cleaved. Two sgRNA/Cas9 complexes will then be needed to cut the DNA molecule in two and they must bind to the complementary strands next to each other in order to bring about a double strand break. This method reduces the occurrence of off-target effects because two binding sites are needed to introduce a double strand break. In addition, research is being carried out into reducing off-target effects by varying the length of the sgRNA.[70]

However, how much of a risk is actually posed by off-target effects remains in doubt. Research into the use of CRISPR-Cas in stem cells has shown that off-target effects are rare.[71,72,73,74] Most of the identified genomic changes, such as deletions and insertions, were found at random places in the

genome and were not the result of the CRISPR-Cas application, but occurred during the cell culture.

4.2 Modifications in the genome

The CRISPR-Cas system is used to induce changes in the genome of organisms. The organisms produced in this way will contain insertions, deletions, point mutations, rearrangements or new sequences and genes in their genome. Any environmental risks or adverse effects associated with these genomic changes are not related to the method used to bring about these changes, but to the nature of the changes themselves. Any risks arising from these changes should therefore be assessed in relation to the nature of the change rather than as part of a safety assessment of the technique.

5. GMO legislation and CRISPR-Cas

European directives have been adopted to protect humans and the environment against potential adverse effects that could result from working with GMOs. These directives have been transposed into Dutch law in the Environmental Management Act, the Genetically Modified Organisms Decree (Environmentally Hazardous Substances Act) and the Ministerial Regulation on GMOs. In the current legislation a GMO is defined as ‘an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination’. In addition, genetic modification is involved when certain techniques are used: recombinant DNA and RNA techniques that make use of host/vector systems, techniques in which genetic material prepared outside the organism is introduced directly into the organism (for example by microinjection), and cell fusion or hybridisation techniques.

The GMO legislation was drawn up on the basis of the state of the art twenty years ago. At that time a number of techniques were exempt from the legislation, the argument being that they had been used for many years without any adverse effects. Some of these techniques have been in commercial use for plant breeding since the 1930s. Exempt techniques are cell fusion and protoplast fusion between crossable relatives, self-cloning of non-pathogenic microorganisms, and the use of chemical mutagens and radiation to induce mutation.

The question is whether all applications of the CRISPR-Cas system fall under the definition of genetic modification, and therefore under the GMO legislation. There is not always a simple or clear-cut answer to this, partly because both legal and scientific arguments are involved.

5.1 CRISPR-Cas products cannot be conclusively assessed

There are a number of considerations that support the argument that the CRISPR-Cas system and its products do fall under the current GMO regulations:

1) Changes are made in the sequence of the genome in ways that do not naturally occur.

2) A ‘recombinant nucleic acid molecule’ (RNA, on its own or in combination with a protein (Cas9)) prepared outside the organism is introduced into the cell.
3) For some applications not only is the desired change made to the genome, but parts of the CRISPR-Cas system are also inserted into the genome.
4) For other applications a vector system, for example one based on a genetically modified virus, is used to introduce the CRISPR-Cas into the cell of the target organism.

On the other hand, a number of considerations support the opposite argument:
1) Deletions, rearrangements, point mutations and random small insertions can occur spontaneously in nature or can be induced by chemical mutagens or radiation. The ‘classic’ mutagenesis techniques are exempt from the GMO regulations because the products of these techniques have a ‘history of safe use’. In view of the fact that the targeted induction of mutations and such like involves considerably less risk than inducing random genome changes using mutagens, COGEM has previously advised exempting ‘site-directed mutagenesis’ from the GMO regulations, irrespective of the method used.[75]
2) Organisms with point mutations and indels in the genome cannot be distinguished from ‘non-modified’ organisms. This raises serious questions about the enforceability of the GMO regulations. COGEM has previously pointed out that this can lead to a loss of confidence in government.
3) Applications in which CRISPR-Cas is used to influence gene expression by binding with regulatory signals, and for which the Cas9 nuclease activity is switched off, do not lead to changes in the genome sequence and therefore cannot fall within the scope of the GMO regulations.

Regarding the question of whether the sgRNA should be deemed to be a recombinant nucleic acid, the previous COGEM advice and topic report ‘The status of oligonucleotides within the context of site-directed mutagenesis’ is relevant.[76] In response to a request for advice from the then Ministry of Housing, Spatial Planning and the Environment, COGEM defined an oligonucleotide as ‘a single-or double-stranded molecule consisting of different nucleotides (or analogues) of DNA and/or RNA with a length up to approximately 120 nucleotides (or base pairs), which may or may not be produced synthetically’. COGEM’s opinion was that an oligonucleotide with a sequence identical to or just a few nucleotides different from genome sequences of the recipient cell cannot be deemed to be a recombinant nucleic acid. This is one of the reasons why COGEM considers that site-directed mutagenesis using oligonucleotides can be exempted from the GMO regulations.

The sgRNA consist of a combination of two RNA molecules. The first component consists of a fragment of 20 nucleotides identical to the binding site on the genomic DNA, and the second component in needed to bind the Cas9 protein. The active parts of the RNA–protein complex therefore consist of an RNA sequence identical to the genomic sequence and a protein that cuts the DNA. In line with the GMO regulations and the COGEM advice mentioned above, it can be argued that on the basis of these elements the GMO regulations do not apply and the CRISPR-Cas can be

deemed equivalent to chemical mutagens. On the other hand, the RNA molecule in the CRISPR-Cas complex consists of two components, one of which has no sequence equivalence with the genomic DNA of the recipient cell. This can qualify the sgRNA as a ‘recombinant nucleic acid’.

5.2 Various applications need a different approach

The above section highlights the limitations of the present GMO regulations previously noted by COGEM. The European approach in which organisms fall under the GMO regulations if certain ‘recombinant DNA techniques’ have been used is inadequate for new techniques like CRISPR. If CRISPR is used to insert a transgene into an organism there can be no doubt that the GMO regulations apply and that the resulting GMO must be subject to a risk assessment. However, if CRISPR is used to induce point mutations or indels, the resulting organism will be ‘safer’ than a product made using ‘classic’ mutagens. In such cases it would seem appropriate to exempt the organism form the regulations (as for the products of chemical mutagens and radiation). This is discussed further in Chapter 6.

If CRISPR-Cas is used as a drug to influence the expression of a gene by binding to the sequence, but not to modify it, this is comparable to medicines that have an effect on gene expression. In such cases, therefore, the GMO regulations should not apply.

6. Additional considerations: possible consequences for society, the economy and policy

6.1 Public debate

Public resistance to genetic modification is directed towards its use in agriculture and to GM foods. So far the debate about new biotechnological techniques has been largely restricted to agricultural applications. Exemption from the GMO regulations and other assessments of the new techniques have therefore taken place within the context of public disapproval, either real or assumed. However, CRISPR has a much wider range of possible applications, with those in the medical sector likely to come about sooner than in the agricultural sector. This may ease the tension in the public debate about the status of these and similar techniques.

6.2 'Process-based' regulations versus 'product-based' regulations

In recent years COGEM has at several times drawn attention to the fact that technological developments are outpacing European legislation on genetic modification.[75,76,77]

The EU has chosen to establish specific GMO regulations. The EU legislation, as set out in EU Directive 2001/18, is called process-based legislation because the reason for passing it in the first place is the method of production – the process. Inherent in this type of legislation is that what is covered by the regulations and what falls outside their scope depends on the techniques used.

In North America GMOs have been placed under existing general legislation. This type of legislation is called product-based legislation because it is the characteristics of the product or organisms themselves that determine whether they are subject to regulation. In Canada, for example, if a crop has a new characteristic a permit or licence must be obtained for it and it must be subjected to a risk assessment, irrespective of whether the crop is the product of a conventional breeding process or genetic modification. The opposite applies as well: a permit is never required for a crop with a known characteristic.

The COGEM report 'Update the EU legislation?' examines the philosophies underlying these legislative systems and reviews several options for bridging the gap between the EU GMO regulations and scientific developments in the field.[77]

Developments such as CRISPR-Cas reinforce the calls to change the EU legislation to a product-based system. Product-based legislation does not have to be adapted in response to the latest technological and scientific developments.

Given the polarised climate in Europe regarding genetic modification and the disagreement between the EU member states on this topic, making a fundamental change to the EU legislation will not be an easy task. Moreover, under a new legislative regime some applications and products

not covered by the current legislation (because they are produced in the conventional manner) may have to be licensed, which would meet with resistance from various quarters, particularly industry.

Process-based legislation has to be regularly revised to ensure the legal definitions keep pace with GM technologies and technologies that can lead to the production of a GMO. A technology like CRISPR-Cas raises questions about the feasibility of this approach and how long it can continue, but the decision-making process in the EU on these matters appears to have become deadlocked. Partly in response to the COGEM report on new plant biotechnologies published in 2006, the European Commission established an EU working group to look into the status of these 'new' techniques, the EFSA has been asked to identify the risks associated with these new techniques and a legal analysis is being carried out. Six years after the working group's first meeting a decision has yet to be made about the status of the new techniques. It increasingly looks as if coming to a decision on the status of these techniques in the EU will be just as difficult as completely revising the principles underlying the legislation.

While there may be little prospect of the legislation being adapted or revised, the scientific and technical developments continue unabated, as demonstrated by the emergence of CRISPR-Cas and TALEN. The gulf between science and legislation and the associated problems for industry, scientists and society are becoming ever larger.

With no clear definitions of what should be considered to be GMOs, European industry remains hesitant about making or adopting certain innovations. As a result, European industry faces an uneven playing field compared with its counterparts in North America. Trade conflicts and import problems are increasingly likely because of the growing differences of opinion between the EU and countries with a product-based legislative system about what should be classified as GMOs and therefore subject to regulation. And because imported products are not identifiable as GMOs under the European legal definition, and may not be labelled as such, consumer choice and the credibility of government are being undermined.[77]

6.3 Economic consequences

The CRISPR-Cas technique has been embraced by workers in the field as a quick and simple genome editing method for prokaryotic and eukaryotic organisms and for both medical and agricultural research. The technique also opens up considerable opportunities for industrial production ('white biotechnology') because of the possibility it offers for improving production organisms. CRISPR-Cas is also considered to be an essential technique for the upcoming field of synthetic biology.[78,79,80] CRISPR-Cas will therefore play an important role in innovation across the whole field of biotechnology, which in turn has considerable economic implications.

If the use of CRISPR-Cas is viewed in a different light in Europe than outside Europe (in other words, as falling under the GMO regulations), it could lead to the loss of both commercial and research activity from the EU. In response to public resistance to GM crops and the laborious

licensing policy, companies in Europe have given up on genetic modification of agricultural crops and GM research at European universities and research institutes has more or less ground to a halt. Financing for this type of research activity (both public and private) has dried up.

The same scenario could occur for new techniques such as CRISPR. It should be said that the genetic modification of agricultural crops is just one part of the market and so plant breeding companies can still concentrate on conventional breeding practices, and that just a few EU member states have a significant plant breeding industry. Nevertheless, techniques such as CRISPR-Cas affect the whole biotechnology sector, including the medical sector. The potential economic consequences are therefore much greater.

6.3.1 Intellectual property

The issue of intellectual property rights for this technology has not yet been resolved.[81,82] In 2014 a patent for a CRISPR-Cas application was awarded to the start-up company Editas Medicine.[83] Various other institutes and scientists have also submitted patent applications for the technology, improvements (such as higher specificity) and applications. Researchers at the University of Minnesota (US) have submitted an application for a patent for genome engineering in plants[84] and there are probably many more patent applications that have not been published yet.[81] Patent applications made earlier for similar technologies or mechanisms of action may also have an influence on the situation regarding intellectual property rights for CRISPR-Cas.

Uncertainty about intellectual property rights or control over the technology by one or more people or organisations may inhibit implementation of the technology and hold up commercial applications. It can also generate public resistance to the idea that a potentially crucial technology could fall into the hands of a small group of scientists, institutes or companies.

6.4 Germline gene therapy in apes and humans

Genetic modification of humans by germline gene therapy in which progeny are genetically modified is not permitted in Europe. Besides ethical objections to genetic modification of humans, there are also practical objections. It is not certain that germline gene therapy is actually possible (and how efficient it would be); moreover, the risks of randomly (so far) inserting the introduced gene into the genome of the progeny are great.

In early 2014 researchers at the Model Animal Research Center of Nanjing University in China reported that they had succeeded in genetically modifying apes using CRISPR-Cas targeted mutations in embryos.[27] Various research groups have announced that they also want to make GM apes to use them as model systems – in addition to the much used GM rats and mice – for research into human diseases, etc. Besides the questionable desirability of more widespread use of GM apes as a model system for humans, there are serious ethical objections among the public to research on apes.

The development of a system to make targeted genetic modifications in primates negates many of the practical objections to genetic modification of humans. From a scientific point of view the step from apes to humans is a small one, and scientists and patients (or patient organisations) will begin to question why germline gene therapy is not allowed in certain cases. Do the ethical objections to changing and possibly 'enhancing' humans outweigh the possibilities of eradicating hereditary diseases and giving carriers of hereditary diseases the opportunity to have children of their own?

A similar debate surrounds methods of preventing mitochondrial diseases that lead to 'three-parent children'. Mitochondrial diseases are inherited from the maternal DNA and can be prevented by transplanting a cell nucleus into an egg cell of a healthy donor, which is subsequently fertilised in vitro with sperm from the father. The UK Nuffic Council on Bioethics concludes that this is ethically acceptable.[85] In early 2014 the British Government published draft guidelines on permissible cell nucleus transplantation.

7. Conclusions

- CRISPR-Cas is a new technique for gene or genome editing and is being adopted in the field of biotechnology at a rapid rate. As a consequence, genome editing is becoming a standard technique that can be used in every research laboratory within the life sciences.
- The technology is still new and in the future as yet unforeseen applications will be developed.

- Within the current EU legal framework, applications of CRISPR-Cas fall under the legislation on GMOs.
- However, CRISPR-Cas can be used for various purposes, several of which should qualify for exemption from the regulations.

- New techniques such as CRISPR-Cas show that the current EU GMO legislation is due for revision.
- COGEM concludes that, given the scientific and technological developments, the Dutch Government and the European Commission should consider revising or amending the EU legislation on GMOs.

- A decision on whether or not applications and products of CRISPR-Cas should be subject to the GMO regulations can have major consequences for innovation within the EU and the economic position of European companies.
- Given the great importance of this technology for both industry and society (medical applications, etc.), COGEM advises the Dutch government to press for a rapid decision on the legal status of this technique and its applications and products in the EU.

- The growing technical possibilities for making targeted modifications in the genome of plants, animals and humans raise ethical questions, such as why it is not permitted to use genetic modification techniques to eradicate hereditary diseases.

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